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INVESTIGATIONS INTO THE EFFECTS OF PLANT DERIVED CYSTEINE PROTEINASES ON TAPEWORMS (CESTODA)

by

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Dedication:

This thesis is affectionately dedicated to my father, my mother, my wife and my children; Emil and Emily.

ABSTRACT

Gastrointestinal (GI) helminths pose a significant threat to the livestock industry and are a recognized cause of global morbidity in humans. Control relies principally on chemotherapy but in the case of nematodes is rapidly losing efficacy through widespread development and spread of resistance to conventional anthelmintics and hence the urgent need for novel classes of anthelmintics. Cysteine proteinases (CPs) from papaya latex have been shown to be effective against three murine nematodes *Heligmosomoides bakeri, Protospirura muricola and Trichuris muris in vitro* and *in vivo* and against the economically important nematode parasite of sheep *Haemonchus contortus*. Preliminary evidence suggests an even broader spectrum of activity with efficacy against the canine hookworm *Ancylostoma ceylanicum*, juvenile stages of parasitic plant nematodes of the genera *Meloidogyne* and *Globodera* and a murine cestode *Hymenolepis microstoma in vitro*. This project focused on tapeworms. Using 2 different rodent cestodes *Hymenolepis diminuta* and *Hymenolepis microstoma* and 1 equine cestode *Anoplocephala perfoliata* I have been able to show that CPs do indeed affect cestodes whether young newly hatched scoleces *in vitro* (by causing a significant reduction in motility leading to death of the worms) or mature adult worms *in vitro* (by causing a significant reduction in motility leading to death of the worms) and *in vivo* (resulting in a significant, but relatively small, reduction in worm burden and biomass), despite no effects on worm fecundity. Although only minimally efficacious against *Hymenolepis microstoma* and moderately efficacious against *Hymenolepis diminuta in vivo*, efficacy was enhanced by the synergistic effects of the immune system demonstrated against *Hymenolepis diminuta* in the non-permissive host. The results offer the possibility that with further refinement, CPs may be developed into broad spectrum anthelmintics that in addition to their marked effects on nematodes also remove any concurrently residing tapeworms.

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ABBREVIATIONS

µm	Micrometer
µM	Micromolar
µmol	Micromol
ANOVA	Analysis of variance
CP	Cysteine proteinase
CPL	Crude papaya latex
DER	Diglycidyl ether of polypropylene glycol
DMAE	Dimethylaminoethanol
E-64	L-trans-epoxysuccinyl-leucylamido (4-guanidino)-butane
ED_{50}	Effective dose 50
ED_{100}	Effective dose 100
ELISA	Enzyme-linked immunosorbent assay
EPG	Eggs per gram faeces
ERL	3,4 Epoxy Cyclohexyl Methyl 3,4 epoxy cyclohexyl carboxylate
g	gravity
GI	Gastrointestinal
HC	Hanks' cysteine
IC_{50}	Half maximal inhibitory concentration
kg	kilogram
Log_{10}	logarithm base 10
mg	miligram
ml	mililitre
mmol	milimol
MW	Molecular weight
nmol	nanomol
NSA	Nonenyl succinic anhydride
p.i.	Post infection
PLS	Papaya latex supernatant
rmGLM	Repeated measures general linear model
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy

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CHAPTER 1

1.0 Summary

This chapter provides background information pertinent to this research project,highlighting key problems, as well as current knowledge on the subject, before taking the reader to the main aims and objectives of the work described in this thesis.

1.1 Background

Intestinal helminths pose a significant threat to the livestock industry (Nieuwhof and Bishop, 2005) and are a recognized cause of global morbidity in humans (Chan, 1997a). Control relies principally on chemotherapy (Behnke et al., 2008) but in the case of nematodes is rapidly losing efficacy through widespread development and spread of resistance to conventional anthelmintics (Kaplan, 2004) and hence the urgent need for novel classes of anthelmintics (Stepek *et al*., 2006a). One such alternative may be the use of natural plant products as anthelmintics, as traditionally used for centuries in the past in local native medicine among indigenous communities of the tropics (Robbins, 1930).

Earlier observations demonstrated potent anthelmintic activity of papaya latex, pineapple and figs (Berger and Asenjo, 1939, 1940; Robbins, 1930), whose active components were later identified as cysteine proteinases (Walti, 1938). Interest in this subject resumed much later with the demonstration that worm burdens in pigs infected with *Ascaris suum* could be greatly reduced by treatment with papaya latex (Satrija *et al*., 1994) and that mice infected with *Heligmosomoides bakeri (polygyrus*) could also be effectively treated (Satrija *et al*., 1995). There was even a successful clinical trial in Brazil (Hansson *et al*., 1986). However, unequivocal confirmation of the effect of papaya latex on the nematode cuticle was quite recent (Stepek *et al*., 2005).

Cestodes are also important parasites of livestock and humans (Muller, 2001) and preliminary evidence suggests that cysteine proteinases do affect the cestode tegument (Stepek *et al*., 2007e). If this is verified it will greatly extend the usefulness of cysteine proteinases as anthelmintics, because the broader the spectrum of activity, the greater their chances of acceptance by the end-user community and therefore the more likely they are to be produced commercially (Behnke *et al*., 2008).

1.1.2 Cestodes as a global problem

Collectively gastrointestinal nematodes which affect half of the world's population have been shown to cause more morbidity than malaria or even motor vehicle accidents (Chan, 1997a). However, the importance of other parasitic helminth infections such as trematodes (Utzinger *et al*., 2000) and cestodes has been largely underestimated (Budke *et al*., 2009). One particular group which fails to gain world attention, despite its often lethal prognosis, encompasses the zoonotic larval cestodes (Hotez *et al*., 2007) and only recently did the World Health Organization (WHO) included echinococcosis and

cysticercosis in its list of neglected tropical diseases as part of an integrated strategy to alleviate poverty (WHO, 2006).

Overall global estimation of disease burden arising from cestode infections is difficult due to limited data (Budke et al., 2009). Nonetheless, sporadic data support the fact that cestode infections are an impediment to human and livestock health (Benner *et al*., 2010; Flisser *et al*., 2003; Li *et al*., 2006). For example, the number of disability adjusted-life years (DALYs) estimated to be lost as a result of cystic echinococcosis puts this species at least at the same level as the better known neglected tropical diseases such as dengue fever, onchocerciasis and trypanosomiasis (Budke *et al*., 2006).

1.1.3 Taeniasis

T. solium and *T. saginata* are two most important taeniasis affecting humans (WHO, 2006). The discovery of *T. asiatica* as a distinct species (Eom and Rim, 1993) despite sharing morphological similarities with *T. saginata* but using pigs as intermediate host and parasitizing visceral organs (Galan-Puchades and Fuentes, 2009) is forcing us to rethink the current methods of control (Ito et al., 2006).

The consumption of raw and undercooked meat containing the infective cysticerci results in the completion of the *Taenia spp*. lifecycle (Fan et al., 1992). Intestinal taeniasis are generally mild but reports of massive *T. saginata* specimens causing obstruction are not uncommon (Bordon, 1992; Karanikas et al., 2007). The experience of motile proglottids crawling out of the anus often causes psychological distress (Muller, 2001). Control measures rely heavily on abattoir inspection for the presence of cysticerci or 'measley' meat (Scandrett et al., 2009) because to change eating habits is almost impossible as they are ancestral traditions rather than personal (Dupouy-Camet and Peduzzi, 2004). Though intestinal taeniasis due to *T. saginata* is not a cause for concern, the diagnosis of *T. solium* taeniasis is a medical emergency due to the possibility of autoinfection that may lead to the potentially fatal neurocysticercosis (Muller, 2001).

1.1.4 Neurocysticercosis

Neurocysticercosis is the commonest helminthic disease of the central nervous system and a leading cause of acquired epilepsy worldwide (Sinha and Sharma, 2009). In spite of its fatal prognosis it is potentially eradicable (Alarcon, 2006) and control has been successfully achieved in Europe and North America through improvement in sanitary conditions and functional abattoir control systems (Gonzalez et al., 2003). It is caused by infection with the larval stage of *T. solium* also known as *cysticercus cellulosae* mainly from food contaminated with pig faeces containing *T. solium* eggs (Roberts and Janovy, 2006). This is not surprising when humans live closely with pigs under poor sanitary conditions in the endemic areas of Mexico, South America and Asia (Muller, 2001). The main symptoms include seizures and space occupying lesion symptoms due to raised intracranial pressure and unless treated this infection is almost always fatal (Sinha and Sharma, 2009).

1.1.5 Cystic echinococcosis

Although not essentially confined to the tropics, echinococcosis has been listed among the neglected tropical diseases which warrant serious attention (WHO, 2006). Cystic echinococcosis with an approximate worldwide prevalence of 6 million (Siracusano et al., 2009) is caused by *E. granulosus* sensu stricto (sheep strain), now known to comprise 5 independent species (Nakao et al., 2007). Together with the sylvatic polycystic forms found exclusively in Central and South America, caused by either *E. vogeli* or *E. oligarthrus* the latter of which had been recently reclassified as unicystic (D'Alessandro and Rausch, 2008), echinococcosis remains as one of the most lethal helminthic zoonoses (Knapp et al., 2009). Humans become infected by handling infected dogs or items contaminated with their fresh faecal products (Thompson, 1986). As ruminants, especially sheep, are perfect intermediate hosts, it is mostly endemic in impoverished pastoralists community worldwide such as for example North Africa, Argentina and Central Asia (Muller, 2001) but echinococcosis has also been a problem in recent decades among pastoral communities in the islands off the coast of Scotland and in Wales. The majority of cysts develop in the liver followed by the lungs (Bal et al., 2008) but rarely in other organs such as the heart and brain (Aleksic-Shihabi and Vidolin, 2008). Apart from exerting pressure as the cyst enlarges within the organs that are invaded, the infected host is at risk of anaphylaxis when there is spontaneous rupture of the cyst as can occur under particular circumstances (Castanares-Zapatero and Laterre, 2009).

1.1.6 Alveolar echinococcosis

In contrast to the well encapsulated and benign cyst of *E. granulosus,* alveolar echinococcosis caused by *E. multilocularis* is aggressive, invading adjacent structures like a cancer (Muller, 2001) and in fact is often misdiagnosed as liver carcinoma (Stefaniak, 2006). This is essentially a sylvatic species with transmission involving wild canid species such as foxes as definitive hosts and wild rodents as intermediate hosts (Roberts and Janovy, 2006). There is also increasing evidence of domestic dogs playing an important role in its life cycle (Antolova et al., 2009). It is prevalent in communities in the northern hemisphere where hunting and contact with wild canids are inevitable (Deplazes, 2006). Despite this, there is increasing evidence that alveolar echinococcosis is becoming cosmopolitan due to foxes encroaching into urban perimeters (Kamiya, 2007).

1.1.7 Diphyllobothriasis

Diphyllobothriasis is the most important fish-borne zoonosis (Chai *et al*., 2005) with an estimated 20 million cases worldwide (Scholz *et al*., 2009). Traditionally endemic to Scandinavia, Russia and the great lakes of North America (Ruttenber *et al*., 1984), where currently incidence is decreasing, cases are emerging in Alpine lake regions of Central Europe (Dupouy-Camet and Peduzzi, 2004). It is caused by the pseudophyllidean cestode *Diphyllobothrium spp.* and is associated with the consumption of raw and undercooked fish, which are the second intermediate hosts containing the plerocercoid larvae (VonBonsdorff, 1977), such as sushi or sashimi in Japan (Arizono et al., 2009) and salted or marinated

raw fish dishes in Europe (Dupouy-Camet and Peduzzi, 2004). Fourteen species of the genus *Diphyllobothrium* are known to infect man with *D. latum* and *D. nihonkaiense* being the most important (Scholz et al., 2009) of which the latter is now recognized as a separate species from *D. latum* (Yamane et al., 1986). Apart from man as its definitive host, a wide range of piscivorous carnivores can also act as reservoir hosts (VonBonsdorff, 1977) possibly contributing to the continuation of its life cycle (Dupouy-Camet and Peduzzi, 2004). The adult worm, which is possibly the largest common parasite of man, has been reported to grow to up to 25m in length with 4,000 proglottids comprising the strobila (Scholz et al., 2009). The adult worm which resides in the small intestine has an extraordinary affinity for vitamin B_{12} which it obtains by dissociation of the vitamin B_{12}-intrinsic factor complex and competitive absorption of dietary vitamin B_{12} often leading to megaloblastic anaemia in infected subjects (Vuylsteke et al., 2004).

1.1.8 Rare cases of cestode infections

1.1.8.1 Dipylidiasis

There are a number of rarer cestode species known to affect man and this includes species from the genus *Dipylidium* which are both found in dogs and cats as their definitive hosts. Infection is acquired through accidental ingestion of the flea intermediate host *C. felis* or *C. canis* which contains the cysticercoid larvae and mainly affects young children (Neira et al., 2008). Pathology is minimal if not asymptomatic and incidence is limited to sporadic case reports from cosmopolitan areas worldwide (Molina et al., 2003).

1.1.8.2 Sparganosis

Another important infection to note is sparganosis which arises through tissue infection of the genus *Spirometra* and other non-human *Diphyllobothrium* species, mainly occurring in the far east (Muller, 2001). Drinking water contaminated with cyclops harboring the infective procercoid larvae was found to be an important risk factor apart from other modes of infection such as eating frog and snake meat or the traditional practice of poultice where dried parts of snake or frog is put in contact with the mucous membrane like the conjunctiva (Wiwanitkit, 2005).

1.1.8.3 Coenurosis

Coenurosis is one of the rarer forms of cysticercosis caused by infection with the larvae of *Taenia multiceps,* also known as *Coenurus cerebralis,* resulting in cystic lesions in the brain. Reported cases are rare but often lethal without surgical intervention (Benifla *et al*., 2007). Wild canids complete the sylvatic cycle and involve a wide range of wild rodent intermediate hosts (Muller, 2001).

1.1.9 Cestodes of veterinary importance

1.1.9.1 Cestodes of small ruminants

While gastrointestinal nematodes cause significant losses in the livestock industry (Nieuwhof and Bishop, 2005), the pathogenecity of cestodes in small ruminants is continuously being debated (Schoenian, 2009) because there is a steady prevalence of infection in many places (Moazeni and Nili, 2004; Panitz *et al*., 2009). Nevertheless, extreme monieziosis has been known to cause intestinal obstruction (van Schalkwyk *et al*., 2005). While most agree that *Moniezia expansa* infection in sheep are not associated with productivity loss (Elliott, 1986; Love, 2007; Mason *et al*., 2002), a productivity trial using 300 undrenched Romney lambs in New Zealand saw a significant weight gain in the group treated with praziquantel + levamisole compared to the groups treated with levamisole only or untreated controls (Southworth *et al*., 1996). A more recent report from Kenya showed that the intensity of *M. expansa* infection in goats was associated with poor body condition and hence productivity loss (Kanyari *et al*., 2009). Small ruminants like sheep and goats are important intermediate hosts to *E. granulosus* causing cystic echinococcosis and *T. ovis* causing ovine cysticercosis (Smyth, 1994). Despite an overall falling trend in incidence of cystic echinococcosis in sheep worldwide (Cabrera et al., 2003; Singh and Dhar, 1988), this parasite still continues to affect the sheep industry in many different regions (Benner *et al*., 2010; Kassem, 2006; Theodoropoulos *et al*., 2002).

1.1.9.2 Cestodes of large ruminants

The most important cestode affecting large ruminants such as cattle is *T. saginata* which causes millions in lost profits due to condemned carcasses (Dorny and Praet, 2007; Jahed Khaniki *et al*., 2010). Cattle become the intermediate host of the larval *cysticercus bovis* when grazing on pasture contaminated with human manure (Smyth, 1994). Bovine cysticercosis is still prevalent in many parts of the developing world due to poor sanitary conditions but surprisingly is still a problem in Europe due to the use of sewage sludge to fertilise pastures (Cabaret *et al*., 2002; Dorny and Praet, 2007). Monieziosis on the other hand is only important in calves (Borthakur and Das, 2006).

1.1.9.3 Equine cestodes

It was not until the last decade that infection with the equine cestode *Anoplocephala perfoliata* was recognized as a significant cause of morbidity in horses due to the long held belief of its rather questionable pathogenicity and the unavailability of accurate diagnostic tools (Proudman and Trees, 1999). Intestinal problems may range from mild colic (Proudman *et al*., 1998) to localised necrosis (Rodriguez-Bertos *et al*., 1999). Recently, an ELISA based method has been made commercially available for diagnosis but as with other antibody based diagnostics, it only detects exposure rather than actual infection (Matthews et al., 2004).

1.1.10 Cestodes in companion animals

1.1.10.1 Feline cestodes

In companion animals like cats, prevalence of cestode infections is high especially in feral populations (Abu-Madi et al., 2009; Millan and Casanova, 2009). The commoner species affecting cats include *Taenia taeniaformis, Joyeuxiella pasqualei*, *Dipylidium caninum*, *Diplopylidium spp.* and *Diphyllobothrium spp*. Despite the high prevalence of infection, they rarely cause clinical disease (Conboy, 2009). However, there have been reports of intestinal obstruction (Wilcox et al., 2009) as well as unusual cestode infections in cats like *Echinococcus multilocularis* that are lethal to humans (Nonaka *et al*., 2008)

1.1.10.2 Canine cestodes

Dogs play a very important role as definitive hosts to many cestodes notably, *Echinococcus granulosus, Dipylidium caninum*, and *Taenia ovis* (Muller, 2001). However, as definitive hosts, clinical disease is rare (Smyth, 1994).

1.1.11 Controlling cestode infections

As with many other infectious diseases, effective and long term control of cestode infections depends on collateral strategies including education and sanitation (Muller, 2001), as evident for example in the west where cysticercosis has been virtually eradicated through improvements in sanitation and efficient meat inspection (Gonzalez et al., 2003).

1.1.11.1 Surgical intervention

Unlike in the case of gastrointestinal nematodes where control is principally based on chemotherapy, surgery plays an important part in the management of most cestode infections mediated by larval stages. Symptomatic neurocysticercosis often requires surgery alongside chemotherapy and treatment with corticosteroids due to space occupying lesions in the central nervous system (Sinha and Sharma, 2009). Coenurosis, a rare zoonoses affecting the brain, is often lethal without surgery (Benifla et al., 2007). Large cystic echinococcosis will need specialized surgery as the threat of anaphylaxis is not uncommon (Castanares-Zapatero and Laterre, 2009). However, smaller uncomplicated cysts may just benefit from albendazole therapy (Stojkovic et al., 2009). Alveolar echinoccosis often needs radical resection of the liver and only in palliative cases does chemotherapy play some role (Stefaniak, 2006). In the special case of sparganosis, surgery is the only known treatment (Muller, 2001).

1.1.11.2 Arthropod control

Unlike in soil-transmitted gastrointestinal nematodes, where grazing management involves rotation of pastures and mixed species flocks/herds to limit transmission to livestock (Stepek et al., 2006a), control of exposure to the *oribatid* mite intermediate associated with ruminant cestode infections (Jimenez et al., 2007; Meana et al., 2005) is more difficult. It has been suggested that the application of acaricides on pasture may be an effective method for controlling moneziosis (Haq, 1998). Another example of arthropod control which would be useful in limiting cestode infections is the elimination of dog and cat fleas *Ctenocephalides canis* and *Ctenocephalides felis* that serve as intermediate hosts to *Dipylidium caninum*.

1.1.11.3 Immunotherapy

Although there are no available vaccines yet for humans against cestode infections, or in fact any helminth infections (Muller, 2001), indirect protection is now available through livestock vaccination against the metacestodes of taeniid and echinococcus species (Smith and Zarlenga, 2006). It was the recombinant antigen vaccine derived from the oncospheres of *T. ovis* that led to subsequent successes in *T. saginata, E. granulosus* and more recently *T. solium* and *E. multilocularis (*Lightowlers *et al., 2003)*

1.1.11.4 Synthetic chemotherapy

While the control of nematodes may rely on a wide variety of anthelmintic classes, cestodes are only susceptible to salicylanilides, isoquinolines and benzimidazoles (Muller, 2001; Roberts and Janovy, 2006; Smyth, 1994).

1.1.11.5 Niclosamide

Niclosamide is an effective intestinal taenicide under the class salicylanilide. It is widely used for intestinal taeniasis but is not effective against other helminths. It acts by inhibition of mitochondrial oxidative phosphorylation (Weinbach and Garbus, 1969). It is however not effective against larval tissue cestodes as it is not significantly absorbed from the gastrointestinal tract. It is also used as a molluscide in schistosomiasis control programmes (Muller, 2001).

1.1.11.5 Praziquantel

Originally jointly developed by Bayer and Merck in the 1970s as the much anticipated broad spectrum fluke treatment (Seubert et al., 1977), praziquantel remains as the primary treatment for schistosomiasis (Doenhoff et al., 2008). As part of its broad spectrum activity it is being used extensively for the treatment of other trematodes, and cestodes (King and Mahmoud, 1989). Its usage in treatment of cestode infections (Muller, 2001) includes tissue metacestode infections such as echinococcosis (Craig and Ito, 2007) and cysticercosis (Sinha and Sharma, 2009). Despite its poor water solubility and lipophilia, it is well absorbed from the GI tract (Cinto et al., 2009). Praziquantel is

known to induce muscle contraction and tegumental disruption that leads to surface antigen exposure to the host immune system (Tallima and El Ridi, 2007a) but the exact mechanism of action however, is still unclear. The only plausible theory to date is that it targets calcium channels causing a rapid influx of calcium ions and subsequently muscle contraction and damage. This idea, however, has been challenged by experiments using cytochalasin D in which praziquantel induced worm death and calcium influx are not correlated (Doenhoff et al., 2008).

1.1.11.6 Benzimidazoles

Benzimidazoles are effective against nematodes, cestodes and trematodes making this family of drugs the best choice as broad spectrum anthelmintics (McKellar and Scott, 1990). Benzimidazoles act by binding to the microtubular subunit protein β-tubulin disrupting microtubule structure and functions, which are essential components for cell integrity, mitosis, motility and active transport. When impaired by the drug, death of the organism follows soon (Lacey, 1988). In the treatment of cestodes, it is the drug of choice for the adjunctive treatment of alveolar echinococcosis (Mantion *et al*., 2008) and hydatid cyst (Junghanss *et al*., 2008) but has also seen an increase in use as a complete alternative to surgery in uncomplicated cases of cystic echinococcosis (Stojkovic *et al*., 2009). The resistance to benzimidazoles among veterinary nematodes has been identified as being mediated by the single amino acid substitution from phenylalanine to tyrosine in β-tubulin gene (Schwab et al., 2005) but no such mechanisms have been proposed with regards to resistance to among cestodes.

1.1.12 Problems associated with synthetic chemotherapy

Control measures are not without problems (Behnke *et al*., 2008) and the most important being the accelerated development and spread of drug resistance (Kaplan, 2004) which arises essentially from the recurrent excessive use of the main drug classes (Wolstenholme *et al*., 2004). Worsening the matter, resistance in one drug inextricably results in resistance to all the drugs with similar mechanisms of action within the same class (Michel, 1985). Unlike in nematodes which have reached a multiple drug resistance level (van Wyk *et al*., 1997), evidence of resistance in cestodes is limited. Nonetheless, a controlled slaughter trial using lambs in New Zealand revealed a lack of efficacy of praziquantel against *Moniezia expansa* suggesting resistance (Southworth *et al*., 1996). Another efficacy trial of albendazole and fenbendazole against *Moniezia expansa, Moniezia benedeni* and *Thysanosoma actinioides* in three farms in Bolivia revealed a disturbing trend of clear resistance to febendazole while albendazole was losing efficacy (Linares and Rojas, 2009). However benzimidazoles are not the treatment of choice for platyhelminth infections (Mansour, 2002). In humans, just as there have been reports of nematocidals losing efficacy (Albonico et al., 2003), an Ethiopian patient infected with *T. saginata* was unsuccessfully treated with niclosamide (Vermund *et al*., 1986). In view of the unlikelihood of a new synthetic anthelmintic in the near future, alternative strategies are urgently needed (Stepek et al., 2006a).

1.1.13 Usefulness of plant extracts as cestocidals

1.1.13.1 Earliest works

Since helminths have been known to afflict humans and livestock since antiquity (Cox, 2004), it might be expected that traditional remedies mostly from plant extracts have been used to treat them (Behnke et al., 2008). While ethno-medical evidence of use of plants as anthelmintics abounds (Prakash and Mehrotra, 1987), specific indications of use of cestocidals remain elusive apart from the use of powdered fern extract by the ancient Greeks (350-250 B.C.), mentioned in Nordic literature (Waller et al., 2001). Evidence of efficacy of plant extracts as cestocidals are rather recent compared to the earlier trials using ascarid models with fig species (Robbins, 1930), pineapple juice (Berger and Asenjo, 1939) and crystalline papain (Berger and Asenjo, 1940).

1.1.13.2 More recent works

One of the earliest supportive evidence was from experiments using the *H. nana* model and assessing the anthelmintic effects of whole extracts of coleus leaves and croton twigs (with ED_{100} values of 0.5ml of 42% in distilled water and 0.5ml of 72.46% in distilled water respectively)(He et al., 1992). One of the more convincing findings was the ability of chloroform extracted Indian mulberry fruit to significantly reduce egg counts and worm counts in *H. nana* infected mice with an ED_{50} value of 0.669g per kg body weight (Widdhiasmoro, 2000). The most convincing evidence by far is the 100% reduction in worm counts of *H. nana* in infected mice treated with a papaya seed infusion at a dose of 1.2g kg^{-1} BW for 3 consecutive days (Lamtiur, 2000).

1.1.13.3 More convincing evidence

Using murine cestode models, mature *Hymenolepis microstoma* incubated in papaya latex, ficin and stem bromelain showed a significant reduction in motility compared to controls *in vitro* (Stepek et al., 2007e). In screening the efficacy of various *Cassia* plant species for cestocidal activity Kundu and Lyndem (2012) observed through scanning electron microscopy studies tegumental damage on *Raillietina tetragona* that was comparable to those mediated by praziquantel.

1.1.13.3 Controversial evidence

Despite these promising results, there is also contradictory evidence. *H. microstoma* incubated in kiwifruit extract did not show a significant reduction in motility compared to controls (Stepek *et al*., 2007e). *H. nana* infected mice treated with *L. leucephala* seed infusion showed an intriguingly significant increase in *H. nana* population (Kustiawan, 2001). Treatment with juice from young pineapples was not effective *in vivo* in *H. nana* infected mice despite positive *in vitro* result (He *et al*.,

1992). *H. nana* infected mice treated with the lattices of *Ficus carica* and *Ficus insipida* failed to produce significant elimination of worms (de Amorin *et al*., 1999).

1.1.14 Cysteine proteinase as anthelmintics?

As the threat of drug resistance is real (Kaplan, 2004) and prospects of a synthetic anthelmintic are unlikely in the near future (Behnke et al., 2008), a potential alternative would be the use of natural plant latices and fruits containing the enzyme cysteine proteinase of which papaya, pineapple and fig species are good examples (Behnke *et al*., 2008). Reports of their use as anthelmintic by indigenous people go back more than a 100 years (Robbins, 1930) and they were even used in Europe decades ago (Jonxis and Bekius, 1953). Pilot observations demonstrated their ability to digest nematodes (Berger and Asenjo, 1939; Robbins, 1930) after which its enzymic basis was known (Walti, 1938). This was followed by the demonstrations of reductions in worm burden in pigs with *A. suum* (Satrija *et al*., 1994) and mice with *H. bakeri* (Satrija *et al*., 1995) and a clinical trial (Hansson *et al*., 1986). However, it was not until recently that these effects was proved to be mediated by the enzyme (Stepek *et al*., 2005). However, evidence demonstrating their wide spectrum of activity are mostly limited to gastrointestinal nematodes; *H. bakeri* (Stepek *et al*., 2005), *T. muris* (Stepek *et al*., 2006b) and *P. muricola* (Stepek *et al*., 2007b).

1.1.14.1 Cysteine proteinase

While we are still learning more about their many therapeutic use, cysteine proteinases may have evolved primarily as a defence mechanism against arthropod attacks (Konno *et al*., 2004) or even parasites where they have been shown to be effective against them (Stepek *et al*., 2007a). However, their more critical role in plant biology, such that in wound sealing, leaf senescence and ripening, should not be dismissed (El Moussaoui *et al*., 2001). Phylogenetically, cysteine proteinases that have been shown to have anthelmintic activity are classified under the papain family C1 and clan CA (MEROPS, 2009). At a molecular level, they work by the target polypeptide binding to the active site cleft where the important cysteine and histidine residues are present and mediate hydrolysis of the target (Behnke *et al*., 2008). (see Fig 1.1 (a) & (b))

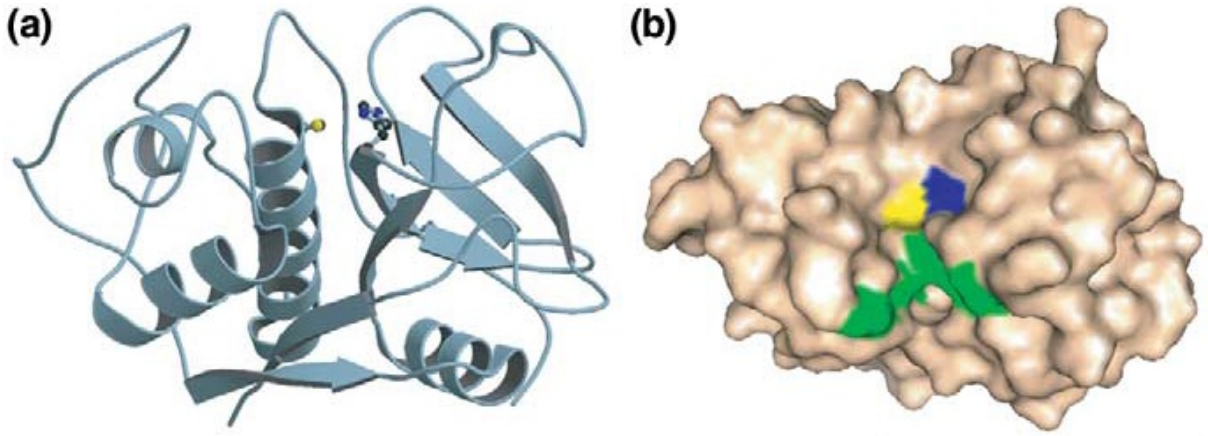


Fig 1.1 The three dimensional structure of papain*
(a) Ribbon diagram of a papain molecule. Cysteine (yellow) and histidine (blue) residues are shown in ball and stick representation (b) The three dimensional surface view of a papain molecule revealing the active site substrate-binding cleft, running from top to bottom. The main hydrophobic-binding pocket (green) where the side-chain of a hydrophobic amino acid in the substrate polypeptide binds. * adapted from (Stepek et al., 2004)

This property coupled with the fact that they are irreversibly inhibited on a 1:1 molar basis by trans-epoxysuccinyl-L-leucylamido-butane (E-64), makes quantification of enzyme activity possible through titration assays using commercially available substrates. The substrate benzoyl-arginyl-p-nitroanilide (Bz-Arg-pNA) is useful when used with papain, chymopapain, and crude papaya latex, while pineapple juice, kiwifruit extract, Ficin, *F. carica* and *F. benjamina* latex will cleave benzyloxycarbonyl-phenylalanyl-arginyl-p-nitroanilide (Z-Phe-Arg-pNA) (Stepek et al., 2005). Stem bromelain however binds reversibly (Ritonja *et al*., 1989) to the substrate benzyloxycarbonyl-arginyl-arginyl-p-nitroanilide (Z-Arg-Arg-pNA) (Stepek *et al*., 2007e). (see table 1.1)

Table 1.1 Naturally occurring cysteine proteinases and their plant source *

Plant species	Enzyme	Artificial substrate
C. papaya(papaya)	Papain	Bz-Arg-pNA
	Chymopapain	Bz-Arg-pNA
	Caricain	Bz-Arg-pNA
	Glycyl endopeptidase	Bz-Arg-pNA
A. comosus(pineapple)	Stem bromelain	Z-Arg-Arg-pNA
	Fruit bromelain	Z-Phe-Arg-pNA
	Ananain	Z-Phe-Arg-pNA
	Comosain	Z-Phe-Arg-pNA

A. chinensis (kiwifruit)	Actinidain	Z-Phe-Arg-pNA

*Adapted from (Stepek *et al*., 2004)

1.1.14.2 Cysteine proteinase as cestocidals?

The prospect of cysteine proteinase as a broad spectrum anthelmintic started with the observation of effects of pineapple juice on a porcine acanthocephalan (Berger and Asenjo, 1939) but this did not have the same effect using papain (Berger and Asenjo, 1940).

To date, the effects of cysteine proteinase affecting cestodes have not been fully evaluated and existing evidence of efficacy against cestodes is limited and to a certain extent controversial (de Amorin et al., 1999; He et al., 1992). The earliest work using cysteine proteinase on cestodes was that of pineapple juice on *H. nana* (He *et al*., 1992) which was successful *in vitro* but failed *in vivo*. More convincing evidence was published quite recently showing that *Hymenolepis microstoma* is susceptible to papaya latex, ficin and stem bromelain *in vitro* and tegumental damage occurs and can be detected by electron microscopy (Stepek *et al*., 2007e). Controversial evidence showed that kiwifruit extract did not affect *Hymenolepis microstoma in vitro* and trials using *Hymenolepis nana* models failed to reduce worm burden using *ficus* spp latex (de Amorin *et al*., 1999) and young pineapple juice (He *et al*., 1992).

1.1.15 Animal models used in this project

Although many different species of cestodes parasitize humans (e.g. *Taenia solium, T. saginata*) and livestock (e.g. *Monezia expansa* of sheep), most are host specific and cannot be grown in rodents. Experiments using livestock are expensive and are not permitted unless likely agents have been tested first in laboratory animals. Likewise, human trials would be unethical without evidence of efficacy and safety from animal trials. Laboratory facilities at Nottingham are suitable for rodent trials. For these reasons rodents and rodent helminths are the best starting point for this line of research. Furthermore, rodents are the lowest vertebrate group in which well characterised host-parasite relationships with minimal severity have been developed. So the rodent specific species are the best available models and certainly the best studied cestodes of all. *Hymenolepis diminuta* a natural parasite of rats (model of gut lumen cestodes) and *Hymenolepis microstoma,* a natural parasite of *Mus* spp. (model of cestodes that attach outside the gut lumen where its scolex is located in the bile duct in the liver) are both currently maintained in Nottingham and have been so since 1976 through the regular passaging of the rodent host and maintenance of the intermediate host *T. confusum.* Due to the availability of obtaining horse tapeworms from certain abbatoirs (Turner's abbatoir in Cheshire and Lawrence J. Potter's abbatoir in Somerset), the horse cestode *A. perfoliata* will also be used to test the efficacy of CPs. But this is limited only to *in vitro* experiments as I do not have license to conduct animal experiments involving horses.

1.1.16 Cestode biology

Cestodes belong to the phylum Platyhelminthes as they are dorsoventrally flattened. Cestodes possess a scolex which is the organ of attachment to the host which in the case of *H. diminuta* are unarmed but is well equipped with hooks in *H. microstoma.* Unlike trematodes, they do not possess any alimentary tract and nutrition is obtained by means of diffusion through their tegument and by active transport mechanisms in the tegument that selectively carry certain amino acids, sugars and other molecules internally. Unlike the tough cuticular layer of nematodes, the tegument is a syncitial ectocytoplasmic layer that facilitates the process of nutritive uptake through the presence of microtriches which increase the absorptive surface area, much like the microvilli of the intestinal mucosa (Holy and Oaks, 1989). It has been clearly demonstrated in nematodes that cysteine proteinase exert damage to the cuticles as early as 30min (Stepek *et al*., 2005) by initially causing it to crinkle before the whole architecture is lost eventually. At the weakest point the high hydrostatic pressure within the pseudocoelomic cavity bursts open the worm, releasing internal organs and this results in death of the organism (Behnke *et al*., 2008). On further incubation of nematodes they become completely digested (Berger and Asenjo, 1939, 1940; Robbins, 1930). However, the exact proteolytic mechanism and target proteins on the cuticle are still not known (Behnke *et al*., 2008). It is therefore quite reasonable to suspect that the cestode tegument might also be the target site of damage by cysteine proteinase, based upon limited preliminary observations in *H. microstoma* (Stepek *et al*., 2007e) and *H. diminuta* (Bolton, 2007). However, it is quite premature to make any conclusions about this since the tegument is a completely different structure from that of the cuticle and hence the basis of the current project was to clearly demonstrate firstly whether or not cestodes are susceptible to the effects of cysteine proteinse and then to elucidate the mechanistic basis of this process. With respect to reproduction, cestodes are hermaphroditic and their functional reproductive organs of both sexes are contained in the mature proglottids which are shed as they become gravid after cross fertilization from other proglottids or self fertilization (Muller, 2001).

1.1.17 Murine cestode life cycle

The definitive host of *H. diminuta* is the rodent *R. norvegicus* but it can also grow in other rodents such as hamsters and *Mus* spp. but in most abnormal hosts destrobilisation occurs after a few days of infection, rendering the worms sterile (Smyth, 1994). Although rare, they have also been reported to infect humans (Wiwanitkit, 2004). In the natural environment, eggs released together with gravid proglottids in the faeces of the infected rodent will be eaten by the intermediate host. The most important intermediate hosts are the flour beetles *Tribolium confusum* and *Tenebrio molitor*. In the intermediate beetle host, the hexacanth embryo within the egg hatches and migrates to the haemocoele cavity of the beetle where it differentiates and then lies dormant until the insect is consumed by a suitable mammalian host. When a suitable host accidentally ingests the infected beetle, the cysticercoids become activated on stimulation by the correct temperature, Ph and proteolytic enzymes in the stomach. When this is followed by exposure to bile salts and trypsin in the small intestine, they

excyst . The newly excysted scolex then attaches itself to the intestinal mucosa where it matures into adulthood and reproduction (Smyth, 1994). *H. microstoma* a cestode of *Mus* spp. shares the same life cycle as *H. diminuta.* However, *H. microstoma* occupies an extra-intestinal location namely the common bile duct and extra hepatic ducts (Moss, 1971).

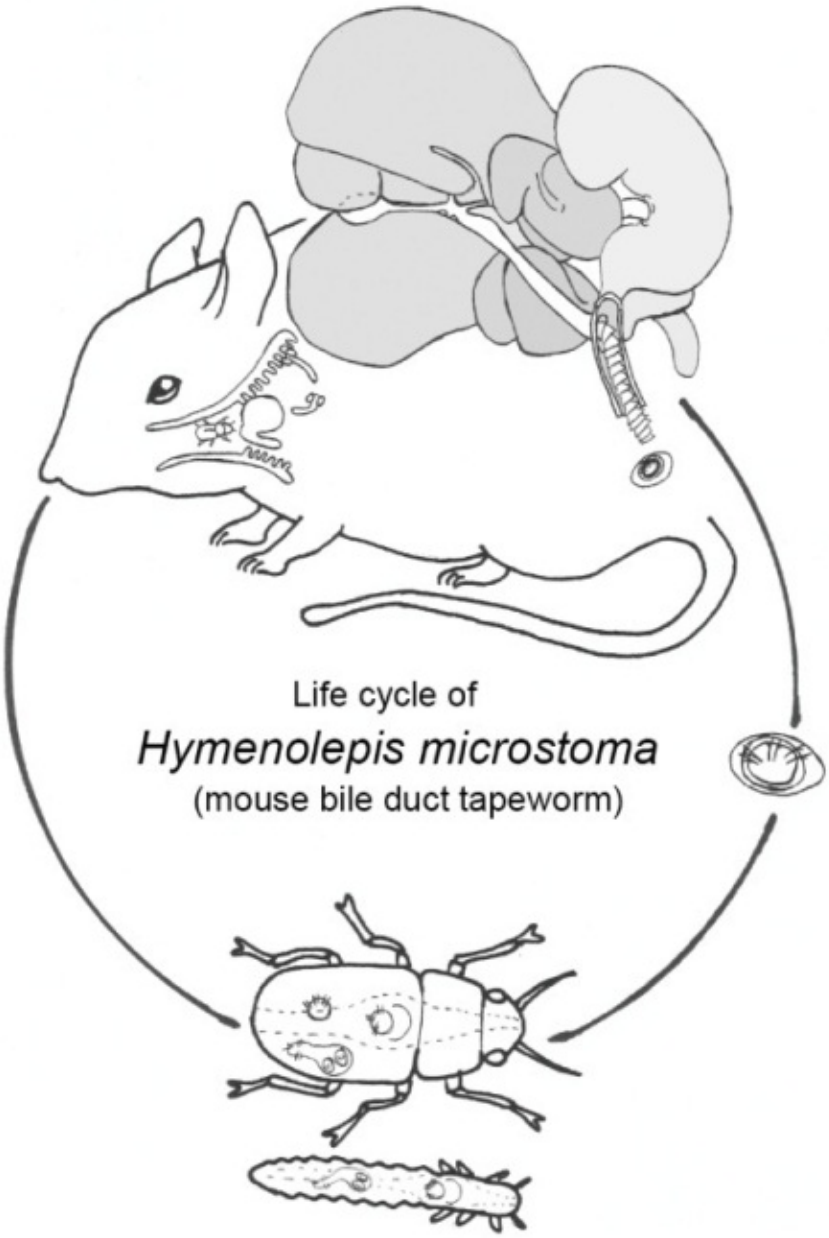


Fig 1.2 The life cycle of *H. microstoma* (Cunningham and Olson, 2010).

1.1.18 Equine cestode lifecycle

Recently equine cestode infections was recognized as a significant risk factor for the problem of intestinal colic in horses (Proudman et al., 1998). Three species of cestodes parasitize the intestines of horses with *Anoplocephala perfoliata* being the most prevalent from the rarer *Anoplocephala magna* and *Paranoplocepahala mamillana* (Gasser et al., 2005). All species exploit the oribatid mite species which infests pastures as their intermediate host.

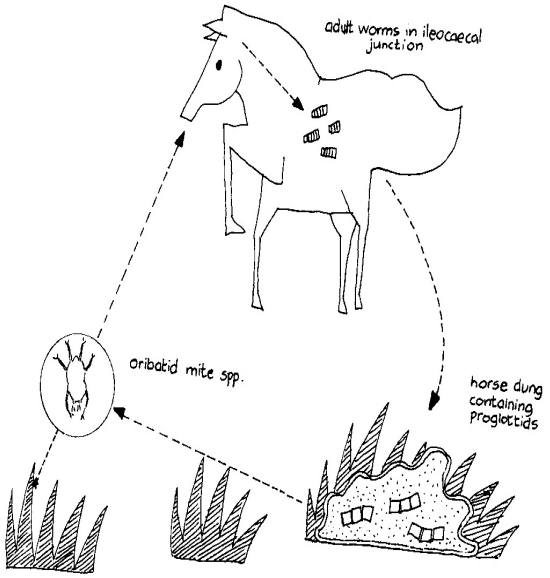


Fig 1.3 The lifecycle of equine cestodes

1.2 Aims and objectives

It has been clearly demonstrated in nematodes that CPs exert damage to the cuticles by hydrolysing certain proteins present in the nematode cuticle before the whole architecture is lost at the weakest point, causing the high hydrostatic pressure within the pseudocoelomic cavity to burst open the worm usually releasing internal organs and resulting in death of the organism (Behnke et al., 2008). On further incubation the nematodes become completely digested (Berger and Asenjo, 1939, 1940; Robbins, 1930). However, the exact proteolytic mechanism and target proteins on the cuticle remain unknown (Behnke et al., 2008). It is therefore quite reasonable to suspect that the cestode tegument might also be the target site of CPs based upon limited preliminary observations in *H. microstoma* (Stepek et al., 2007e). However, this remains speculative since the cestode tegument is completely different in structure from that of the nematode cuticle.

The experiments described in this thesis address the key hypothesis that cestodes are susceptible to digestion by CPs, and that therefore naturally occurring CPs from plants and their fruits constitute a source of novel anthelmintic drugs for the treatment of cestode infections in people and in our domestic livestock.

Using *H. diminuta* and *H. microstoma* as main laboratory model systems I aim to:

- clearly determine first whether or not adult cestodes are susceptible to the effects of CPs *in vitro*.
- describe in detail the nature of the damage taking place in the tegument after exposure to CPS, through careful observation of the changes occurring as evident by scanning (SEM) and transmission electron microscopy (TEM).
- determine whether or not the oral administration of CPs reduces parasite burdens as reflected in changes in faecal egg counts and worm burdens recovered at autopsy
- assess whether or not the juvenile stages of cestodes are affected by CPs by using an assay in which freshly *in vitro* hatched cysticercoids will be exposed to CPs. Scoleces of cestodes are known to be considerably more resilient in the presence of anthelmintics so this will be a particular challenge and there is contradictory evidence on the ability of CPs to affect juvenile stages of *C. elegans* (Behnke et al., 2008).
- assess the efficacy of CPs against cestodes of horses*, s*ince cestode infections are particularly pathogenic in these animals (Proudman and Trees, 1999), and resistance to the current anthelmintic is a big problem in the equine livestock industry (Matthews et al., 2004).

In all this work, I will explore the effects of natural plant products from sources such as for example papaya latex, extracts from pineapple as well as commercially available purified cysteine proteinases such as papain and stem bromelain.

CHAPTER 2

Materials and methods

2.0 Summary

This chapter details the general materials and methods used throughout the thesis. It is important, however, to refer to specific experiments for detailed experimental design and results.

2.1 Enzyme preparations

The enzymes used for *in vitro* experiments were in the supernatant of papaya latex extract (PLS) or pineapple extract, purchased papain and stem bromelain provided from Hong Mao Biochemicals Co. Ltd., while the enzymes used for *in vivo* experiments were crude papaya latex (CPL) and PLS only.

2.1.1 Crude Papaya Latex

The same CPL (catalogue no P3250, Sigma-Aldrich UK) used in previous published works (Stepek et al., 2005; Stepek et al., 2007a; Stepek et al., 2006b, 2007b, c, e) was used in the work described in this thesis. Five mg of CPL were dissolved in 8mls of water as first described by Stepek *et al*. (2005). To measure the operational molar concentration of this preparation the amount of active CP present in a 1ml assay was determined by titration against the CP inhibitor E-64 (Sigma-Aldrich). This preparation was made fresh on the day of use and was only used in Experiment 1 in Chapter 5.

2.1.2 Papaya Latex Supernatant

Papaya latex was sourced from Enzymase (Brussells, Belgium; http://www.enzymase.com/). The latex was in the spray-dried form which had undergone several patented processes of mechanical filtration under lower temperatures to remove impurities and contaminants before finally being spray-dried. Four kg of spray-dried papaya latex were dissolved in 12 litres of water. The solution was centrifuged at 17,700 *g* at 4 ºC (Beckman rotors, UK model J2-21) to obtain the supernatant. The papaya latex supernatant (PLS) was dialysed against polyethylene glycol 20,000 (VWR international) through dialysis membrane [MW Cut-off 3,500 (SpectraPOR® 45mm diam.)] concentrating it to a third of its original volume. The concentrated PLS was aliquoted into individual containers and frozen at -80 ºC. The operational molar concentration of active CPs for this preparation and throughout the purification process was determined by measuring the amount of active CPs present in a 1 ml assay through titration against the CP inhibitor E-64 (Sigma-Aldrich). The frozen PLS was thawed and diluted with Hanks' saline to the exact molar concentration of active CPs used in individual *in vitro* experiments or with water to the exact amount of active CPs present in a single dose for *in vivo* experiments. Part of this frozen PLS was freeze-dried using Chemlab instruments, England SB4 freeze-drier at 4°C. The freeze-

dried PLS was only used in Experiments 1&3 in Chapter 3. The frozen PLS however was used throughout the thesis. This PLS preparation was also used in recent publications (Buttle *et al*., 2011; Luoga *et al*., 2011).

2.1.3 Pineapple extract

Fresh and ripe pineapples *Ananas comosus* were purchased from Sainsbury's supermarket. The skin, leaves and stem were removed and the fruit portion was juiced using a Philips juicer HR1861. The juice was then filtered through Whatman filter paper under vacuum suction to remove impurities. The filtered juice was concentrated through dialysis against polyethylene glycol 20,000 (VWR international) through a dialysis membrane [MW Cut-off 3,500 (SpectraPOR® 45mm diam.)] All the purification steps were conducted in the presence of ice whenever possible, to avoid enzyme denaturation. No further extraction method such as for example acetone precipitation was applied because of the substantial loss of active CPs encountered during earlier attempts. The pineapple extract was then frozen at -80°C. The operational molar concentration of active CPs for this preparation and throughout the purification process was determined by measuring the amount of active CPs present in a 1 ml assay through titration against the CP inhibitor E-64 (Sigma-Aldrich). The frozen pineapple extract was thawed and diluted with Hanks' saline to the exact molar concentration of active CPs used in individual *in vitro* experiments. The pineapple extract was only used in the *in vitro* experiments described in Chapter 4.

2.1.4 Stem bromelain

Purified stem bromelain was sourced from Hong Mao Biochemicals Co., Ltd. (Rayong, Thailand). Two hundred gm of purified stem bromelain were dissolved in 1 litre of water. The solution was then centrifuged at 17,700 *g* at 4°C (Beckman rotors, UK model J2-21) to obtain the supernatant. The supernatant was frozen in -80°C. The operational molar concentration of active CPs for this preparation was determined by measuring the amount of active CPs present in a 1 ml assay through titration against the CP inhibitor E-64 (Sigma-Aldrich). The frozen stem bromelain was thawed and diluted with Hanks' saline to the exact molar concentration of active CPs used in individual *in vitro* experiments. Purified stem bromelain was only used in the *in vitro* experiments in Chapter 4.

2.1.5 Papain

The same purified papain (catalogue no P3125; Sigma-Aldrich UK) used in earlier publications (Stepek et al., 2005; Stepek et al., 2007a; Stepek et al., 2006b, 2007b, c, e) was used in the experiments described in this thesis. The lyophilized papain was dissolved proportionately in Hanks' saline to the exact molar concentration of active CPs required for individual *in vitro* experiments. Purified papain was only used in the *in vitro* experiments in Chapter 4.

2.1.6 CP active-site titration

The method of determining the operational molar concentration of active CPs was adapted from Zucker *et al*. 1985 using E-64 as titrants. Ten µl (depending on CP; neat for pineapple extract and 1:100 for PLS and stem bromelain) of the enzyme solution were added to 250 µl of 0.2 M sodium phosphate buffer/16mM L-cysteine (pH 6.85). From 0 to 100 µl of 20 µM E-64 were then added (11 tubes, 10 µl increments). 1mM EDTA was added to make up the remaining assay volume of 1ml (975 µl for PLS; 987.5 for pineapple extract and stem bromelain) before vortexing and incubating the mixture at 40°C for 15 min. Either 25 µl of 10 mM Bz-Arg-pNA for PLS or 10mM 12.5 µl of Z-Phe-Arg-pNA for pineapple extract or 10 mM 12.5 µl Z-Arg-Arg-pNA for stem bromelain were added making the final assay volume 1 ml. After 10 min at 40 °C the reaction was stopped by the addition of 1ml 0.1M sodium chloroacetate/ 0.2 M sodium acetate buffer (pH 4.3) (see appendix 1). The released 4-nitroaniline was determined by spectrophotometric absorbance measurement at 410 nm (Mole and Horton, 1973b). Since E-64 inhibition results in a stoichiometric inactivation of CPs on a 1:1 molar basis, the result was a linear fall in the concentration of 4-nitroaniline released with increasing concentrations of E-64. The intercept of the line with the abscissa denotes the molar concentration of active CPs present (see appendix 2 for an example of this).

2.1.7 Measurement of enzyme activity in the rodent GI tract

This protocol was modified from (Stepek *et al*., 2007b). Fifty µl sample from the mouse GI tract or 100 µl from the rat GI tract were added to 250 µl of phosphate buffer, pH 6.85, containing 16 mM L-cysteine with and without 20 µM E-64 (final volume of 1ml). After 15 min, the reaction was stopped by the addition of 1ml 0.1M sodium chloroacetate/ 0.2M sodium acetate buffer (pH 4.3) and the absorbance at 410 nm was measured on a spectrophotometer. Enzyme activity was calculated using a molar coefficient of 8800 $M^{-1}cm^{-1}$(Mole and Horton, 1973a). One unit of enzyme activity was defined as that which produced 1 nmol min^{-1} of product under these assay conditions. The addition of E-64 to half of the assays was necessary in order to distinguish between CP and trypsin which also cleaves Bz-Arg-pNA.

2.2 *In vitro* experiments

2.2.1 Animals

Male C3H mice from Harlan, U.K. and male BKW mice from B&K, U.K., were used for the production of *H. microstoma* worms for *in vitro* experiments. Male Wistar and Lister hooded rats from Charles River, UK were used for the production of *H. diminuta for in vitro* experiments.

2.2.1 Parasites

The parasite models used in this project were the rat tapeworm *H. diminuta*, the mouse tapeworm *H. microstoma* and the horse tapeworm *A. perfoliata*.

2.2.2 *H. diminuta* parasite production

Rats were infected with 50 *H. diminuta* cysticercoids dissected from infected flour beetles *T. confusum* which were suspended in 1 ml of distilled water. The rats were culled on day 14 of infection by an overdose of CO_2 and cervical vertebrae dislocation. The small intestine was removed and worms were flushed out with Hanks' saline using a 50 ml syringe. The flushed out worms were transferred into a collecting Petri dish containing pre-warmed at 37^{o}C Hanks' saline before being transferred into individual treatment wells for motility assays or imaging experiment.

2.2.3 *H. microstoma* production

Mice were infected with 12 *H. microstoma* cysticercoids, dissected from infected flour beetles *T. confusum,* which were suspended in 0.2 ml of distilled water. Mice were culled on day 14 of infection by an overdose of CO_2 and dislocation of cervical vertebrae. Because the scoleces of *H. microstoma* are mainly attached to the bile duct, the liver was removed first from its attachments to the diaphragm and other gut structures in order to expose an intact bile duct. The bile duct was then opened up carefully using sharp-ended dissecting scissors starting proximally from its base at the liver and then moving distally away from the liver towards the duodenum. This incision was then continued in both directions of the duodenum, one way towards the stomach and the other all the way towards the ileo-caecal junction. The opened intestine was then placed in a glass Petri dish containing pre-warmed Hanks' saline (37 ^{o}C) for 30 min to allow the worms to move away from the tissues. Worms were identified and transferred into another collecting Petri dish containing pre-warmed at Hanks' saline (37^{o}C) before being transferred to individual treatment wells for motility assay or imaging experiments.

2.2.4 Collection of *A perfoliata*

The horse tapeworm *A. perfoliata* was collected from Turner's abattoir, Cheshire and from the Lawrence J. Potter abattoir, Somerset. Upon receipt of the whole horse GI tract which was removed by slaughterhouse personnel, a small incision ~5 cm was made at the ileocaecal junction so as to create an opening. About 500ml of caecal fluid were collected and stored in a flask, the fluid acting as a maintenance medium during transportation of the worms to the laboratory. Worms attached to the mucosal surface of the intestine were removed and transferred into the flask containing the caecal fluid. The flask was then stored inside a polystyrene box pre-warmed with water bottles containing water which had been boiled earlier. Worms were then conveyed rapidly back to the University of Nottingham and transferred immediately into Hanks' saline at 37°C before commencing *in vitro* motility assays or imaging experiments.

2.2.5 Production of rodent cestode cysticercoids

Gravid proglottids at the terminal ends of the strobila (~2 cm) of either *H. microstoma* or *H. diminuta* were fed to *T. confusum* beetles which had been starved for at least 5 days. After a few days, when the proglottids have been consumed, the beetles were maintained with their regular diet of a 50/50 mixture of wholemeal flour and white flour with additional Brewer's yeast. After 30 days a few representative beetles from either infection were dissected to ascertain the presence of fully developed cysticercoids. Fully developed cysticercoids are ready for use in juvenile cestode motility assays by artificial excystation.

2.2.6 Artificial excystation of cysticercoids

This process was conducted based upon an amended protocol by (Goodchild and Davis, 1972). Dissected cysticercoids from infected *T. confusum* beetles were incubated in acid-pepsin solution pre-warmed at 37°C for 15min in the 37°C incubator. Acid-pepsin solution was then removed and washed 3x with Tyrodes' saline. Trypsin-tyroglycocholate solution, pre-warmed at 37°C, was added and incubated at 37°C for 5 min after which scoleces could be seen evaginating and leaving their cysts.

2.2.6 *In vitro* motility assays

For adult worm motility assays, BD Falcon™ 12 wells tissue culture plates were used with individual assay consisting of 2ml volume. For motility assays using juvenile artificially excysted scoleces, BD Falcon™ 48 wells plastic tissue culture plates were used with individual wells consisting of 0.5ml volume. Different concentrations of active CPs were prepared by proportionate combination of CP, Hanks' saline (see appendix 1) and L-cysteine. E-64 was also used as a control treatment to block the effects of CPs.

2.2.7 *In vitro* motility grading for adult worms

A semi-objective motility grading system was designed, based upon Stepek *et al*. 2005 in which incubated worms were observed and graded for their motility on a scale of 0 to 5.

Table. 2.1 Motility grading scale for adult cestodes

Score	Motility
0	Motionless
1	Motile only when prodded
2	Motile only at end parts of worm

3	Marked reduction in motility
4	Slightly less motile
5	Full motility

2.2.8 *In vitro* motility grading for artificially excysted scoleces

Due to the relative difference in motility compared to adult worms an amended grading system was formulated, based upon the system mentioned above for adult worms.

Table 2.2 Motility grading scale for artificially excysted scoleces

Score	Motility
0	Motionless
1	Motile only when prodded
2	Reduction in motility
3	Full motility

2.2.9 Data storage

Data from motility experiments were recorded manually during experiments and were then entered into Microsoft Excel 2010 documents using a Samsung laptop P510. Data was also converted to Graphpad prism files to generate figures.

2.2.10 Data analysis

Data from motility assays were analysed using repeated measures general linear models (rmGLM) using the statistical software SPSS (version 21.0). Time was fitted as a within subject factor while the concentration of active CPs was fitted as a between subject factor. Where the data did not meet the Mauchley's sphericity test the Huynh-Feldt adjustment was used to interpret data on the side of caution. In gauging variation in CP efficacy motility data at 30min incubation were used to generate concentration-inhibition curves in Graphpad prism 5 software.

2.2.11 Light microscopy imaging

Images of artificially excysted juvenile cestodes were digitally captured using MA88 300 Hirocam digital microscope eyepiece 3.0 Megapixels which was fitted to a light microscope viewed at

magnifications of x20. Digitally captured images was processed by the accompanying software TSview.

2.3 *In vivo* experiments

2.3.1 Animals

C3H mice from Harlan, UK and Wistar rats from Charles River, UK were used in the *in vivo* work described in this thesis. The animals were provided with water and food *ad libitum*. All animal procedures were carried out using UK Home Office license number 40/9618, 40/9617, 40/6326 and 40/1313 under the regulations of the Animals (Scientific Procedures) Act 1986.

2.3.2 Faecal egg counts

Rodent faeces were collected for egg counts using the McMasters flotation technique every other day for 3 days before treatment and every other day for 3 days during treatment. To collect individual faecal samples, animals were temporarily separated into individual cages without bedding for 2 hours, early in the morning, after which the faeces were collected and transferred into pre-weighed universals. During faecal collection, all animals had access to water but not food, which could have contaminated the faeces. The faecal samples together with their universals were then weighed and recorded. Ten ml of saturated sodium chloride solution were added to each universal and allowed to soak and to break up for 1h using a rotary mixer. For egg counting, each homogenised faecal sample was washed through a sieve with 50mls of saturated sodium chloride solution to remove excessive fibre and debris. Using a Pasteur pipette, a small volume was transferred out from the 60mls faecal-sodium chloride mixture, whilst maintaining a gentle stir of the solution, to ensure randomised dispersion of eggs. The sample solution was transferred into a 2-chamber McMaster slide with each chamber holding a volume of 0.15 ml. Using a light microscope at magnification of 10x, eggs that float within the designated grid lines were counted for each chamber. The mean value from both chambers counted was used to calculate the number of eggs per gram of faeces (EPG) using the following formula which is a modified formula (Behnke and Parish, 1979) originally developed for counting nematode eggs in sheep (Gordon and Whitlock, 1939).

$$\text{EPG} = \frac{\text{number of eggs counted}}{\text{weight of faeces}} \text{x} \frac{\text{total volume}}{\text{volume counted}} = 400 \text{ x} \frac{\text{mean number of eggs counted}}{\text{weight of faeces}}$$

2.3.3 Assessment of worm burdens and worm biomass

On the final day of each experiment all the animals were autopsied to ascertain worm burdens and worm biomass. The intestine was opened up using blunt ended scissors, starting from the lower half of the stomach right up to the caecum, concentrating mainly around the bile duct and duodenum where *H. microstoma* usually reside. The opened up intestine was then transferred into a Petri dish containing

pre-warmed Hanks' saline (37°C) to allow worms to detach from the tissues of the intestine. Individual worms were first identified by their scoleces under the light microscope before being counted and transferred into pre-weighed aluminium foil, that had been fashioned into cups. They were weighed in these cups and the weight of empty cups was subtracted to obtain the value for the wet biomass of the parasite burdens from individual mice. Apart from the wet biomass, the dry biomass was determined by baking the worms in an oven at 60°C continuously for 5-10 hours daily for 2 consecutive days, as opposed to the usual method of baking continuously for 24 hours (Hopkins et al., 1972b), followed by reweighing and subtraction of the weight of the empty cups. The two periods of drying were necessary to conform to local safety regulations aimed at avoiding a fire hazard, when could have occurred had the oven been left unattended overnight.

2.3.4 Induction of immunosuppression in mice

In order to achieve delayed rejection of *H. diminuta* in the non-permissive mouse host, mice were injected subcutaneously with dexamethasone (Voren^{TM} suspension Boehringer-Ingelheim, UK). Mice were also given oxytetracycline hydrochloride (Terramycin, Pfizer Ltd) in their drinking water at a concentration of 3g/litre for the duration of the experiment to prevent opportunistic infection during periods of immunosuppression.

2.3.5 Data storage

Data from faecal egg counts and autopsy indices were first recorded manually during experiments and were then entered into Microsoft Excel 2010 documents using a Samsung laptop P510. Data was also converted to Graphpad prism 5 files to generate figures.

2.3.6 Data analysis

Data from *in vivo* experiments were analysed using SPSS software (version 21.0). For Faecal egg counts data were analysed using repeated measures general linear models (rmGLM). In experiments assessing treatment efficacy the non-parametric tests, Mann-Whitney U and Kruskal-Wallis, were used depending on number of treatment groups being tested. In experiments assessing dose-dependency, data were analysed using Spearman's correlation test.

2. 4 Observations of changes and structural damage of cestodes by SEM

2.4.1 Preparation of fixation reagents

Worms were incubated in different treatments or control media at 37°C. At timely intervals of 10min, 20min and 60min one worm was removed and fixed in 2.5% glutaraldehyde for one hour. Glutaraldehyde was then replaced with 0.1M phosphate buffer pH 7.2 for another hour or overnight at

4°C. In the case of *in vivo* experiments, proglottid segments found in the colon were directly fixed in glutaraldehyde 2.5% and the same steps as detailed above were repeated.

2.4.2 Staining

Osmium tetroxide stain was prepared by breaking a 1 glass vial containing osmium tetroxide into a glass bottle containing 10ml 0.1M phosphate buffer, under the fume hood. Fixed worms in 0.1M phosphate buffer pH7.2 were stained in osmium tetroxide solution just enough to submerge the whole worm or segment for an hour. Osmium stained worms turn black in colour and the osmium solution was then removed. Stained worms were washed in distilled water 3 times before dehydration.

2.4.3 Critical point drying

On the day of drying, dehydrated worms or segments in 70% ethanol were removed and dehydrated further in 90% and 100% ethanol with 15min intervals in between. The dryer Polaron E1000 was adjusted to an optimal temperature of 18-20°C by running tap water through the system. The dryer was also tested for leaks. The specimens were placed in wire mesh baskets which was placed into a metal container filled with 100% ethanol. The metal container was then placed into the drying chamber and was tightly closed. The pressure was turned up to approximately 800 bars so that the CO_2 was liquefied and entered the chamber. The meniscus (the level of liquid CO_2 visible) then rose to the top. Ethanol was released by opening the ethanol valve for 20 seconds and the CO_2 valve was opened so that liquid CO_2 would replace the ethanol within the worm. This process was repeated several times until no more wet ethanol could be detected upon releasing the ethanol valve. The hot water bath was now turned on to reach a temperature of 40°C. The hot water was run through the system until the critical point drying had been achieved, when the meniscus disappeared.

2.4.4 Sputter coating with gold

Dried worms were mounted onto aluminium stubs with the use of quick drying silver paint (Agar scientific ltd, Essex, UK). The mounted worms on stubs were placed onto the disc in the Polaron E5100 sputter coater. The pump was turned on to create a vacuum and left for 10min. When the Argon indicator rose and passed the mark indicating a vacuum, the right knob was set to HT. Then the voltage (middle knob) was set clockwise to 2.5 volts and was left to rise until 18-20 volts and then left for about 60 seconds for coating to occur. Then the voltage was turned off and the vacuum was released. The gold coated worms were then ready for viewing under SEM.

2.4.5 Scanning electron microscopy (SEM)

Gold coated worms were viewed using JEOL JSM-840 scanning electron microscope.

2.5 Observation of changes and structural damage of cestodes by transmission electron microscopy (TEM)

2.5.1 Fixation

Adult worms were incubated in different treatments or control wells at 37°C. At timely intervals of 10min, 20min and 60min 1 worm was removed and immersed in 2.5% glutaraldehyde for 5min before being taken out again. The partially fixed worm was inspected under light microscopy for signs of damage. Where damage on the tegument was apparent, a cross sectional incision was made with a sharp blade and another cut was made adjacent to the first cut, practically reducing the worm to a mere ~5mm x5mm segment of interest and the rest of the worm was discarded. The segment of interest was again fixed in 2.5% glutaldehyde for 1 hour. Glutaraldehyde was then replaced with 0.1M phosphate buffer pH 7.2 and left again for another hour or overnight at 4°C.

2.5.2 Staining

Osmium tetroxide stain was prepared by breaking 1 glass vial containing osmium tetra oxide into a glass bottle containing 10ml 0.1M phosphate buffer under the fume hood. Fixed segments in 0.1M phosphate buffer pH 7.2 were stained in osmium tetroxide solution, just enough to submerge the whole segment for an hour. Osmium stained segments turn black in colour and the osmium solution was then removed. Stained segments were washed in distilled water 3 times before dehydration.

2.5.3 Dehydration

Dehydration with ethanol was carried out by adding 30% ethanol. This process was repeated with 50% ethanol with 15min duration in between, before finally leaving the worms at 70% ethanol for an indefinite period.

2.5.4 Embedding in resin

Spurr's resin was prepared by mixing 10g ERL 4206 (3,4 Epoxy Cyclohexyl Methyl 3,4 epoxy cyclohexyl carboxylate), 6g DER 736 (Diglycidyl ether of polypropylene glycol), 26g NSA (Nonenyl succinic anhydride) and 0.4g DMAE (Dimethylaminoethanol) in the fume cupboard. The resin solution was then refrigerated overnight at 4°C. On the day of embedding, dehydrated worms in 70% alcohol were dehydrated further in 90% ethanol and then in 100% ethanol. Fresh resin was poured into mould blocks (1 block per sample) until level with the top of each mould. The mould was then kept at 4°C overnight. The hardened resin block was sectioned using a Reichert Ultracut microtome.

2.5.5 Staining the sections

The sections were stained in lead citrate and uranyl acetate before viewing.

2.5.6 Transmission electron microscopy (TEM)

Lead citrate and uranyl acetate stained sections were viewed under JEOL 1010 transmission electron microscope.

CHAPTER 3

Thermostability profile of papaya latex supernatant

3.0 Summary

Papaya latex supernatant (PLS) is a partially refined form of papaya latex which was used in recent publications (Buttle *et al*., 2011; Luoga *et al*., 2011) and throughout the experiments reported in the following chapters of this thesis. In order to develop PLS into a useful anthelmintic it is important for it to be thermostable, and although the thermostability profiles of CPs have been well documented in earlier publications, nevertheless it was important here also to establish clearly the rate at which activity is typically lost in CP preparations held in various storage conditions for use in the present program of research. In this chapter PLS was found to be stable for almost 1 year when frozen or if freeze-dried and refrigerated. It was also stable at 37 ºC for the entire 2 hours duration of typical *in vitro* motility assays. Active PLS was detectable along the whole gastrointestinal tract of both rodent model systems used in this project; *M. musculus* and *R. norvegicus*, and it was confirmed that the levels of activity dropped rapidly in the stomach and upper small intestine whilst increasing in the large intestine and accumulating over a period of 2-3 hours post oral administration.

3.1 Introduction

Plant products from pineapple (Berger and Asenjo, 1939), papaya (Berger and Asenjo, 1940) and figs (Robbins, 1930) have been shown to possess anthelmintic properties and the active principles mediating the effects have long been identified (Walti, 1938) but only recently confirmed to be CP (Stepek *et al*., 2005). In order to develop natural plant products into useful anthelmintic agents, isolation and characterisation of their active principles are essential (Behnke *et al*., 2008). However, the processes of isolation and purification of proteins are often detrimental to their biological activity and structure (Arakawa *et al*., 2001) rendering them thermo specific and heat labile (Somero, 1995). Many factors may contribute to protein inactivation but most notably extremes of temperature and pH.

Heat denaturation of proteins is well-known and starts with reversible structure unfolding and later due to prolonged heat exposure leads to permanent structural change such as aggregation and mispairing of thiol groups (Roy and Gupta, 2004). Protein inactivation due to cold denaturation is equally important as most purified proteins are often freeze-dried for longer shelf life and logistics. Cold denaturation is however a multifaceted phenomenon centred around ice crystal formation with the resulting abrupt changes in solute concentration and pH (Arakawa et al., 2001; Cao et al., 2003; Roy and Gupta, 2004) and due to physical disruption by ice crystals.

Various methods of quantifying enzyme activity have been developed ranging from classical substrate labelled chromogenic assays (Goddard and Reymond, 2004) to real-time assays (Konarzycka-

Bessler and Bornscheuer, 2003). Specific inhibition of CPs by E-64 has enabled convenient chromogenic based assay through titration (Zucker *et al*., 1985) which have been used to date throughout all the work on CP anthelmintic development starting from crude papaya latex (Stepek et al., 2005; Stepek et al., 2007a; Stepek et al., 2006b, 2007b, c, e) to the more refined papaya latex supernatant (Buttle *et al*., 2011; Luoga *et al*., 2011). Due to its commercial value the thermostability profile of papaya latex proteases have been investigated by many of which most observed an obvious heat lability (Chaiwut, 2007; Hinkel, 1951; Ortiz *et al*., 1980). Within the context of anthelmintic development CPs have been shown to survive stomach acidity to reach the colon (Stepek et al., 2007b) to affect *T. muris* (Stepek et al., 2006b) despite controversial *in vitro* work suggesting irreversible damage to papain subjected to low pH (Huet *et al*., 2006). The aims of this chapter are to investigate the thermostability of PLS because it is being used throughout the thesis and questions of whether the enzyme is still active or not after storage, freeze-thawing and even as it is being administered in the animal is obvious. In this chapter I report experiments in which I have investigated the basic thermostability profile of PLS within the context of the question .

3.2 Experimental design and results

3.2.1 Experiment 1

Processing PLS

3.2.1.1 Experimental design

This experiment was conducted primarily for the papaya latex used in the sheep trial at the Moredun Institute (Buttle *et al.*, 2011). Papaya latex was sourced from Enzymase (Brussells, Belgium; http://www.enzymase.com/). This enzyme is in the spray-dried form which had undergone several patented processes of mechanical filtration under lower temperatures to remove impurities and contaminants before finally being spray dried and is also known as Papain P1. For this experiment 4 kg of spray-dried papaya latex was reconstituted into solution by addition of 12l distilled water. The papaya latex solution was centrifuged at 17,700 x *g* at 4 ºC to obtain the supernatant. The papaya latex supernatant (PLS) was dialysed against polyethylene glycol 20,000 through dialysis membrane MWCO 3500 SPECTRA/POR® 7, concentrating the enzymes and removing water to facilitate freeze-drying and logistics. The resultant dialysed PLS was then frozen at -80 ºC before being freeze-dried in a SB4 freeze-drier (Chemlab, England). The amount of active CP present in a 1ml assay (see chapter 2 for methodology) was determined in the different forms of processed papaya latex as it underwent refinement from solution to the final freeze-dried product.

3.2.1.2 Results

There was no loss of active CP during centrifugation of papaya latex solution to obtain the supernatant. However, during dialysis of papaya latex supernatant there was a 33.9% loss of active enzymes (Fig 3.1). There was also further loss of active CP (33.8%) when the dialysed PLS was frozen at -80°C (Fig 3.1). During freeze-drying however, the amount of active enzymes actually increased by 20.2% (Fig. 3.1).

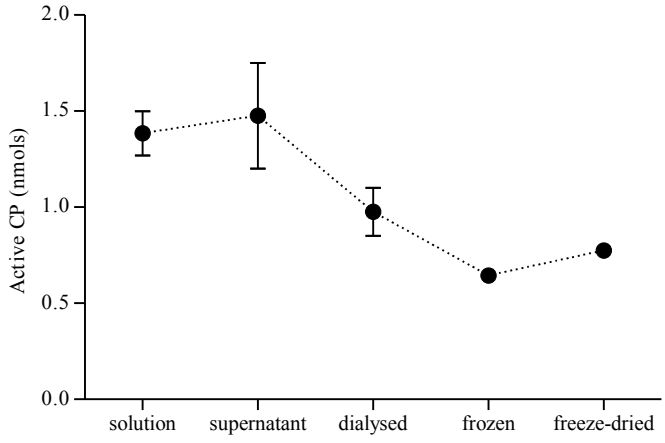


Fig 3.1 PLS processing.
Amount of active CPs in different forms of processed papaya latex as it underwent refinement from solution to the final freeze-dried product. The amount of active CPs expressed in nmols are the molar equivalent of the inhibitor E-64 used to block active CP in a 10µl volume in a titration assay (see chapter 2). The solution was made by reconstitution of spray dried papaya latex from Enzymase (Brussels, Belgium) with water at a ratio of 1:3. The solution was then centrifuged at 17,700 *g* at 4°C to obtain the supernatant. Papaya latex supernatant (PLS) was then dialysed against polyethylene glycol to concentrate active CPs. The concentrated PLS was frozen at -80°C before finally being freeze-dried ready for use in the sheep trial. Note the 33.9% loss of active CPs during dialysis and also the further 33.8% loss during freezing. Freeze-drying however saw a 20.2% increase in active CPs. Error bars represent the standard error of the mean.

3.2.2 Experiment 2

The stability of PLS during storage

3.2.2.1 Experimental design

This experiment was carried out to determine the stability of PLS which was used throughout the thesis in terms of amount of active CP retained or lost when stored at different storage conditions against time. Papaya latex was processed in the same manner as in Experiment 1 starting from reconstitution of the same spray dried powder from Enzymase (Brussells, Belgium) into solution before centrifugation to obtain the supernatant. Unlike in Experiment 1 here PLS was not dialysed because there was no need to

concentrate the enzymes for logistical reasons and was immediately aliquoted into small 1.5ml micro centrifuge tubes in a batch of 5 for each storage conditions ; ambient temperature, 4ºC, -20 ºC and -80 ºC. Each vial receiving 1ml of the solution. The amount of active CP present in a 1ml assay (see Chapter 2 for methodology) from the samples from different storage conditions were determined at 1 week, 1 month, 6 months and 1 year post processing.

3.2.2.2 Results

The original amount of active CPs in this PLS preparation (post centrifuge) was 0.825nmols (the amount of active CP present in 1ml assay). This amount then declined by around 70.1% (Fig 3.2) from its original value after 1 week of storage at ambient temperature after which rather gradual further loss of active enzymes commenced until only 1.3% (Fig 3.2) of the original amount of active CPs remained at 1 year. When stored in the refrigerator at 4ºC the rate of loss was rather gradual with an initial loss of 3.0% after 1 week followed by 15.2% after 1 month and 39.4% after 6 months (Fig 3.2). After 1 year storage at 4ºC, the amount of active CPs has been reduced by 71.2% from its original value (Fig 3.2). When PLS was frozen in the freezer at -20 ºC the loss was rather minimal with an initial loss of about 9.1% after which the subsequent loss of 15.2% after 1 month stabilised up to 1 year. Similarly when PLS was deep frozen at -80 ºC the amount of active CP stabilized at around 80% of the original starting value and stayed at this level without further loss for up to 1 year post processing.

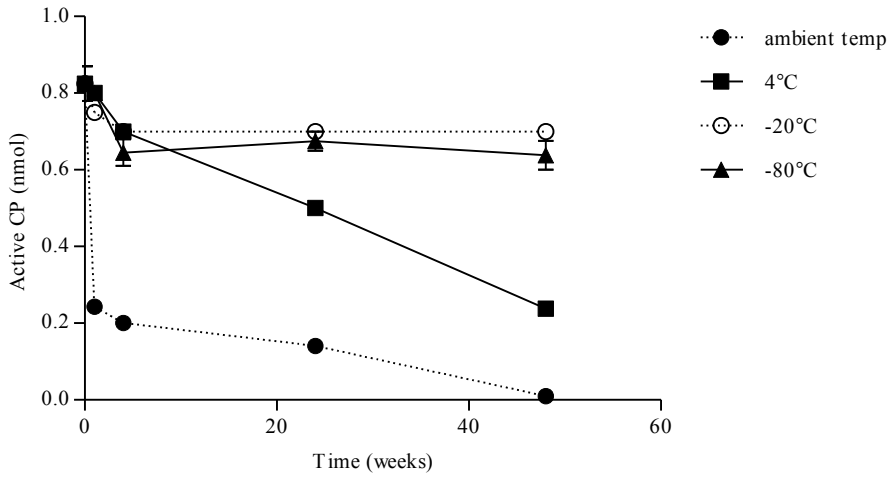


Fig 3.2 PLS storage stability
Amount of active CP in post centrifuge PLS as it was being stored at different temperature conditions at different time points. The amount of active CPs expressed in nmols are the molar equivalent of the inhibitor E-64 used to block active CP in a 10µl volume in a titration assay (see chapter 2). Note that the amount of active CPs decreased when PLS was stored at ambient temperature and at 4ºC. The amount of active CPs was relatively preserved when PLS was stored at lower temperatures such as -20 ºC and -80 ºC. Error bars represent the standard error of the mean.

3.2.3 Experiment 3

Thermostability of freeze-dried PLS

3.2.3.1 Experimental design

This experiment was basically an extension of Experiment 2 assessing enzyme stability of papaya latex in the freeze-dried form. For this, data were collected from 2 different papaya latex enzyme preparations intended for use in 2 different livestock trials; the sheep trial in Moredun Institute, Scotland of which the results have been published recently (Buttle et al., 2011) and the pig trial in University of Ghent, Belgium which is still an on-going trial. In both preparations, papaya latex were sourced from Enzymase (Brussels, Belgium) and were processed the same way (see chapter 2 for methodology) until they were in the freeze-dried form.

3.2.3.2 Results

The process of freeze-drying alone caused loss of active enzyme by 10-15% in both preparations (Fig 3.3). After 1 week the amount of active enzyme in the freeze-dried PLS stored at ambient temperature deteriorated further by about 30% from the original level after which it stabilized (Fig 3.3). After 1 year of being stored at ambient temperature, the amount of active enzymes retained was just 50% from the original level (Fig 3.3). When freeze-dried PLS was stored at 4ºC however, apart from the initial loss following the freeze-drying process, the amount of active enzymes present was relatively preserved with just about 15% loss from the original level up to 1 year (Fig 3.3).

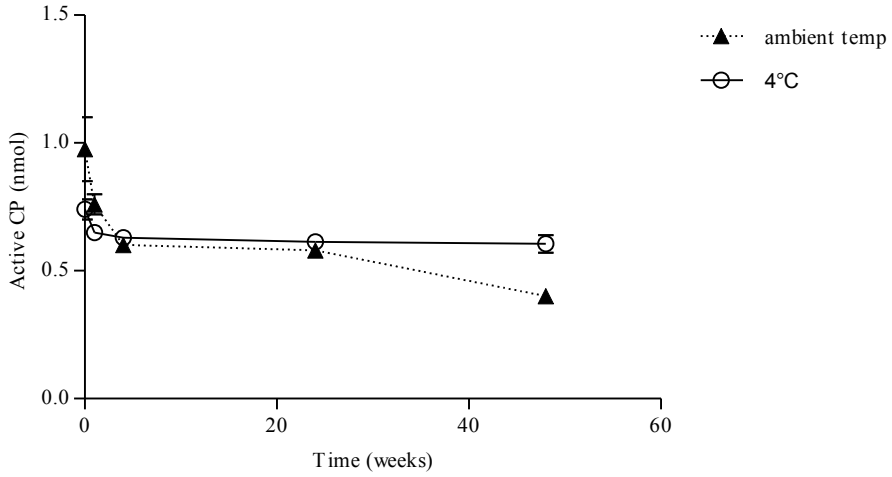


Fig 3.3 Freeze-dried PLS storage
Amount of active CP present in freeze-dried PLS as it was being stored at different temperature conditions at different time points. The amount of active CPs expressed in nmols are the molar equivalent of the inhibitor E-64 used to block active CP in a 10µl volume in a titration assay (see chapter 2). Note that the amount of active CPs decreased when freeze-dried PLS was stored at ambient temperature but was relatively preserved at 4°C. Error bars represent the standard error of the mean.

3.2.4 Experiment 4

Thermostability of PLS during *in vitro* motility assay condition

3.2.4.1 Experimental design

This experiment was conducted to determine the stability in terms of amount of active CPs retained or lost when PLS was incubated at 37ºC for the duration of *in vitro* motility assay. PLS from Experiment 1 was used throughout the whole experiment.

3.2.4.2 Results

PLS was relatively stable throughout the duration of 120min at 37°C which is the standard duration for *in vitro* motility assays used in Chapter 4. There was no obvious loss in the amount of active CP throughout the whole experiment.

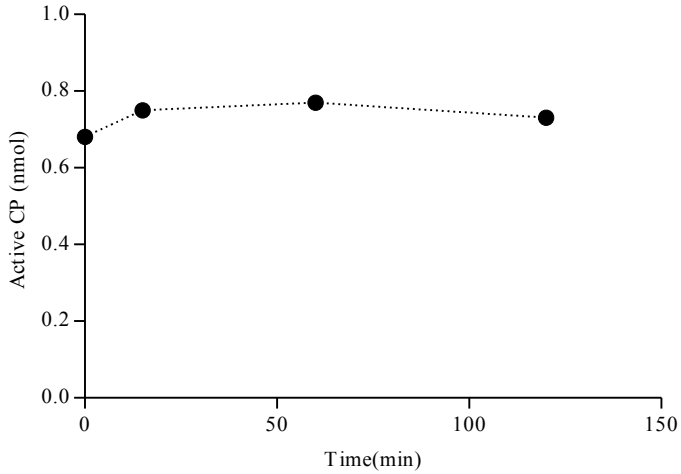


Fig 3.4 PLS stability during in vitro assay
The amount of active CP present in PLS incubated at 37ºC for 120min. The amount of active CPs expressed in nmols are the molar equivalent of the inhibitor E-64 used to block active CP in a 10µl volume in a titration assay (see chapter 2). No obvious loss in amount of active CP was noted. Error bars represent the standard error of the mean.

3.2.5 Experiment 5

Thermostability of PLS undergoing repeated freeze-thaw cycles

3.2.5.1 Experimental design

This experiment was conducted to determine the PLS stability in terms of amount of active CPs retained or lost when going through repeated freeze-thaw cycles. If papaya latex is to be used in the frozen form it is important to determine its stability because frozen PLS obviously needs to be thawed before use and sometimes left over from the initial thaw can be refrozen for next use to avoid waste. PLS from Experiment 1 was used in this experiment. Dialysed PLS kept at -80ºC from Experiment 1 was thawed and the amount of active CP present in 1ml assay (see chapter 2 for methodology) determined. The same sample was refrozen at -80ºC and left for about 1 month before repeating the same freeze-thaw cycle with amount of active CPs determined each time.

3.2.5.2 Results

Apart from the initial loss of active CPs following the first freezing, post dialysis, there was no further loss of active CPs after repeated freeze-thaw cycles of up to 5 times.

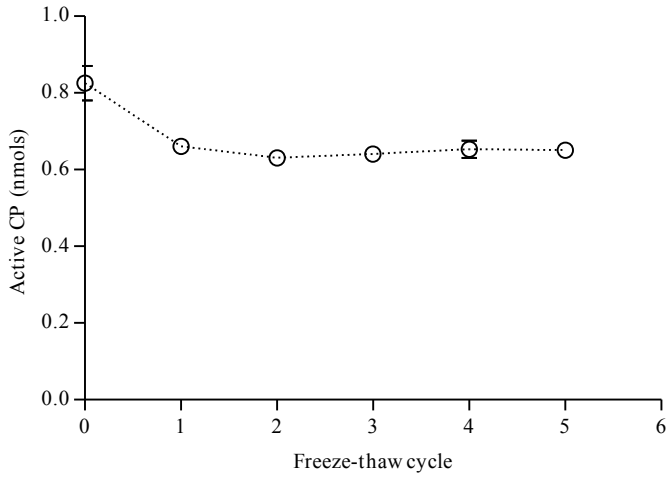


Fig 3.5 Freeze-thawing PLS
Amount of active CPs in PLS deep frozen at -80ºC which underwent repeated freeze-thaw cycles. The amount of active CPs expressed in nmols are the molar equivalent of the inhibitor E-64 used to block active CP in a 10µl volume in a titration assay (see chapter 2). No obvious deterioration of the amount of active CP noted up to 5 freeze-thaw cycles. Error bars represent the standard error of the mean.

3.2.6 Experiment 6

PLS activity in the mouse GI tract

3.2.6.1 Experimental design

This experiment was conducted to reconfirm the earlier observation that PLS was active along the mouse GI tract (Stepek et al., 2007b). Six C3H mouse were purchased from Harlan. Mice were treated to a single dose of PLS at 240nmols each at 0min. 1 mouse was killed at 10min, 20min, 60min, 90min, 120min and 180min. Each was dissected to remove their whole GI tract intact and washed in PBS buffer. The GI tract was divided into 4 segments; stomach, upper intestine, lower intestine (the small intestine was divided into 2 equal length portions), and the colon (the caecum was not included). Each of the segments was opened and its contents washed in 5mls of PBS before being filtered. The enzyme activity of each segment was then determined by enzyme activity assay using Bz-Arg-pNA as substrate. Naturally occurring trypsin in the mammalian GI tract also cleaves the Bz-Arg-pNA substrate. Hence E-64 was used to block any PLS activity, the residual activity providing an indication of trypsin activity. Thus PLS activity could be determined by subtracting trypsin activity from total activity. The concentration of enzyme activity was calculated using a molar extinction coefficient of 8800 $M^{-1}cm^{-1}$(Mole and Horton, 1973a) (see chapter 2 for methodology).

3.2.6.2 Results

In the mouse GI tract enzyme activity in the stomach and upper small intestine rapidly declined within 90min of treatment after which only negligible amounts persisted. In contrast, enzyme activity in the lower small intestine and the colon slowly rose within 90min after which it was relatively stable until the end of the experiment at 3hrs.

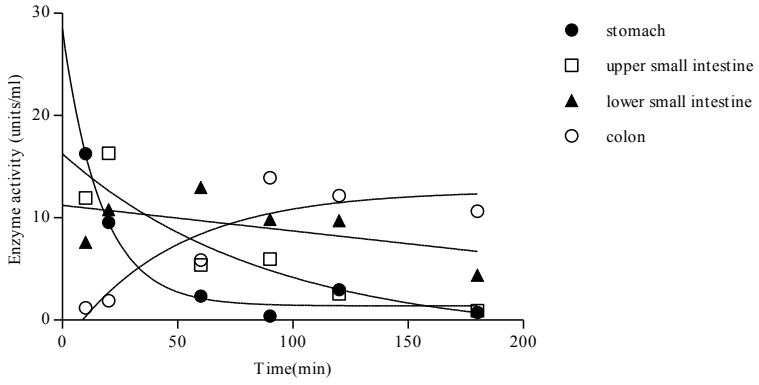


Fig 3.6 PLS activity in mouse GIT
The enzyme activity (units/ml) of PLS throughout the C3H mouse GI tract over time. The unit of activity was defined as that which produced 1 nmol min^{-1} of product (see chapter 2 for methodology).

Note the rapid decline of PLS enzyme activity in the stomach and small intestine and a steady increase in the colon. Trendlines are fitted to guide the eye.

3.2.7 Experiment 7

PLS activity along the rat GI tract

3.2.7.1 Experimental design

This is a similar experiment to Experiment 6 but to observe PLS activity along the rat GIT since the rat model is also used in some chapters of this thesis. Six Wistar rats were purchased from Harlan. Rats were treated to a single dose of PLS at 2.4μmols each at 0min. 1 rat was killed at 10min, 20min, 60min, 90min, 120min and 180min. Each rat was dissected to remove their whole GIT intact and washed briefly in PBS buffer. The GIT was divided into 4 segments; stomach, upper intestine, lower intestine (the small intestine was divided into 2 equal length portions), and the colon (the caecum was not included). Each of the segments was opened and its contents washed in 5mls of PBS before being filtered. The enzyme activity of each segment was then determined by enzyme activity assay using Bz-Arg-pNA as substrate. Naturally occuring trypsin in the mammalian GI tract also cleaves the Bz-Arg-pNA substrate. Hence E-64 was used to block PLS activity in order to get pure PLS only activity by subtracting the PLS blocked (trypsin) values from the combination value (PLS and trypsin).

3.2.7.2 Results

In the rat GI tract enzyme activity in the stomach and upper small intestine declined rapidly within 60min after which only negligible amounts persisted (Fig 3.7). In contrast, enzyme activity in the colon only rose after 90min and was well maintained until the end of the experiment at 3hrs (Fig 3.7) The enzyme activity in the lower small intestine was not stable with a sharp rise within 60min and a sudden plummet by 90min after which it rose sharply for a second time peaking at 2hrs (Fig 3.7).

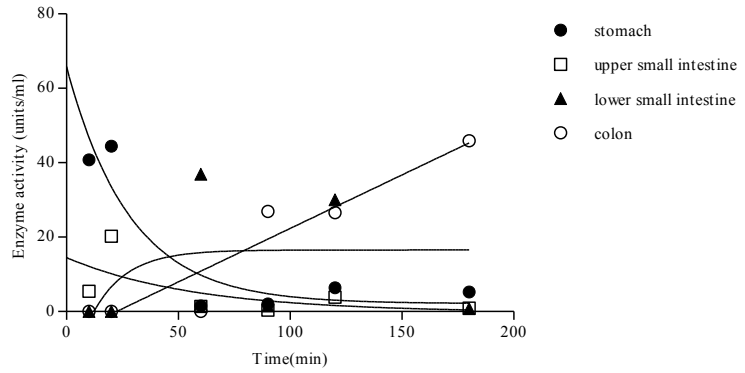


Fig 3.7 PLS activity in the rat GIT
The enzyme activity (units/ml) of PLS throughout the Wistar rat GI tract over time. The unit of activity was defined as that which produced 1 nmol min^{-1} of product (see chapter 2 for methodology). Note the rapid decline of PLS enzyme activity in the stomach and small intestine but increased steadily in the colon. Trendlines are fitted to guide the eye.

3.3 Discussion

Stepek et al. (2007b) have shown that crude papaya latex was active along the mouse GI tract and it was also effective *in vivo* at reducing worm burdens of nematodes residing in different regions of the gut (Stepek et al., 2006b, 2007b, c). However, crude papaya latex lacks refinement and hence in Experiment 1, papaya latex was taken a step further in refinement by centrifugation to obtain the supernatant. This process has been shown to retain enzyme activity (Fig 3.1) whilst removing some contaminants present in the latex. However, this approach is not entirely new since in fact PLS was shown to be more active than its precursor form in an earlier publication (Frankel et al., 1937). Nonetheless, it is important to highlight this fact again since PLS is going to be used throughout the following chapters of this thesis. Dialysis was carried out only to concentrate active enzymes for logistical reasons (it is easier to transport and store a small volume of concentrated stock and later dilute to final desired concentrations rather than vice versa) and to facilitate freeze-drying. This process however resulted in a 33.9% loss of active CPs (Fig 3.1). It is unclear as to how this could have happened but postulations include oxidation (Kimmel et al., 1955) and possibly even osmotic loss through defective membrane pores. Oxidation in particular is perhaps more plausible as the amount of active CPs was increased in the final freeze-drying step suggesting that active enzymes presumed lost earlier might only be temporarily inactivated rather than irreversibly denatured (Stephen et al., 1993). It was also evident that PLS underwent further loss of active CPs during freezing which will be discussed later in this discussion. It was then imperative for the now partially refined PLS to be kept stable before use.

Due to its commercial value the papaya latex thermostability profile has been investigated by many of which most observed its heat lability (Chaiwut, 2007; Hinkel, 1951; Ortiz et al., 1980) just like most proteins (Somero, 1995). More detailed studies on the thermostability of each of the 4 different proteinases that constitute papaya latex have also been investigated (Sumner et al., 1993). Experiment 2

was carried out to justify the use of PLS from the same stock over long periods of storage duration besides comparing the different possibilities for storage of PLS to enable better logistics in practical usage. For this, the exact preparation protocol as used for Experiment 1 (see chapter 2 for methodology) which has now been published (Buttle et al., 2011; Luoga et al., 2011), was carried out to produce PLS which was then stored at different temperatures ranging from ambient temperature to -80 ºC, the latter being used for the entire thesis. It was apparent that PLS when stored at relatively higher temperatures such as 4 ºC and higher underwent a gradual loss of active CPs over the span of 1 year (Fig 3.2). When PLS was stored at much higher temperatures such as ambient temperature the amount of active CP was drastically reduced by 70% within the first week post processing after which it gradually fell to almost no detectable active CPs by 1 year (Fig 3.2). This was in contrast to the considerably more stable profile of PLS stored frozen both at -20 ºC and -80 ºC. The results are quite comparable to the literature where Ortiz et al. (1980) recorded a 20% loss of activity within 24hrs of storage under tropical conditions.

Experiment 4 was carried out to investigate PLS stability maintained at 37 ºC simulating a typical *in vitro* motility assay, such as those employed in different experiments in Chapter 4. It is perhaps surprising that there was no loss in active CPs noted throughout the whole experimental duration of 120min, although in contrast to the above when active CPs were lost at ambient temperature over a week (Fig 3.2), these experiments at 37 ºC were on a much shorter time frame of just 2 hours. The addition of cysteine to mimic exact *in vitro* motility assay conditions may have perhaps contributed to additional stabilisation of PLS as cysteine influencing papain stability has been reported (Homaei *et al*., 2010) and the role of excipients in thermo protection is well accepted (Arakawa *et al*., 2001).

As much as the observations of PLS behaviour at higher temperatures are in agreement with the literature so is its response to lower temperatures. It was evident that freeze-drying (Figs 3.1 & 3.3), freezing (-20ºC) and deep freezing (-80ºC) (Figs 3.1 & 3.2) of PLS resulted in minimal loss of active enzymes amounting to no more than 15-20% after which no further deterioration ensued (Fig 3.2 & 3.3). This loss of active CPs can be explained by cold denaturation. It was interesting to note that there was no apparent difference in the stability of PLS stored frozen in the fridge freezer at -20ºC or deep frozen at -80ºC. This observation is particularly appealing for the prospects of PLS logistics as it would be easier to distribute to remote locations without deep freezing facilities. Unlike heat denaturation which is clearly correlated to temperature (Somero, 1995) the mechanism of protein inactivation by cold denaturation is multifaceted with much damage attributed to crystallisation of water through physical and chemical changes (Arakawa et al., 2001).

Therefore, one would naturally expect that repeated freeze-thawing will inactivate or at least reduce the amount of active CPs as was shown by Cao et al. (2003). Surprisingly, the PLS was fairly stable throughout repeated freeze-thaw cycles of up to 5x. This might be due to the fact that PLS is not a singular purified enzyme but rather a partially refined enzyme complex which readily contains many components other than proteases which apart from physically protecting the proteases also contribute to the stability of CP as excipients. Despite this resistance to repeated freezing-thawing cycles, the PLS used throughout the rest of this thesis never underwent repeated freeze-thaw cycles apart from one

instance of freezing during the initial process of aliquoting and samples were only used once after thawing before being discarded.

A more practical approach for PLS storage and logistics would be lyophilized PLS. Although no experiments conducted throughout the thesis utilised lyophilized (freeze-dried) PLS as a CP source, it is nevertheless interesting to observe the effects of different storage conditions on lyophilized PLS. Lyophilized or freeze-dried PLS stored in the refrigerator at 4 ºC was more stable than when stored at room temperature over the period of 1 year (Fig 3.3). The long term stability of lyophilized proteins has been discussed and the factors affecting it are thought to include moisture and oxidation as suggested by (Arakawa *et al*., 2001; Roy and Gupta, 2004).

Within the context of the objectives of the work reported in this thesis, apart from investigating PLS thermostability it was also important to investigate how PLS behaves in the animal model itself as to date it has been reported only for oral treatment with crude papaya latex (Stepek et al., 2007b). The results from Experiment 5 can only concur with the findings of Stepek et al. (2007b) in which PLS activity declined within 60min in the stomach by 75% before steadily rising in the colon and lower part of the small intestine. Similar observations were also made in the rat model (Experiment 6) confirming at least that active PLS was still detectable in the colons of both rodent model systems used throughout the thesis despite the reported concerns about inactivation of orally administered CPs by stomach acid (Huet *et al*. (2006).

CHAPTER 4

Assessment of the anthelmintic efficacy of cysteine proteinases against cestodes *in vitro*

4.0 Summary

Despite limited literature documenting the efficacy of CPs on cestodes *in vitro*, the experiments described in this chapter showed that both naturally occurring fruit-derived CPs such as PLS and pineapple extract, as well as commercially available purified CPs such as papain and stem bromelain, were efficacious *in vitro* against both juvenile, artificially excysted scoleces, as well as against adult worms of both rodent cestodes, *H. microstoma* and *H.diminuta,*. Significant reduction in motility, ultimately leading to death of the worms, was observed with both species, and against both freshly excysted scoleces and adult worms. CPs appear to attack the tegument resulting in generalised erosions mainly in the strobila. The scolex was more resistant to CP attack but was still affected to some extent.

4.1 Introduction

Parasite motility assays remain the most popular, frequently used methods for assessing antiparasitic drug efficacy (Cox, 1993). Nevertheless, whilst not requiring sophisticated methodologies, such assays, gauging parasite motility visually, either directly or via light microscopy, are laborious, subjective and difficult to standardize (Smout *et al*., 2010). While effort towards standardization has proven costly (von Samson-Himmelstjerna et al., 2009), nevertheless it has led to the developments of some more automated techniques such as the light disruption based micromotility meter (Bennett and Pax, 1986; Folz *et al*., 1987). Other techniques that have helped to address the issue of subjectivity include assays based on migration (Wagland et al., 1992), feeding (Alvarez-Sanchez *et al*., 2005) and development (Kotze *et al*., 1999), but none of these can be easily scaled up sufficiently to enable high throughput screening of hundreds/thousands of new, potentially useful compounds. Nevertheless, the research to develop an automated, objective and high throughput screening method continues, as we seek novel compounds to combat the threat of anthelmintic resistance (Smout *et al*., 2010). In the context of cestodes, assessing motility per se might not be representative of the true stress being experienced by the worms as worm motility not being controlled by central nervous system but rather by the peripheral nervous system because the strobila is still capable of movement after removal of the scolex (Sukhdeo, 1992a). Sukhdeo and Kerr (1992) observed a retrograde peristaltic wave-like movement along the strobila with a frequency of 25 cycles per min in the anterior segments of the worm decreasing linearly to 7 cycles per min in the posterior segments. Ideally cestode motility assays should be designed to grade the change in frequency of cycles per min in the strobili. This however clearly imposes limitation on the number or samples that could be assessed at the same time manually.

Because helminths have been known to afflict humans and livestock since antiquity (Cox, 2004), it is not unexpected that in the past traditional remedies, mostly from plant extracts, have been used to treat them (Behnke *et al*., 2008). While ethno-medical evidence of use of plants as anthelmintics abounds (Prakash and Mehrotra, 1987), historical record of specific use of plant based cestocidals remain elusive, apart from the use of powdered fern extract by the ancient Greeks (350-250 B.C.), mentioned in Nordic literature (Waller *et al*., 2001). Scientific evidence of plant extracts possessing cestocidal properties are rather recent and limited. Few plants with possible active principles suspected of mediating cestocidal activity have been reported, but among these are reports that the tuberous root of the plant *Flemingia vestita* has an active principle thought to be genistein (Tandon et al., 2003), that the alkaloid content of *Adhatoda vasica* is cestocidal (Yadav and Tangpu, 2008) and that *Acacia* spp. contain saponins and tannins with cestocidal properties (Ghosh *et al*., 1996). There are also several reports of plants showing cestocidal activity, but with unknown active principles, as for example in the case of *Strobilates discolour* (Tangpu et al., 2006) and *Cassia* spp. (Kundu and Lyndem, 2012; Kundu et al., 2012) and *Solenostemon* spp. leaves and *Croton* spp. twigs (He et al., 1992). However, despite the exciting prospect that some of these plant sources may eventually yield new cestocidal drugs, so far none have been tested rigorously and in no case have the active principles been proven conclusively to mediate the purported cestocidal activity.

Plant products from pineapple, papaya and figs, which contain the active principles CPs, however, have been systematically tested against nematodes and their anthelmintic properties have been confirmed to be mediated by the CPs (Stepek *et al*., 2005). Therefore, CPs hold a real potential for development into mainstream anthelmintic. In comparison to nematodes, little is known about the effects of CPs on cestode infections. There is some evidence that CPs are detrimental to the survival of cestodes in *in vitro* maintained assays (He et al., 1992; Stepek et al., 2007e), but data also indicate that this is not reflected in a reduction of parasite burdens when CPs are administered to infected hosts (de Amorin *et al*., 1999; He *et al*., 1992).

In this chapter I tested the hypothesis that plant derived CPs are detrimental to cestodes maintained in temporary *in vitro* assays. I used two species of laboratory maintained cestodes, the murine species *H. microstoma* and the rat parasite *H. diminuta*, and in both cases I assessed the effects of a range of CPs, at a range of concentrations, on newly excysted scoleces obtained from cysticercoids derived from beetles and on adult worms extracted from infected rodent hosts.

4.2 Experimental design and results

Effects of CPs against adult cestodes *in vitro*.

4.2.1 Experiment 1

Confirmation of the effects of CPs against cestodes

4.2.1.1 Experimental design

CPs have been shown to be the active principles in papaya latex affecting nematodes since their effect can be totally blocked in the presence of the specific CP inhibitor E-64 (Stepek et al., 2005). Experiment 1 was designed to confirm that the motility decreasing effects seen in earlier pilot experiments (not shown) were indeed mediated by CP. The rat cestode *H. diminuta* was used as a model in this experiment. Fourteen day old adult *H. diminuta* were sourced from infected male Wistar rats from Charles River, UK. Worms dissected from their host were transferred into various treatment wells (1 worm per well) containing either Hanks' saline, 1mM Hanks cysteine (HC), E-64 1600µM, PLS 1500µM+1mM HC+E-64 1600 µM, PLS 1500µM+1mM HC (3 replicates per treatment n=3) and incubated at 37°C for 2hrs. During this period their motility was recorded every 15min using a semi-objective scale of 0-5 (see Chapter 2). Data were analysed using SPSS software.

4.2.1.2 Results

There was a reduction of motility in the worms incubated in either PLS alone or PLS +1mM HC (see Fig 4.1) compared to the other control groups and this was totally inhibited in the presence of E-64. In a 3-way rmGLM with time as the within subject factor and presence/absence of PLS and cysteine as between subject factors, the main effect of PLS was hugely significant ($F_{1,8}$=4900, P<0.001). The main effects of cysteine, however, was not significant ($F_{1,8}$=1.778, P=0.219) and there was no significant interactions between PLS and cysteine ($F_{1,8}$=0.444, P=0.524). In a second rmGLM the effect of PLS+cysteine was compared to that of PLS+cysteine+E-64 and the main effect of presence/absence of E-64 was highly significant ($F_{1,4}$=3267, P<0.001). E-64 alone had no effect on the motility of *H. diminuta* (rmGLM comparing motility in the control treatment of Hanks's alone with Hanks'+ E-64; $F_{1,4}$=0.2, P=0.678).

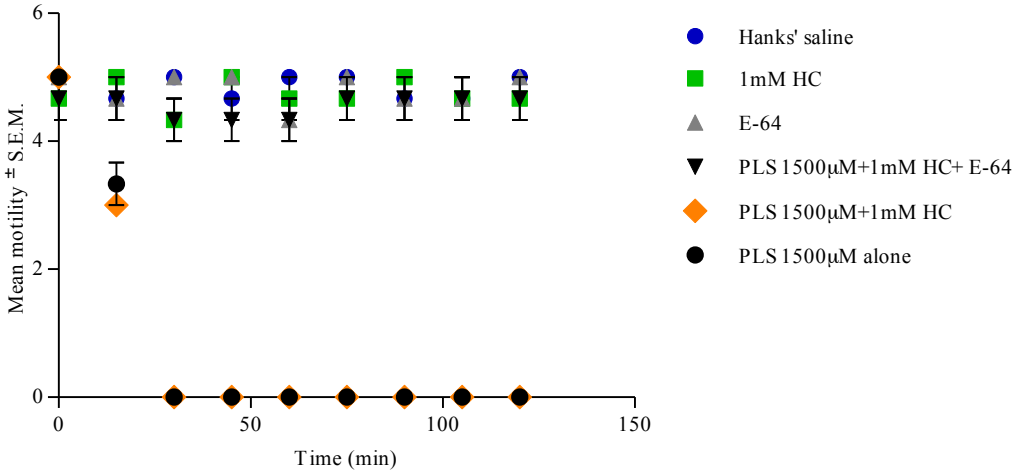


Fig 4.1 The effects of PLS, cysteine & E-64 on cestode motility
Motility of adult *H. diminuta* exposed to 1500µM PLS in an experiment incorporating the presence/absence of CP (PLS), the activator cysteine and the inhibitor E-64. The concentration of active CP expressed in µM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean (*n*=3).

4.2.2 Experiment 2

The efficacy of various CPs against adult *H. diminuta in vitro.*

4.2.2.1 Experimental design

Experiment 2 was a series of experiments assessing the efficacy of various CPs against the adult *H. diminuta in vitro*. *H. diminuta* were sourced from infected male Wistar and male Lister hooded rats from Charles River, UK (see Chapter 2). Adult *H. diminuta* were transferred into various treatment wells (1 worm per well) containing CPs of varying active enzyme concentrations as well as controls (3 replicates per treatment *n*=3) and incubated at 37°C for 2hrs. During this period their motility was recorded every 15min using a semi objective scale of 0-5 (see Chapter 2). Data were analysed using SPSS software and IC_{50} values were generated using GraphPad Prism 5 software.

4.2.2.2 Results

In all CPs tested the motility of adult *H. diminuta* was rapidly reduced in the presence of higher concentrations of active CPs, but more gradually in the presence of moderate concentrations of active CPs (see Figs 4.2 A-D). Pineapple extract, PLS, papain and stem bromelain each caused a significant reduction in motility which in all cases was both time ($F_{8,80} = 18.48$ $P<0.001$; $F_{8,112} = 105.852$ $P<0.001$; $F_{8,96} = 24.674$ $P<0.001$ and $F_{8,112} = 51.307$ $P<0.001$, respectively) and concentration ($F_{4,10} = 159.815$ $P<0.001$; $F_{6,14} = 68.494$ $P<0.001$; $F_{5,12} = 31.995$ $P<0.001$ and $F_{6,14} = 72.023$ $P<0.001$, respectively) dependant. Motility however, was relatively preserved when worms were incubated in the

presence of low concentrations of active CPs or in the control solutions of Hanks' saline with or without cysteine and also in the presence of CP pre-treated with E-64 (see Fig 4.2 A-D). Although all CPs tested were efficacious, efficacy varied between the enzymes with pineapple extract being most efficacious at IC_{50} of 199μM and stem bromelain being the least efficacious with $1C_{50}$ of 1374μM (see Fig 4.3 & Table 4.1).

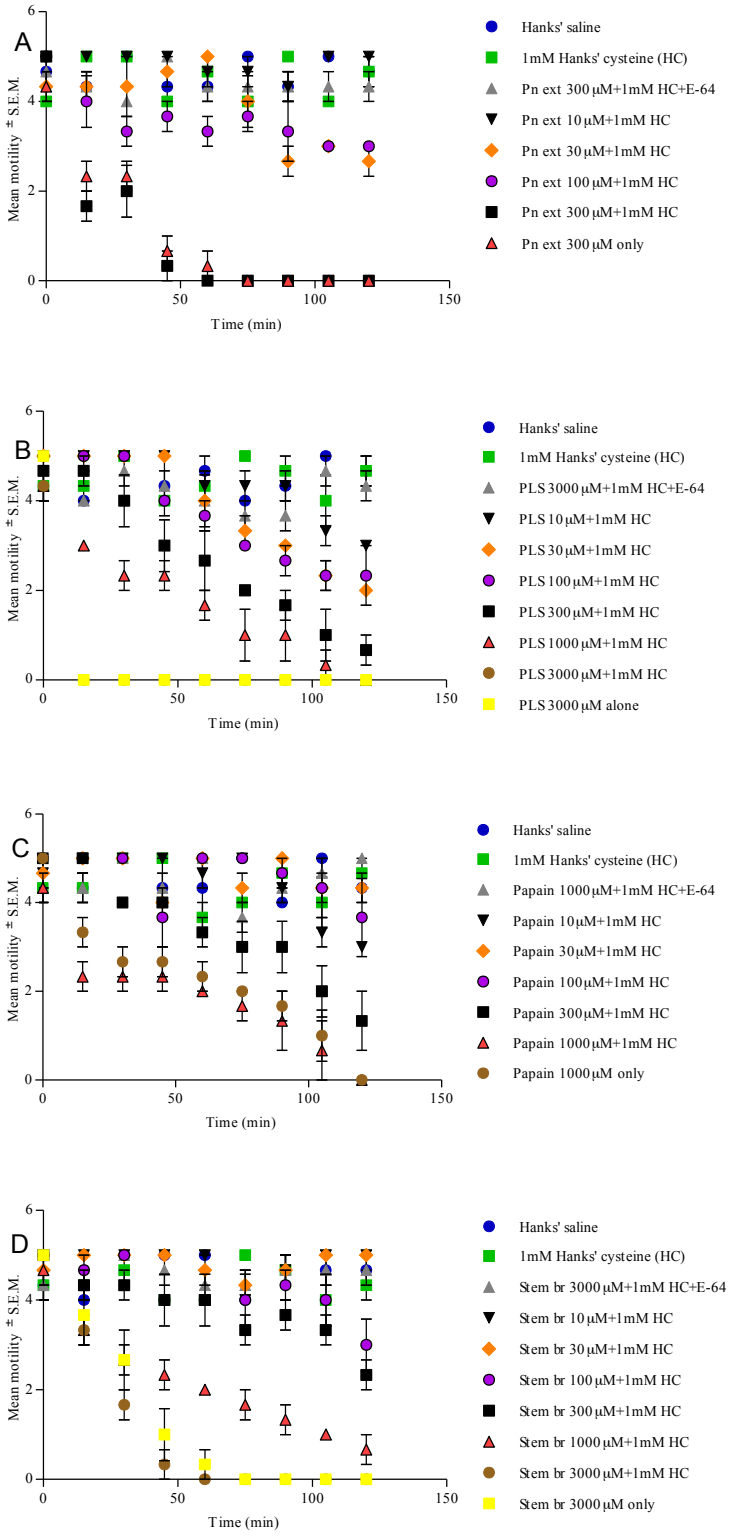


Fig 4.2 Motility of adult *H. diminuta* in various CPs *in vitro*
Motility of adult *H. diminuta* incubated in various CPs *in vitro* (A) pineapple extract (B) PLS (C) papain (D) stem bromelain. The concentration of active CP expressed in µM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean (*n*=3).

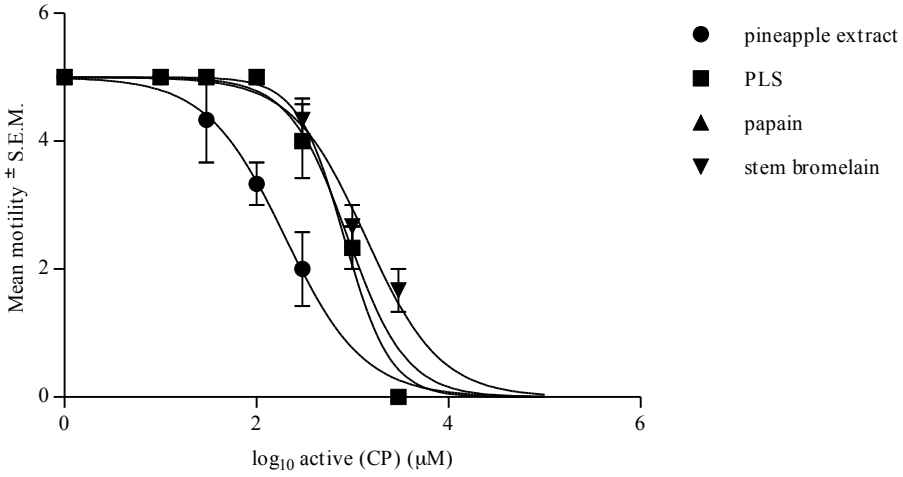


Fig 4.3 Concentration-inhibition curves of the efficacy of various CPs against adult *H. diminuta in vitro*.
The concentration of active CP expressed in μM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10μl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean (*n*=3).

Table 4.1 IC_{50} values of various CPs against adult *H. diminuta in vitro.*

CP	IC_{50}(μM)*
Pineapple extract	199
PLS	818
papain	892
Stem bromelain	1374

* The concentration of active CP expressed in μM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10μl volume in a titration assay (see chapter 2)

4.2.3 Experiment 3

Observation of CP mediated damage under scanning electron microscopy (SEM)

4.2.3.1 Experimental design

Following the promising results from Experiments 1&2, Experiment 3 comprised a series of experiments to document visually the effects of CPs on *H. diminuta in vitro* through scanning electron microscopy. Adult *H. diminuta* were obtained from infected male Wistar and male Lister hooded rats

purchased from Charles River, UK. Worms (3 worms per well) were incubated either in PLS 1500µM. papain 1500µM, pineapple extract 300 µM, stem bromelain 2000µM or Hanks' saline at 37°C. At timely intervals, one worm was removed, fixed in glutaraldehyde, stained in osmium tetraoxide, dehydrated in ethanol, critically point dried, gold coated and viewed under scanning electron microscopy (see Chapter 2 for further details of the methods used).

4.2.3.2 Results

There were progressive changes on the cestode tegument in worms incubated in PLS, papain & stem bromelain. The changes were in the form of blunting of the proglottid edges and a generalised eroded appearance apparent even at 10min post incubation albeit very superficial in nature (Fig 4.4 B,F&N). After 20min there was deeper erosion (Fig 4.4 C,G&O). After 60min post incubation the erosion became so extensive exposing the internal proglottid contents (Fig 4.4 D,H&P). Worms incubated in pineapple extract, however, showed different changes on the tegument albeit the presence of similar very superficial erosions was still apparent. At 10min post incubation there was formation of transverse wrinkling (Fig 4.4 J). At 20min post incubation there was only very superficial erosion and blunting of the proglottid edges (Fig 4.4 K). At 60 min post incubation apart from extensive transverse wrinklings and blunted proglottid edges there were also pockets which appeared to be fluid filled blebs at the worm's right flank (Fig 4.4 L). The worms incubated in Hanks' saline, however, did not show any significant change on the tegument surface even after 60min of incubation (Fig 4.4 R). When imaging was focused on the worm's scolex there was similar progressive erosion but this was comparatively less intense (Figs 4.5 B-D).

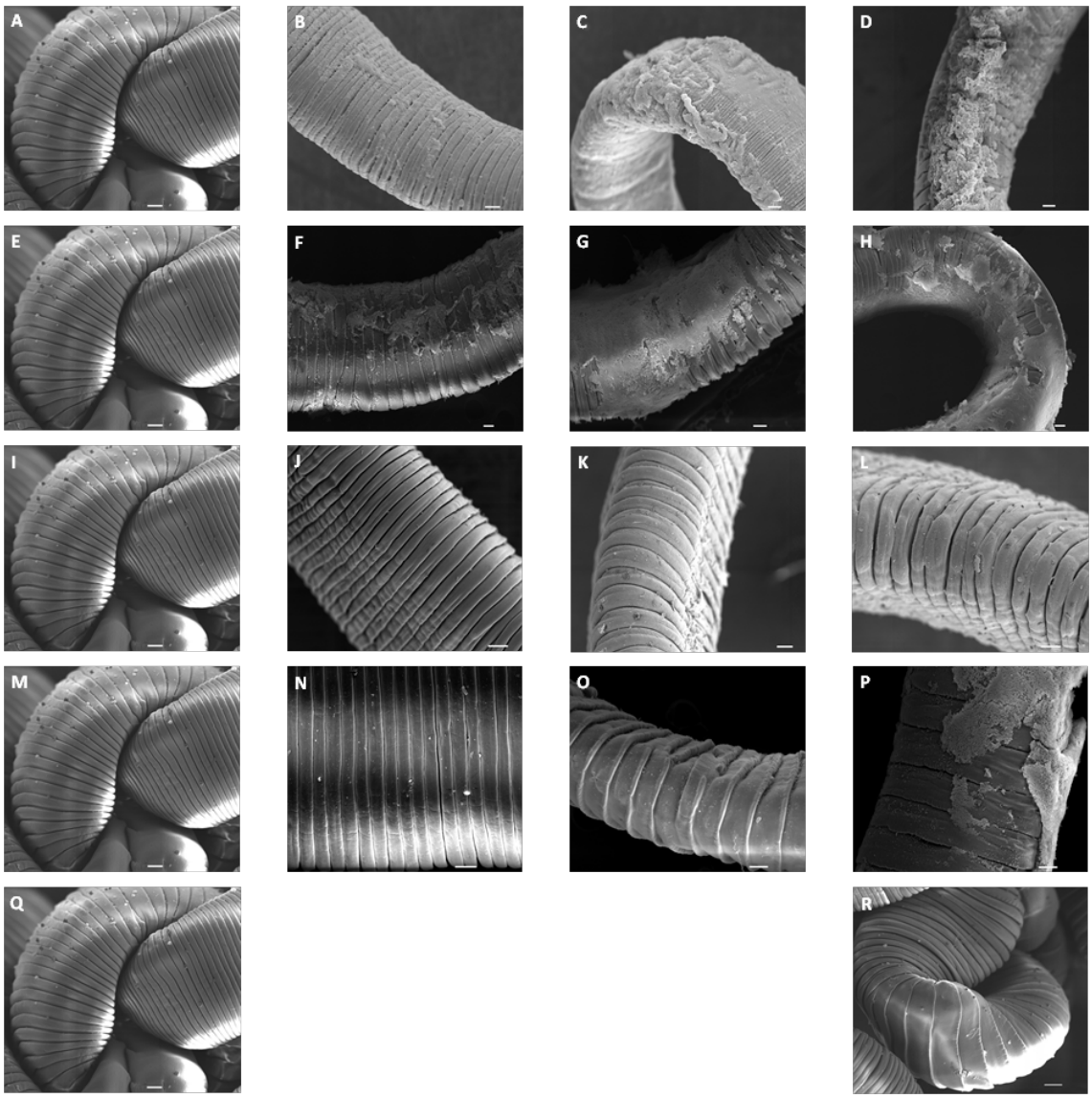


Fig 4.4 SEM images of adult *H. diminuta* in various CPs
Adult *H. diminuta* incubated in various CPs *in vitro*. A-D; in PLS1500µM+1mM HC, E-H; in papain 1500 µM+1mM HC, I-L; in pineapple extract 300µM+1mM HC, M-N; in stem bromelain 2000µM+1mM HC, Q-R; in Hanks' saline. At specific time intervals worms were removed fixed in glutaraldehyde, stained in osmium tetraoxide, dehydrated in ethanol, gold coated and viewed under scanning electron microscope (see chapter 2). The concentration of active CP expressed in µM is the molar equivalent of the inhibitor E-64 used to block active CP in a titration assay (see chapter 2). Scale bars =100 µm.

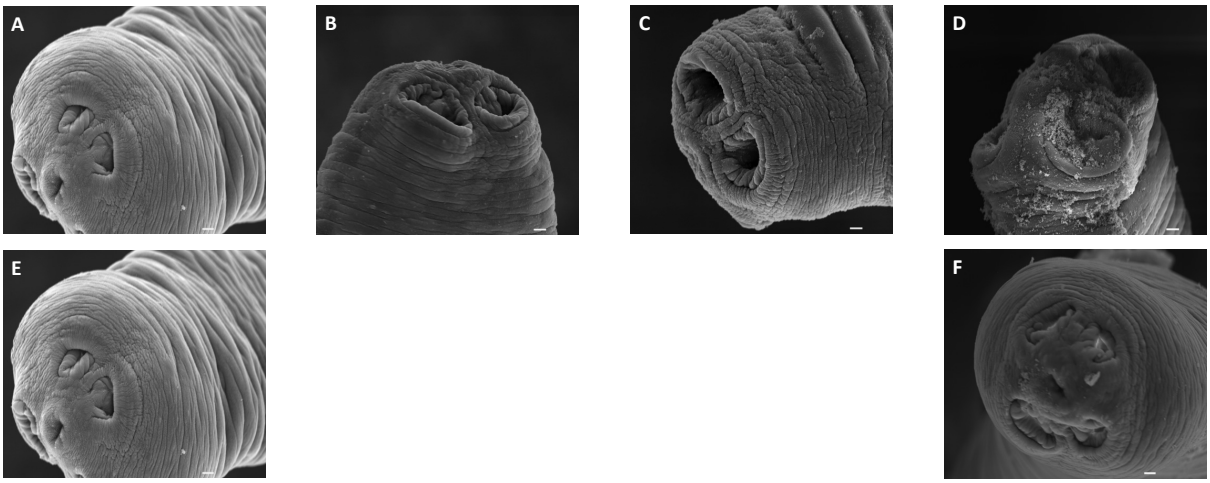


Fig 4.5 SEM images of adult *H. diminuta* scolex in PLS
Adult *H. diminuta* incubated in PLS *in vitro* focusing on the scolex only. A-D; in PLS 1500µM+1mM Hanks; E-F in Hanks' saline. At specific time intervals worms were removed fixed in glutaraldehyde, stained in osmium tetraoxide, dehydrated in ethanol, gold coated and viewed under scanning electron microscope. Note the progressive erosion of the scolex tegument and the collapse of the globular shape by 60min while the scolex is perfectly preserved in Hanks' saline even after 60min. The concentration of active CP expressed in µM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10µl volume in a titration assay (see chapter 2). Scale bars =10µm.

4.2.4 Experiment 4

Observation of CP mediated damage under transmission electron microscopy (TEM)

4.2.4.1 Experimental design

Following the results from Experiment 3, where tegumental damage was observed in worms incubated in CPs, it was felt important to also document the events occurring within the worm through cross sectional images captured by transmission electron micrography. Adult *H. diminuta* were obtained from infected male Wistar rats purchased from Charles River, UK. Worms were incubated in PLS 1000µM at 37°C. At specific timely intervals, 1 worm was removed, fixed in glutaraldehyde, stained in osmium tetraoxide, embedded in plastic, ultrathin cross sections cut, stained in uranyl acetate and viewed under transmission electron microscope.

4.2.4.2 Results

There was formation of vacuoles at the subtegumental layer after 10min incubation in PLS (see Fig 4.6B). It was also noted that the thin top layer coating the brush border had also disappeared (see Fig 4.6B). After 20min there was increased formation of vacuoles and the brush border layer started to become irregular and less dense (Fig 4.6C). After 60min however, there were no more vacuoles seen in

the subtegumental layer and the brush border layer have become regular again but still devoid of the thin top layer (Fig 4.6D). The control worm incubated in Hanks' saline still retains all the exact morphology seen earlier at 0min (Figs 4.6E&F).

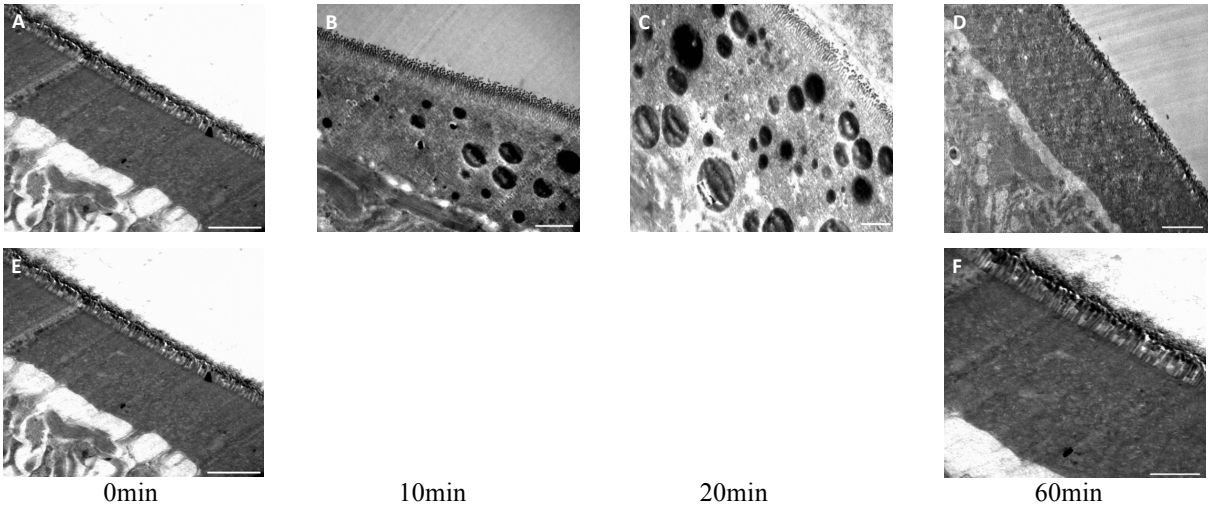


Fig 4.6 TEM images of adult *H. diminuta* in PLS
Transmission electron micrograph images of adult *H. diminuta* incubated in PLS *in vitro*. A-D; in 1000µM PLS, E-F; Hanks' saline. At specific time intervals worms were removed fixed in glutaraldehyde, stained in osmium tetraoxide, embedded in plastic, ultra-thin cross sections cut, stained in uranyl acetate and lead citrate and viewed under transmission electron microscope. The concentration of active CP expressed in µM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10µl volume in a titration assay (see chapter 2). Scale bars = 2µm.

4.2.5 Experiment 5

The efficacy of various CPs against adult *H. microstoma in vitro*.

4.2.5.1 Experimental design

Following the promising results from Experiments 1-4 using the rat cestode *H. diminuta* it was also important to establish whether or not other cestode species are affected and to the same extent. Experiment 5 was a series of experiments assessing the efficacy of various CPs against the mouse cestode *H. microstoma in vitro*. *H. microstoma* were obtained from infected male BKW mice from B & K, UK and male C3H mice from Harlan, UK (see Chapter 2). Adult *H. microstoma* were transferred into various treatment wells (1 worm per well) containing CPs of varying concentrations of active enzymes as well as controls (3 replicates per treatment n=3) and incubated at 37°C for 2hrs. During this period their motility was recorded every 15min using a semi objective scale of 0-5 (see Chapter 2). Data were analysed using SPSS software and IC_{50} values were generated using GraphPad Prism 5 software.

4.2.5.2 Results

In all CPs tested the motility of adult *H. microstoma* was reduced rapidly in the presence of higher concentrations of active CPs, but more gradually in the presence of moderate concentrations of active CPs (see Figs 4.7 A-D). Pineapple extract, PLS, papain and stem bromelain each caused a significant reduction in motility which in all cases was both time ($F_{8,104} = 132.812$ $P<0.001$; $F_{6.883,96.365} = 38.006$ $P<0.001$; $F_{5.811,69.734} = 79.233$ $P<0.001$ and $F_{8,112} = 38.426$ $P<0.001$, respectively) and concentration ($F_{4,13} = 232.316$ $P<0.001$; $F_{6,14} = 95.017$ $P<0.001$; $F_{5,12} = 99.834$ $P<0.001$ and $F_{6,14} = 132.113$ $P<0.001$, respectively) dependant. However, motility was relatively preserved when worms were incubated in the presence of low concentrations of active CPs or in the control solutions of Hanks' saline with or without cysteine and even in the presence of CP pre-treated with E-64 (see Fig 4.7 A-D). Although all CPs tested were efficacious, efficacy varied between the enzymes with pineapple extract being most efficacious at IC_{50} of 260µM and stem bromelain being the least efficacious with $1C_{50}$ 1451µM (see Fig 4.8 &Table 4.2).

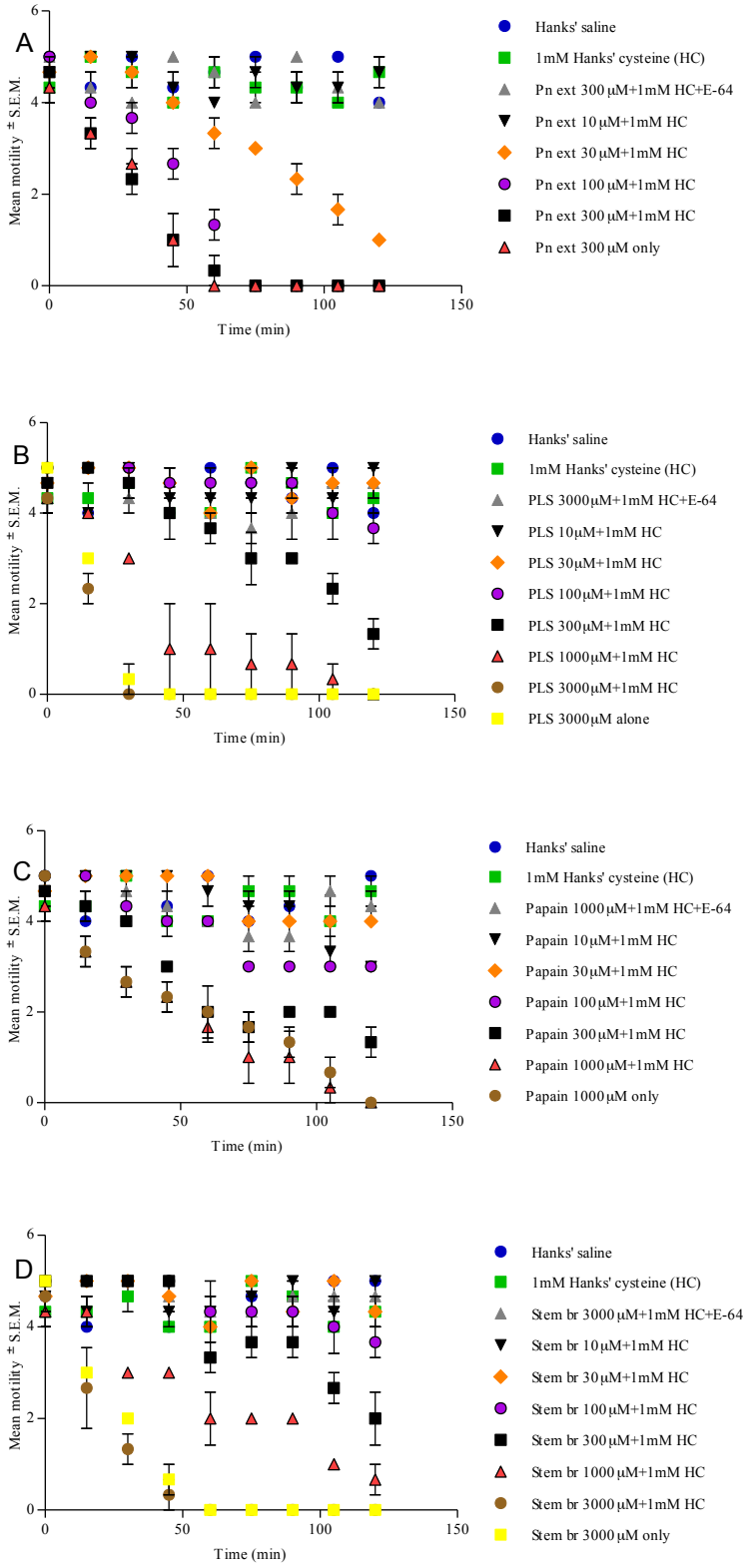


Fig 4.7 Motility of adult *H. microstoma* in various CPs *in vitro*
Motility of adult *H. microstoma* incubated in various CPs *in vitro* (A) pineapple extract (B) PLS (C) papain (D) stem bromelain. The concentration of active CP expressed in µM is the molar equivalent of the inhibitor E-64 used to block active CP in a titration assay (see chapter 2).Error bars represent the standard error of the mean (*n*=3).

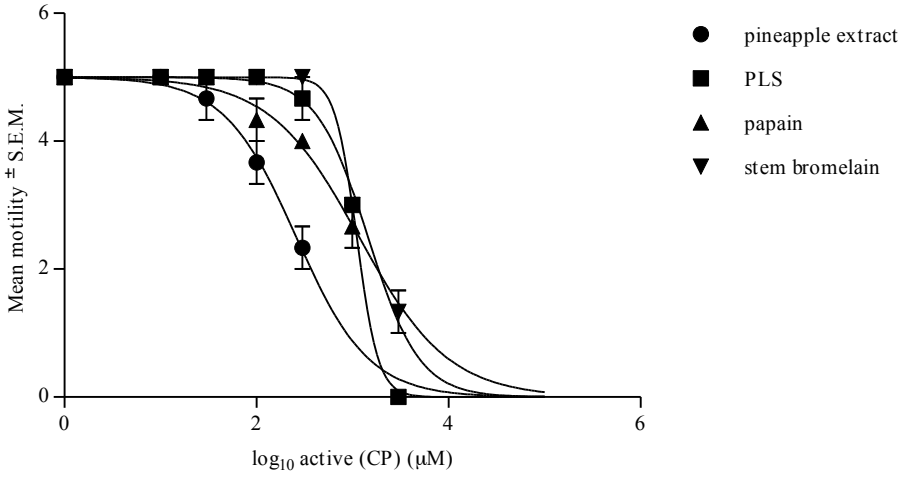


Fig 4.8 Concentration-inhibition curves of the efficacy of various CPs against adult *H. microstoma in vitro*.
The concentration of active CP expressed in μM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10μl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean (*n*=3).

Table 4.2 IC_{50} values of various CPs against adult *H. microstoma in vitro.*

CP	IC_{50}(μM)*
Pineapple extract	260
PLS	1107
papain	1172
Stem bromelain	1451

* The concentration of active CP expressed in μM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10μl volume in a titration assay (see chapter 2).

4.2.6 Experiment 6

Observation of CP mediated damage against *H. microstoma in vitro*.

4.2.6.1 Experimental design

Experiment 6 was an experiment to document visually the effects of CPs on *H. microstoma in vitro* through scanning electron microscopy. Adult *H. microstoma* were obtained from infected male C3H mice from Harlan, UK. Following the results from Experiment 3 where *H. diminuta* was incubated in

1500 µM which resulted in extensive tegumental erosions it was decided that in this experiment a lower dose would be used and hence worms were incubated in PLS 1000µM at 37°C. At timely intervals, one worm was removed, fixed in glutaraldehyde, stained in osmium tetroxide, dehydrated in ethanol, critically point dried, gold coated and viewed under scanning electron microscopy.

4.2.6.2 Results

There were progressive changes seen on the tegumental surface starting from blunting of proglottids seen at 10min post incubation (see Fig 4.9B). At 20min a generalised superficial eroded appearance was apparent (see Fig 4.9C). At 60min there was marked generalised erosion and at certain point there was tegumental discontinuity and the outpouring of internal contents (Fig 4.9D). The worms incubated in Hanks' saline however maintained the same appearance even after 60min with sharp proglottid edges and a smooth tegumental surface (see Fig 4.9E&F).

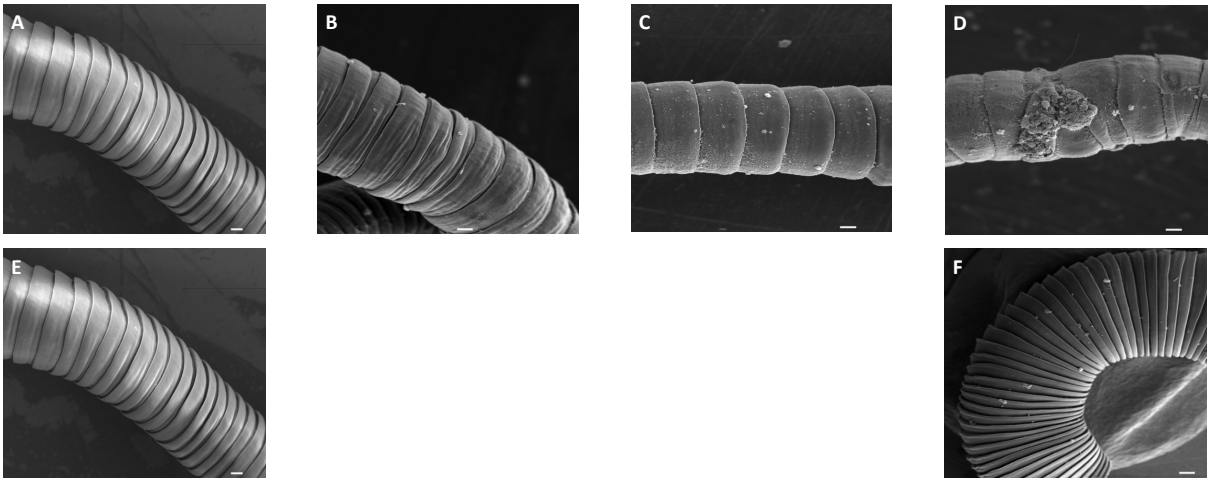


Fig 4.9 SEM images of adult *H. microstoma* in PLS
Adult *H. microstoma* incubated in PLS 1000µM+1mM HC (A-D) and Hanks saline (E-F). At specific time intervals worms were removed fixed in glutaraldehyde, stained in osmium tetroxide, dehydrated in alcohol, gold coated and viewed under scanning electron microscope (see chapter 2). The concentration of active CP expressed in µM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10µl volume in a titration assay (see chapter 2). Scale bars represent 100µm.

Effect of CPs against juvenile stages of cestodes

4.2.7 Experiment 7

The efficacy of various CPs against juvenile *H. diminuta in vitro*.

4.2.7.1 Experimental design

Behnke et al. (2008) reported on the basis of unpublished results that juvenile free-living -stages of *H. bakeri* were not affected by CPs. Hence it was relevant here to assess whether or not juvenile cestodes are affected by CPs. Experiment 7 was a series of experiments assessing the efficacy of various CPs against the juvenile artificially excysted *H. diminuta in vitro*. *H. diminuta* cysticercoids were obtained from infected *T. confusum* beetles maintained at the University of Nottingham. Dissected cysticercoids were artificially hatched following a protocol (Goodchild and Davis, 1972) (see Chapter 2 & Fig 4.10A-D). Fully evaginated scoleces (1 worm per well) were then transferred into various treatment wells containing CPs of varying concentrations of active enzymes as well as controls (3 replicates per treatment n=3) and incubated at 37°C for 2hrs. During this period their motility was recorded every 15min using a semi objective scale of 0-3 (see Chapter 2). Data were analysed using SPSS software and IC_{50} values were generated using GraphPad Prism 5 software.

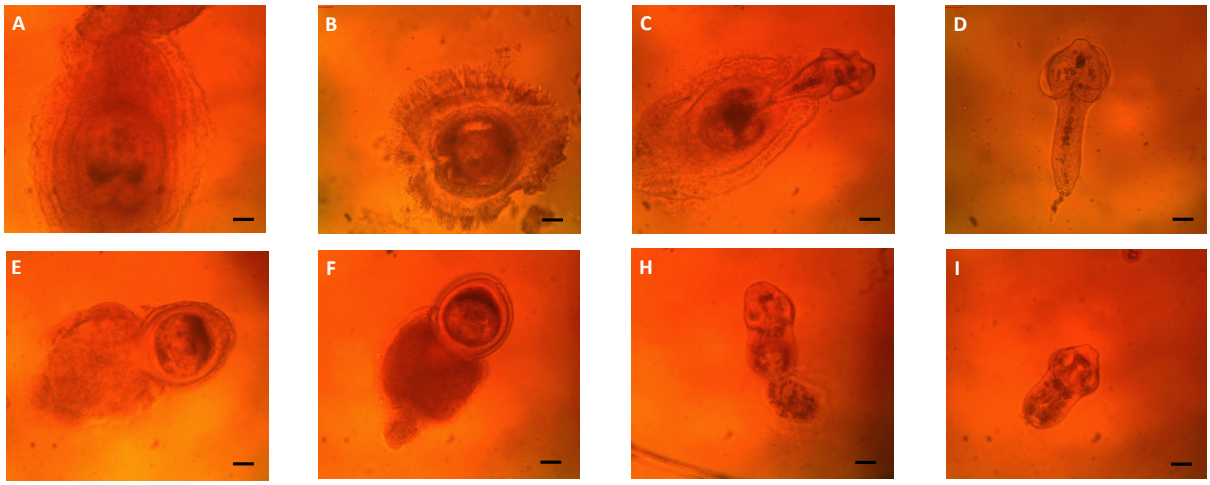


Fig 4.10 Artificial excystation of cestodes *in vitro*
The sequential process of artificial excystation of cysticercoids *in vitro; H. diminuta* (A-D); *H. microstoma* (E-I). (A&E) Freshly dissected cysticercoids from *T. confusum* incubated in Hanks' saline. (B&F) cysticercoids incubated in acid-pepsin solution (C&H) cysticercods incubated in trypsin-bile solution with emerging scolex (D&I) Free scoleces. The concentration of active CP expressed in µM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10µl volume in a titration assay (see chapter 2). Scale bars represent 61µm.

4.2.7.2 Results

In all CPs tested the motility of artificially excysted *H. diminuta* scoleces were rapidly reduced in the presence of higher concentrations of active CPs, but more gradually in the presence of moderate concentrations of active CPs (see Figs 4.11 A-D). Pineapple extract, PLS, papain and stem bromelain each caused a significant reduction in motility which in all cases was both time ($F_{5.54,55.402} = 28.139$ $P<0.001$; $F_{8,128} = 42.996$ $P<0.001$; $F_{8,128} = 24.35$ $P<0.001$ and $F_{8,128} = 16.82$ $P<0.001$, respectively) and concentration ($F_{4,10} = 321.719$ $P<0.001$; $F_{7,16} = 111.516$ $P<0.001$, $F_{7,16} = 30.154$ $P<0.001$ and $F_{7,16} = 23.672$ $P<0.001$, respectively) dependant. Motility, however, was relatively preserved when artificially excysted scoleces were incubated in the presence of low concentrations of active CPs or in

the control solutions of Hanks' saline with or without cysteine and even in the presence of CP pre-treated with E-64 (see Figs 4.11 A-D). Although all CPs tested were efficacious, efficacy varied with pineapple extract being most efficacious at IC_{50} of 63µM and stem bromelain being the least efficacious with $1C_{50}$ 2843µM (see Fig 4.12 & Table 4.3).

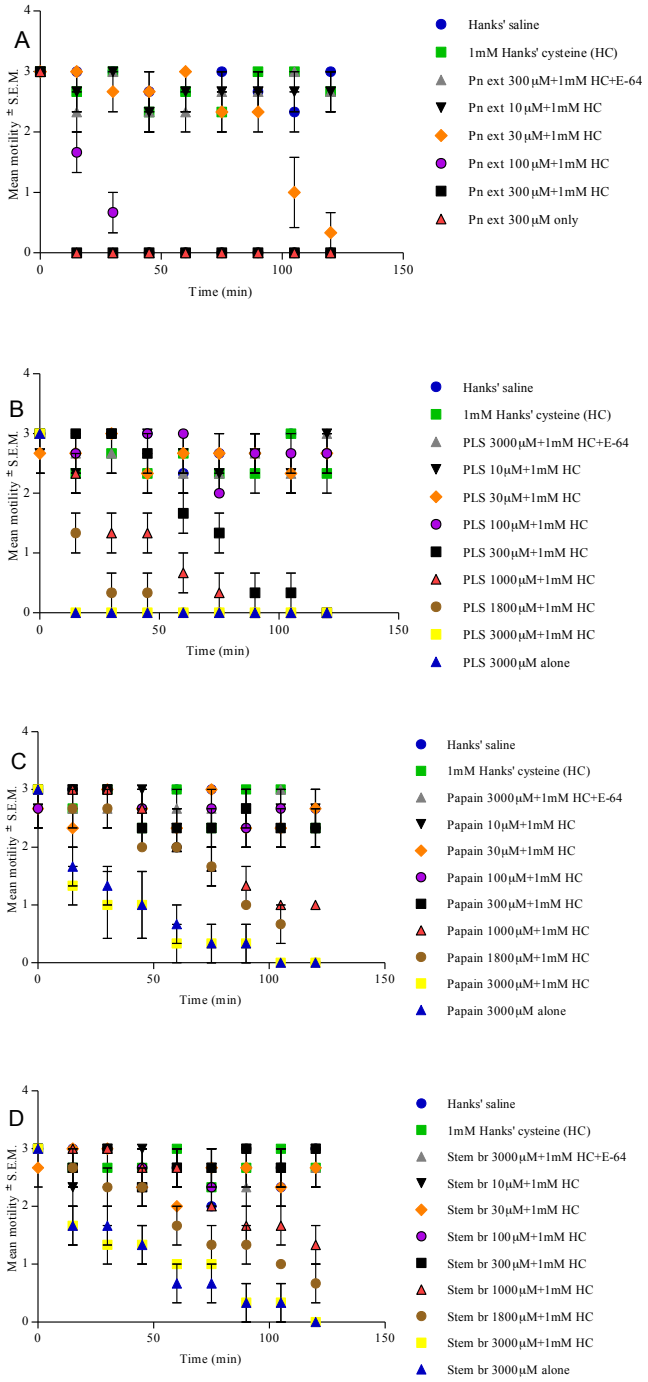


Fig 4.11 Motility of juvenile *H. diminuta* in various CPs *in vitro*
Motility of artificially excysted *H. diminuta* incubated in various CPs *in vitro* (A) pineapple extract (B) PLS (C) papain (D) stem bromelain. The concentration of active CP expressed in µM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean (*n*=3).

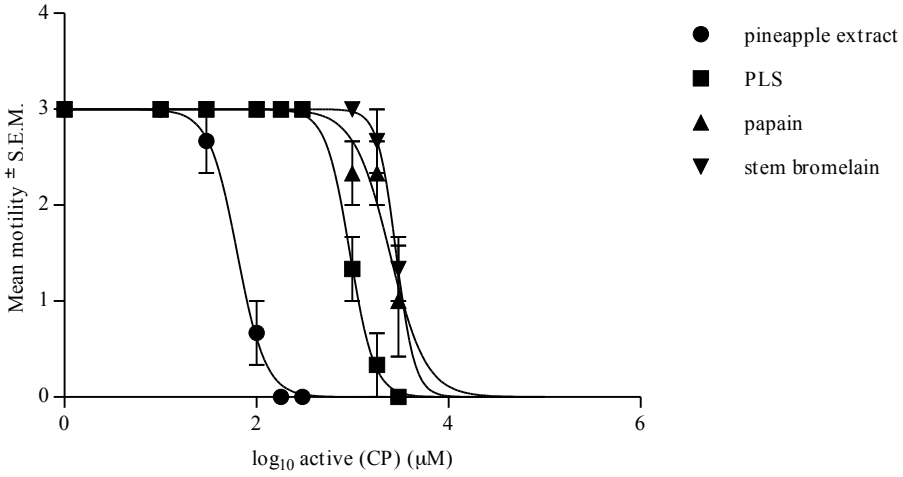


Fig 4.12 Concentration-inhibition curves of the efficacy of various CPs against artificially excysted *H. diminuta in vitro*.
The concentration of active CP expressed in μM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10μl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean (*n*=3).

Table 4.3 IC_{50} values of various CPs against artificially excysted *H. diminuta* scoleces *in vitro.*

CP	IC_{50}(μM)*
Pineapple extract	63
PLS	1283
papain	2727
Stem bromelain	2843

* The concentration of active CP expressed in μM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10μl volume in a titration assay (see chapter 2).

4.2.8 Experiment 8

Observation of CP mediated damage against juvenile *H. diminuta* under light microscopy

4.2.8.1 Experimental design

Following the results from Experiment 7, Experiment 8 was designed to document visually the events taking place when artificially excysted *H. diminuta* scoleces are incubated in CPs. *H. diminuta* cysticercoids were obtained from infected *T. confusum* beetles maintained at the University of

Nottingham. Dissected cysticercoids were artificially hatched following the protocol described by (Goodchild and Davis, 1972) (see Chapter 2 for full details & Figs 4.10A-D). Fully evaginated scoleces (1 worm per well) were then transferred into either a well containing PLS 1500µM+1mM HC, pineapple extract 300 µM+1mM HC, papain 1500µM+1mM HC, stem bromelain 2000µM+1mM HC or Hanks' saline. At specific time intervals worms were photographed under light microscopy at a magnification of x20.

4.2.8.2 Results

After 1 hour of incubation in active CPs, there were notable changes in worm morphology with apparent ballooning of the distal end of worms incubated in PLS, papain and stem bromelain (see Figs 4.13B,H & K). The worm incubated in pineapple extract however appeared to have shrunk and contracted (Fig 4.13E). After 2 hours the worm incubated in PLS appeared to be partially digested (see Fig 4.13C). The worm incubated in pineapple extract remained unchanged in the contracted state observed after 1hr (see Fig 4.13F). The worm incubated in papain appeared to develop shrinkage of the middle and caudal section (see Fig 4.13I). The worm incubated in stem bromelain appeared to develop another ballooning in the middle section (see Fig 4.13L). There were no significant changes in morphology seen in the worm incubated in Hanks' saline (see Fig 4.11E)

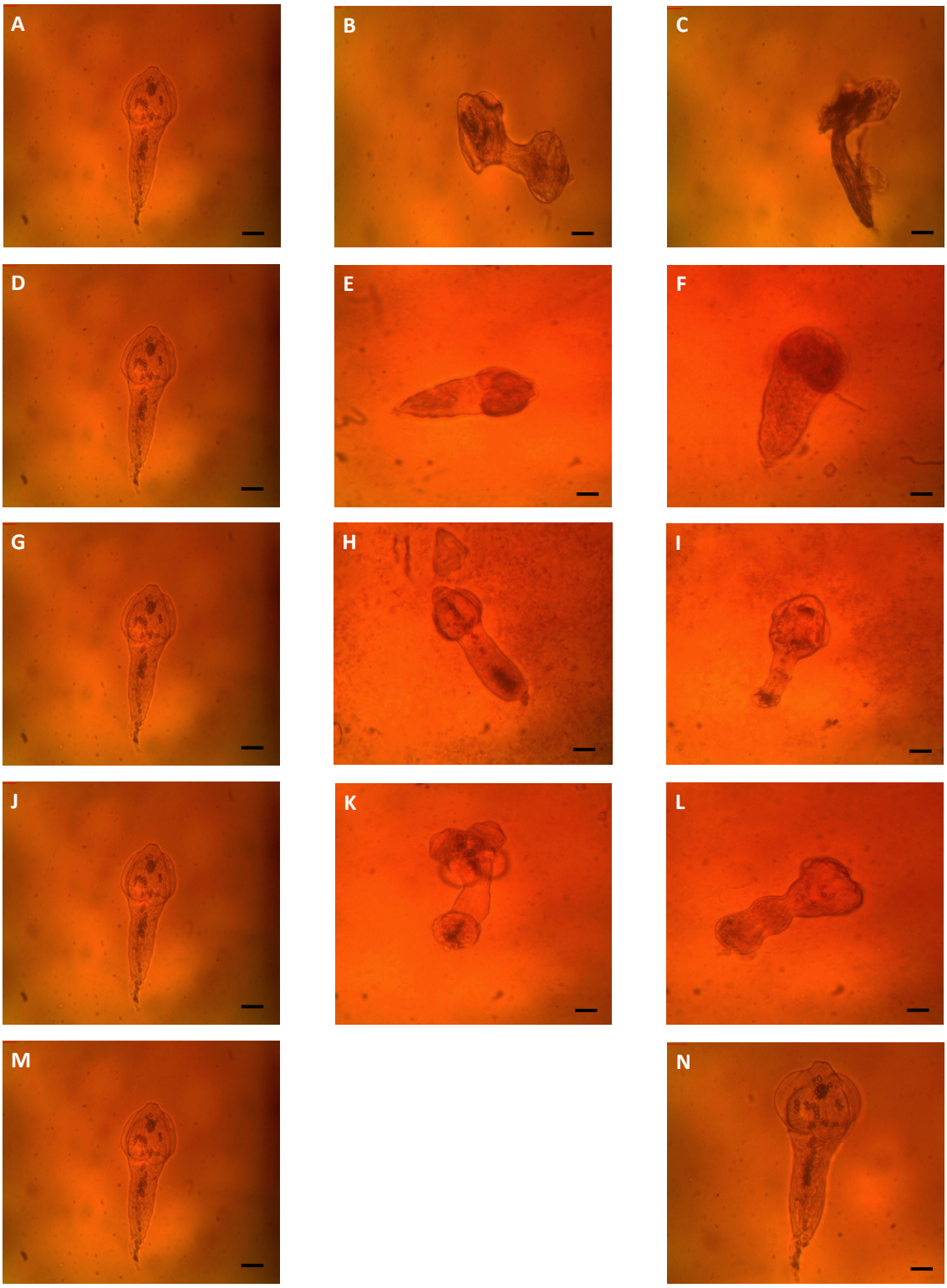


Fig 4.13 Light microscopy images of juvenile *H. diminuta* in various CPs
Artificially excysted *H. diminuta* scoleces incubated in PLS 1500µM+1mM HC (A-C), pineapple extract 300 µM+1 mM HC (D-F), papain 2000 µM+1mM HC (G-I), stem bromelain 2000 µM +1mM HC (J-L) and Hanks' saline (M-N) *in vitro*. The concentration of active CP expressed in µM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2). Scale bars = 61 µm.

4.2.9 Experiment 9

The efficacy of various CPs against juvenile *H. microstoma in vitro*.

4.2.9.1 Experimental design

Experiment 9 was a series of experiments assessing the efficacy of various CPs against the juvenile artificially excysted *H. microstoma in vitro. H. microstoma* cysticercoids were ontained from infected *T. confusum* beetles maintained at the University of Nottingham. Dissected cysticercoids were artificially hatched following the protocol described in Chapter 2(Goodchild and Davis, 1972). Fully evaginated scoleces (1 worm per well) were then transferred into various treatment wells containing CPs of varying concentrations of active enzymes as well as controls (3 replicates per treatment n=3) and incubated at 37°C for 2hrs. During this period their motility was recorded every 15min using a semi objective scale of 0-3 (see Chapter 2). Data were analysed using SPSS software and IC_{50} values were generated using GraphPad Prism 5 software.

4.2.9.2 Results

In all CPs tested the motility of artificially excysted *H. microstoma* scoleces were rapidly reduced in the presence of higher concentrations of active CPs, but more gradually in the presence of moderate concentrations of active CPs (see Figs 4.14 A-D). Pineapple extract, PLS, papain and stem bromelain each caused a significant reduction in motility which was both time ($F_{7.407,74.066} = 32.326$ $P<0.001$; $F_{7.88,126.086} = 38.692$ $P<0.001$, $F_{8,128} = 21.683$ $P<0.001$ and $F_{8,128} = 20.896$ $P<0.001$, respectively) and concentration ($F_{4,10} = 106.278$ $P<0.001$; $F_{7,16} = 180.025$ $P<0.001$; ($F_{7,16} = 14.21$ $P<0.001$and $F_{7,16} = 27.028$ $P<0.001$, respectively) dependant. However, motility, was relatively preserved when artificially excysted scoleces were incubated in the presence of low concentrations of active CPs or in the control solutions of Hanks' saline with or without cysteine and even in the presence of CP pre-treated with E-64 (see Figs 4.14 A-D). Although all CPs tested were efficacious, efficacy varied with pineapple extract being most efficacious at IC_{50} of 74µM and stem bromelain being the least efficacious with $1C_{50}$ 2339 µM (see Fig 4.15 & Table 4.4).

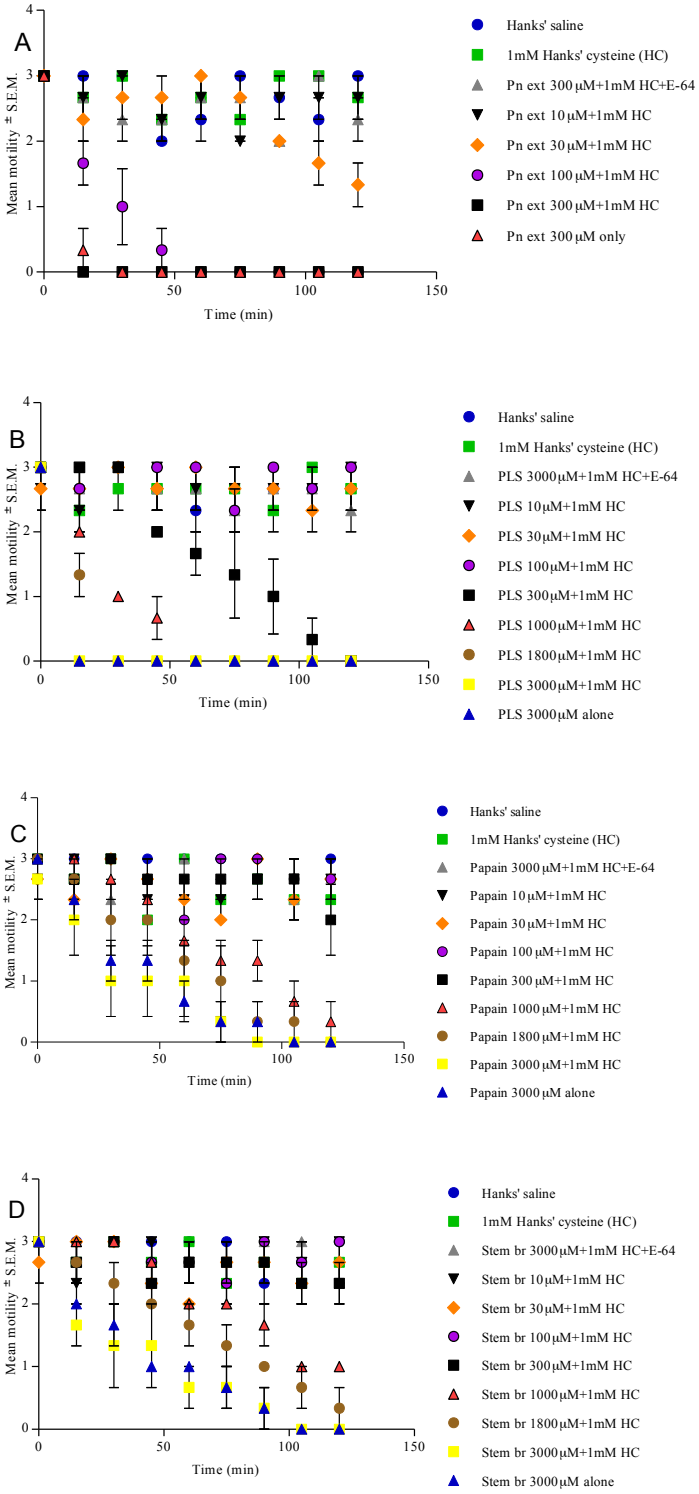


Fig 4.14 Motility of juvenile *H. microstoma* in various CPs *in vitro*
Motility of artificially excysted *H. microstoma* incubated in various CPs *in vitro* (A) pineapple extract (B) PLS (C) papain (D) stem bromelain. The concentration of active CP expressed in µM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean (*n*=3).

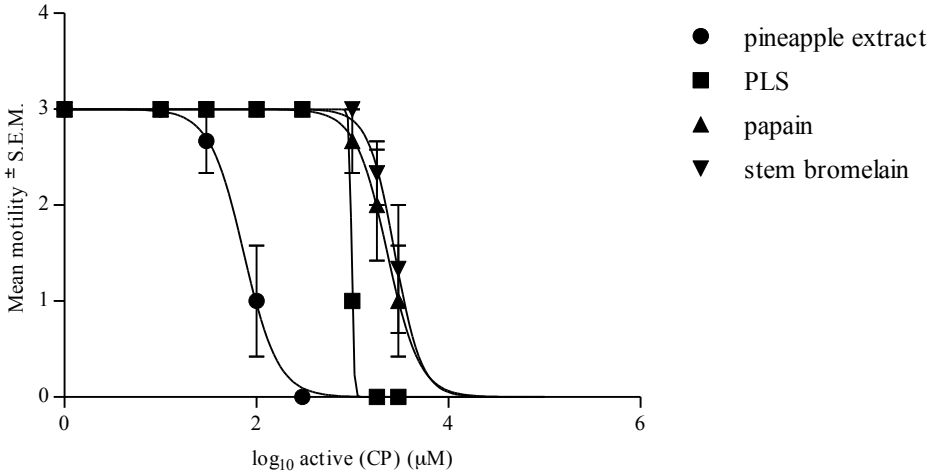


Fig 4.15 Concentration-inhibition curves of the efficacy of various CPs against artificially excysted *H. microstoma in vitro*.
The concentration of active CP expressed in μM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 μl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean (*n*=3).

Table 4.4 IC_{50} values of various CPs against artificially excysted *H. microstoma* scoleces *in vitro.*

CP	IC_{50}(μM)*
Pineapple extract	74
PLS	984
papain	2264
Stem bromelain	2339

* The concentration of active CP expressed in μM is the molar equivalent of the inhibitor E-64 used to block active CP in a titration assay (see chapter 2).

4.2.10 Experiment 10

Observation of CP mediated damage against juvenile *H. microstoma* under light microscopy

4.2.10.1 Experimental design

Following the results from Experiment 9, Experiment 10 was designed to document visually the events taking place when artificially excysted *H. microstoma* scoleces are incubated in CPs. *H. microstoma* cysticercoids were obtained from infected *T. confusum* beetles maintained at the University of

Nottingham. Dissected cysticercoids were artificially hatched following the protocol described in Chapter 2 (Goodchild and Davis, 1972). Fully evaginated scoleces (1 worm per well) were then transferred into either a well containing PLS 1500µM+1mM HC, pineapple extract 300 µM+1mM HC, papain 1500µM+1mM HC, stem bromelain 2000µM+1mM HC or Hanks' saline. At specific time intervals worms were photographed under light microscopy at a magnification of x20.

4.2.10.2 Results

After 1 hour of incubation in active CPs, there were notable changes in worm morphology with apparent shrinkage of worms incubated in PLS, papain and stem bromelain (see Figs 4.16B,H & K). The worm incubated in pineapple extract however appeared to be shortened and contracted (Fig 4.16E). After 2 hours the worm incubated in PLS appeared to be extensively digested (see Fig 4.16C). The worm incubated in pineapple extract appeared to remain similar in a shortened and contracted state similar to that observed at 1hr (see Fig 4.16F). The worm incubated in papain appeared to develop ballooning of its caudal section (see Fig 4.16I). The worm incubated in stem bromelain appeared to develop marked shrinkage of its middle and caudal section (see Fig 4.16L). There was no significant changes in morphology seen in the worm incubated in Hanks' saline (see Fig 4.16N).

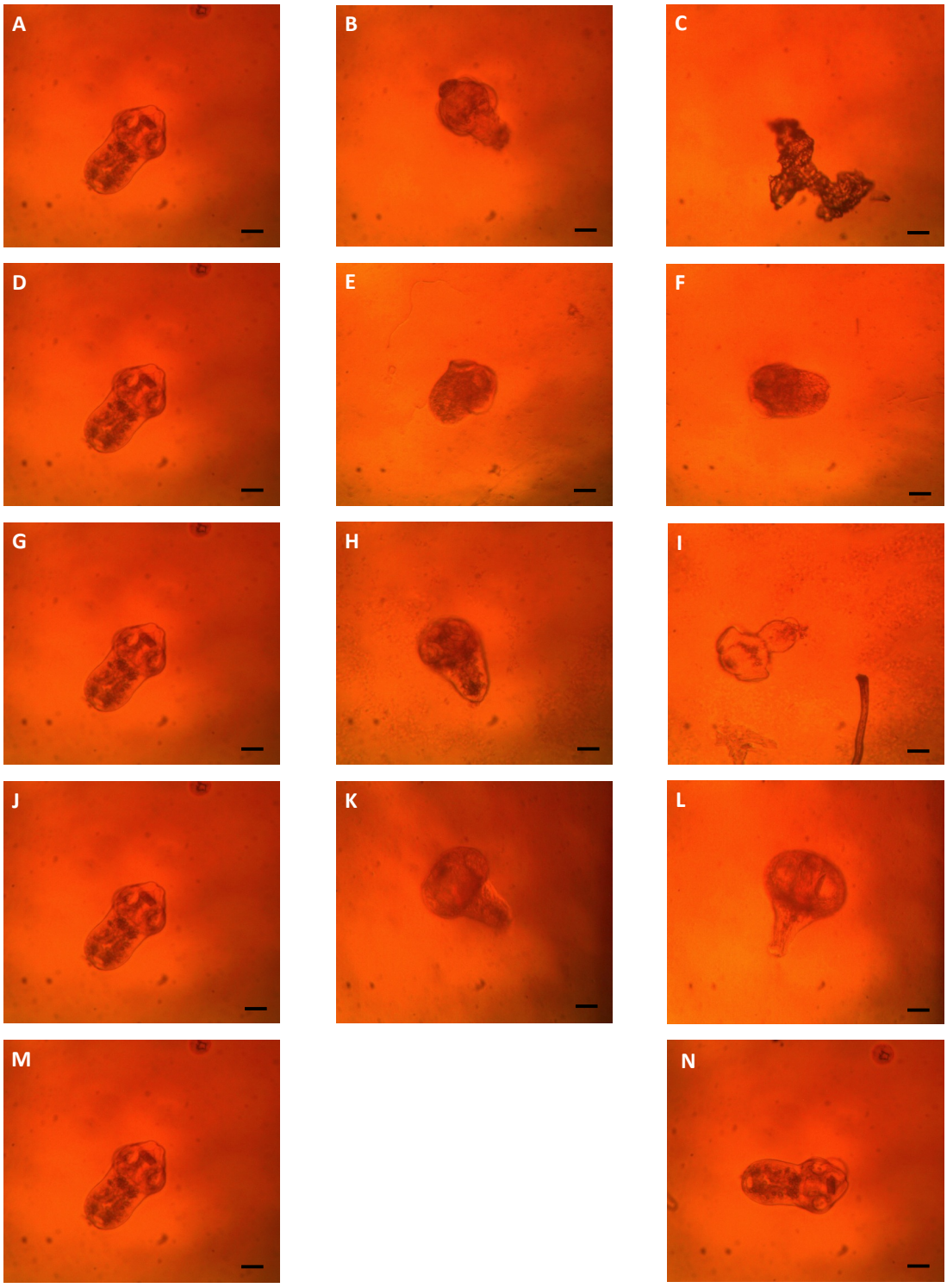


Fig 4.16 Light microscopy images of juvenile *H. microstoma* in various CPs
Artificially excysted *H. microstoma* scoleces incubated in PLS 1500µM+1mM HC (A-C), pineapple extract 300µM+1mM HC (D-F), papain 2000 µM+1mM HC (G-I), stem bromelain+1mM HC (J-L) and Hanks' saline (M-N) *in vitro*. The concentration of active CP expressed in µM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2). Scale bars = 61µm.

4.3 Discussion

Although some preliminary reports of the *in vitro* effects of CPs against cestodes have been published (He et al., 1992; Stepek et al., 2007e) these were either very brief one-off experiments or the effects were never confirmed sufficiently thoroughly, as had been done earlier for nematodes (Stepek et al., 2005). As in nematodes (Stepek *et al*., 2005), a marked reduction in motility of cestodes exposed to CPs was observed here and shown to be mediated by CPs, because worms incubated in CPs pre-treated with E-64 were not affected (Experiment 1). Moreover, the main effects of CP on worm motility were statistically significant while E-64 on its own or in combination with CPs did not reduce worm motility.

Although CPs have been shown to affect various adult nematode species (Stepek et al., 2005; Stepek et al., 2006b, 2007b, e) they were not effective against the juvenile stages of *H. bakeri* (Behnke et al., 2008) but did affect some juvenile plant nematode species (Stepek et al., 2007a). This suggests that the efficacy of CPs could be wider than previously thought and perhaps with further testing and improvements in drug formulation and delivery even nematode juvenile stages may eventually prove to be susceptible to better formulations of CPs. Data from this chapter have shown clearly that the efficacy of CPs are not limited to nematodes. CPs, whether naturally occurring like PLS and pineapple extract, or commercially available purified CPs such as papain and stem bromelain caused a significant reduction in motility leading to death of the worms in both rodent cestode models, and in each case irrespective of whether the worms were juveniles or adults. The reduction in motility on exposure to active CPs was also concentration dependant (Experiments 2,5,7&9) which is similar to the observation made on nematodes (Stepek et al., 2005).

However, there was a striking difference between our observations on cestodes and earlier published reports on nematodes in that the reduction in motility mediated by CPs against cestodes were independent of the presence of the exogenous CP activator cysteine, whilst inclusion of cysteine was crucial in the case of all the nematodes studied to-date (Stepek et al., 2005). Cysteine acts as a reducing agent and the active site on CPs needs to be activated through reduction before the enzyme can digest its target molecules (Chapman et al., 1997). Therefore, a possible explanation for this difference between cestodes and nematodes, the ability of CPs to still mediate damage to the tegument of cestodes independently of the presence of cysteine is perhaps because of the properties of the cestode tegument which differs fundamentally from the nematode cuticle in its structure and component composition. Perhaps through its excretory process, via the tegument, cestodes may be excreting some form of reducing agent that in turn activates CPs by donating electrons.

Various CPs have been screened for their efficacy against *H. bakeri* (a convenient and sensitive rodent nematode model system) *in vitro* ranging from naturally occurring CPs like crude papaya latex, pineapple extract, kiwifruit extract and milkweed latex to purified commercially available CPs like ficin, papain, chymopapain and stem bromelain (Stepek et al., 2005). The efficacy of various CPs against *H. bakeri* varied considerably with the purified enzymes like papain and chymopapain ranking as the most efficacious compared to naturally occurring CPs such as crude papaya latex and pineapple extract, while kiwifruit extract was not efficacious at all (Stepek et al., 2005). An interesting aspect of the current results is that a different hierarchy of efficacy has been observed here for cestodes. When

all the experiments in this chapter were compared it was shown clearly that irrespective of whether adult or juvenile stages were tested, the naturally occurring CPs such as those in pineapple extract and PLS were more efficacious than purified enzymes such as papain and stem bromelain. Commercially available purified enzymes are singular CPs while naturally occurring CPs contain a combination of more than one CP. For example papaya latex contains papain, chymopapain, glycyl endopeptidase and caricain while pineapple extract contains fruit bromelain, ananain and comosain. It is possible that the naturally occurring CPs work in synergy resulting in greater efficacy as compared to singular CPs working individually. A similar phenomenon was also observed *in vivo* when crude papaya latex was found to be more efficacious than papain in reducing *H. bakeri* burdens in mice (Stepek et al., 2007d). Kundu and Lyndem (2012) in screening the efficacy of several species of Cassia plant extracts against the poultry cestode *R. tetragona* observed that worms exposed to a combination of several species of cassia extracts resulted in greater efficacy and damage to the worms compared to singular species exposure. However, at present it is not fully understood as to why purified enzymes were more efficacious against *H. bakeri in vitro* compared to naturally occurring CPs. Perhaps it was the time point at which efficacy was determined by Stepek et al. (2005) who utilised IC_{50} values derived at 90min post incubation in CPs while in all my experiments I derived IC_{50} values at 30min post incubation because the longer the incubation time the more likely that other factors might be influencing the worms such as changes in the osmolarity of the incubation medium for example. Had I chosen a time point earlier than 30min, the effects might not have been so obvious between the different concentrations of active enzymes. It was evident in both rodent cestodes and in both adult and juvenile stages tested that pineapple extract was most efficacious (see tables 4.1-4.4) and this was followed by PLS, the purified enzyme papain and stem bromelain were the least efficacious (see tables 4.1-4.4). It is quite obvious that there was a considerable gap between the efficacy of pineapple extract and the other enzymes tested although this was not reflected proportionately by the apparent damage inflicted upon the tegument. In fact, the effects of pineapple extract on the tegument were different both in appearance under SEM and perhaps also with respect to the mechanistic basis. There appeared to be no marked tegumental erosions (Figs 4.4 J-L) as was observed in worms incubated in other CPs such as PLS (Figs 4.4 B-D), papain (Figs 4.4F-H) and stem bromelain (Fig 4.4N-P). Firstly, the pineapple extract which was used in this chapter was not extracted using the more common method which is through precipitation by acetone or ammonium sulphate. This is because in pilot attempts, both extraction methods yielded low concentrations of active CPs (results not shown). Hence the extraction process was stopped short after juicing and concentrating the fruit (see Chapter 2) to get the maximum amount of active CPs (300μM in the neat stock).While this amended protocol resulted in greater yield of active CPs it did nevertheless result in a less purified extract possibly containing other components of pineapple fruit as for example, sugars and minerals.

One particular component of interest is calcium as pineapples contain a high percentage of this element (Bartolomé et al., 1995). Worms exposed to pineapple juice appeared contracted on light microscopy (results not shown) and showed blebs resembling the tegumental changes described in cestodes incubated in praziquantel (Becker *et al*., 1981) which is the treatment drug of choice in most platyhelminth infection (Mansour, 2002). Praziquantel is thought to affect platyhelminth tegument by

disrupting calcium channels allowing calcium influx causing muscle contraction (Tallima and El Ridi, 2007b). Perhaps in similitude the CPs in pineapple extract may have also affected the tegument disrupting calcium channels allowing calcium influx evident by very superficial erosions appearing only after 20mins of incubation (Figs 4.4 K-L). This limited erosion is perhaps proportionate to the amount of active enzymes present in this particular pineapple extract stock used (300μM) because the extensive erosions seen in worms incubated in other CPs like PLS, papain and stem bromelain correlate to much higher concentrations of active CPs (5-7x more). This idea may be supported by the fact that purified stem bromelain which is also a CP from the pineapple plant exerted similar tegumental erosions comparable to PLS and papain without the contraction and other 'praziquantel- like' changes. Therefore, it can be hypothesized that if one was able to perform a more efficient extraction, yielding pure pineapplefruit CPs, only at much higher concentrations of active CPs, the resultant effect on the worms may be comparable to the other CPs tested in this chapter. Since the high efficacy of pineapple extract in this case cannot be attributable to CPs alone, it is safer to exclude this particular CP variant from further tests like *in vivo* experiments. In fact this particular pineapple extract have been tested twice *in vivo* against *H. bakeri* in the mouse (a very sensitive system) and also against *T. colubriformis* and *H. contortus* in sheep (unpublished work) and in each case it failed to reduce worm burdens.

Although the efficacy of different types of CPs against the same cestode varied, it did not differ substantially between the 2 different rodent cestodes tested in this chapter, whether adults or juvenile stages, and was comparable in both worms (Tables 4.1&4.2 for adult stage Tables 4.3&4.4 for juvenile stage) albeit with slightly more efficacy against *H. diminuta* . This came as a welcomed surprise since *H. microstoma* has been reported to be resistant to benzimidazoles (McCracken *et al.*, 1992). Although there was no substantial difference in the efficacy of CPs between the two different rodent cestodes, there was however a difference in the efficacy of CPs against the equine cestode *A. perfoliata* (Chapter 8) in which PLS was about 4x more efficacious. This was thought to be due to the relative shorter length of *A. perfoliata*.

However, there was a difference seen between the results from the experiments with adult worms and the juvenile worms in that whilst the efficacy differed between naturally occurring CPs and purified CPs when adult worms were tested, despite the difference in efficacy the range was quite narrow (tabs 4.1-4.2). For example, the IC_{50} values for PLS, papain and stem bromelain against adult *H. microstoma* were 1107, 1172 and 1451, respectively. In contrast when juvenile worms were tested the range of efficacy was much wider as for example 984, 2264 and 2339, respectively. There seemed to be a wider range of efficacy with purified CPs and naturally occuring CPs when tested against juvenile worms suggesting that the juvenile worms are more resistant to singular purified CPs but were comparably susceptible to naturally occurring CPs. This is perhaps because juvenile artificially excysted worms has not yet attained a strobila where most of the damage was seen to occur on adult worms. The adult worm scolex although affected by CPs (Figs 4.5 B-D) was comparatively less affected than its strobilar segments. This relative resistance of the scoleces to digestion by CP is in some ways reminiscent of the known resistance of tapeworm scoleces to immune attack. When worms are subjected to immune attack, they destrobilate (Befus and Featherston, 1974) and the scoleces alone survive until the adverse

conditions in the intestinal environment subside and the physiology returns to normal, when the surviving scoleces then proceed to re-grow a new strobila.

In the case of resistance to CPs it is possible that there is a deficiency of CP target proteins in cestode scoleces, which are otherwise present in abundance on the strobila. This assumption, however, will need to be explored and verified by protein studies. This possible explanation may also be reinforced by the observation that damage in juvenile worms was more pronounced after 2hrs of incubation (Fig 4.13 & 4.16) whereas extensive damage had already affected adult worm strobila even at 20min incubation in the same concentrations of active PLS (Figs 4.4 C). These observations are quite different from the observations seen with praziquantel which has a predilection for the neck region, while the strobila remains unaffected (Becker et al., 1981). Apart from this, the type of tegumental damage exerted by CPs is unique because it appears to erode the tegument hydrolysing certain proteins. This observation most certainly concurs with the observations made on nematodes where CPs digest the hard cuticle resulting in the bursting of internal contents due to high internal hydrostatic pressure (Behnke et al., 2008). Most conventional cestocidals however appear to attack the tegument from the inside out. Praziquantel for example disrupts ion channels allowing permeability of elements causing blebbing, that finally bursts the worms (Mansour, 2002) which is also similar to the consequences of exposure to niclosamide (Kumchoo et al., 2007). Benzimidazoles also result in similar tegumental changes but in this case as a result of disruption of the microtubule dependant transport system (Schmidt, 1998b).

The events observed cross-sectionally within the tegument through TEM (Figs 4.6B-C) however, are quite similar to other cestocidals, as most have reported an increase in the formation of vacuoles and vesicles and disruption in the glycocalyx outer layer (Becker et al., 1981; Mansour, 2002; Schmidt, 1998b). Schmidt (1998b) proposed that the vesicles may be transporting materials needed to synthesize and replenish the disrupted or lost glycocalyx layer. On a quick glance the appearance of tegument after 60min post incubation in PLS (Fig 4.6D) appeared to contradict the extensive damage seen in SEMs. On careful consideration, however, the TEM studies were focusing on the outermost layer at a much smaller scale (2µm) since the tegument is almost like a cell membrane while most SEM studies were captured at a larger scale (100µm- 1mm). On closer inspection, despite the generalised normalised appearance due to the lack of multiple vesicles and vacuoles seen earlier (10min & 20min), the outermost glycocalyx layer coating the brush border appear to have disappeared when compared to the control image (Fig 4.6A). This is perhaps suggestive that this particular image despite my attempt at capturing damage exactly where it may have occurred under light microscopy, represented a section of the tegument where rapid repair had taken place thus resulting in an otherwise comparatively normalized depiction.

In summary, the results from the experiments described in this chapter indicate that CPs are indeed efficacious against cestodes *in vitro* by significantly reducing their motility leading to the death of the worms and the effects were indeed mediated by CPs. Unlike in nematodes CPs are able to exert their proteolytic activity against cestodes without the presence of exogenous cysteine. Despite the limited nature and the uncertainty surrounding published reports on CP efficacy against juvenile stages of helminths, CPs were indeed efficacious against juvenile cestodes. Naturally occurring CPs appeared to

be more efficacious than purified CPs. Efficacy also appeared to be dependant upon cestode size since there was no obvious difference in efficacy between the 2 rodent cestodes but CP was more efficacious against the smaller equine cestode *A. perfoliata* (Chapter 8). As in nematode cuticles, CPs appeared to digest the tegument giving rise to a generalised eroded appearance that was evident from SEM studies but which was quite different from the known modes of action of conventional cestocidals.

CHAPTER 5

Assessment of the anthelmintic efficacy of cysteine proteinases against *H. microstoma in vivo*

5.0 Summary

H. microstoma is a natural parasite of house mice, and provides a convenient model system for the assessment of novel drugs for anthelmintic activity against cestodes. The experiments described in this chapter indicate that treatment of mice infected with *H. microstoma* with PLS is only minimally efficacious. The effects seen on worm burdens and worm biomass were only minimally dose dependant, not permanent and the role of cysteine proteinases as the active principles in PLS was not confirmed by specific inhibition with E-64.Worm fecundity was not affected at all.

5.1 Introduction

The anthelmintic effects of cysteine proteinases (CPs) against nematodes *in vivo* have been successfully demonstrated in pigs (Satrija *et al*., 1994) mice (Satrija *et al*., 1995; Stepek *et al*., 2006b, 2007b, c) sheep (Buttle *et al*., 2011) and even chickens (Mursof and He, 1991). They also work effectively against the 3 major human nematode infections (Hansson et al., 1986).

In comparison little is known about the effects of CPs on cestode infections. There is some evidence that CPs are detrimental to the survival of cestodes in *in vitro* maintained assays (He *et al*., 1992; Stepek *et al*., 2007e), but data also indicate that this is not reflected in a reduction of parasite burdens when CPs are administered to infected hosts (de Amorin *et al*., 1999; He *et al*., 1992). Using lattices from the fig species of *F. insipida* and *F. carica* at 3-4mls/kg/day for 3 days, De Amorin *et al*. 1999 failed to demonstrate a significant reduction of parasites in albino mice infected with *H. nana* (also known as *Vampirolepis nana*, *H. frat*erna and *Rodentolepis nana*; 6.3-8.3%). However, the methodology described in this paper was unclear as the worm burdens were expressed as a percentage of the wet weight of proglottids found in faeces in relation to total faecal and intestinal proglottids on autopsy and dry weight was not assessed. Moreover, quantification of enzyme activity was not carried out, which raises the question of enzyme viability. Using young pineapple juice at doses between 0.4-50%, He *et al.* (1992) also found no reduction in worm burdens in Swiss white mice infected with *H. nana*. However, the exact methodology applied in this Indonesian paper could not be ascertained as it is unavailable in the literature. To date, few results based on well designed *in vivo* trials have been published and whether CPs have *in vivo* activity against cestodes is still debatable. Nevertheless, it is very important to establish whether they are effective against cestodes *in vivo*, if potential drugs are to be taken forward to clinical trials and eventually are to be developed into useful therapeutic agents.

Three rodent infective cestode model systems are maintained widely (*H. microstoma*, *H. diminuta,* and *H. nana*) and have been used extensively to study the biology and host-parasite relationships of cestodes (see table 5.1). In my project I have only used the first two species, largely due

to their availability locally, here at Nottingham University. The latter species (*H. nana*), despite being medically important, poses disadvantages in experimental set ups because it has a direct life cycle and hence in conventional animal houses there is always a high probability of cross and re-infection, and spread throughout the mouse colonies.

Table 5.1. Similarities and differences between Hymenolepidids*

Physical characteristics	*H.microstoma*	*H.nana*	*H.diminuta*
Length	8-50mm	25-40mm	20-60cm
width	0.5-4mm	0.25-0.5mm	4mm
Armed rostellum	Yes	Yes	No
Ova size	~85 µm	~40 x 50 µm	~70 µm
Life cycle			
Requires intermediate host	Yes	No	Yes
Location in host	Bile duct	Small intestine (posterior section)	Small intestine
Prepatent period	16-17 days	14-16 days	19-20 days

*adapted from Fox (2007)

The first model is the bile duct dwelling mouse tapeworm *H. microstoma*. This species has attracted considerable interest during its classification history since it was first described by Dujardin in 1845. Much of the confusion about this species is due to the ambiguous use of the generic component of its Latin name which has at various times changed from *Taenia* (Dujardin, 1845) to *Rodentolepis* (Spasskii, 1954) and the more recent *Vampirolepis* (Schmidt, 1986) . Despite the lack of consensus and apparent plasticity of nomenclature, it is considered best to follow the latest proposal of Cunningham and Olson (2010) who prefer the genus *Hymenolepis* (Blanchard, 1891) based upon commonality of usage, until a convincing molecular based phylogeny is generated. Therefore, the parasite is referred to as *Hymenolepis microstoma* throughout this thesis.

While it is generally accepted that *H. microstoma* is specific to the mouse *Mus musculus*, some have reported successful infections in rats (Joyeux and Kobozieff, 1928) and there have also been reports of clinical cases (Macnish *et al*., 2003). However, other workers have reported failure to establish infections in rats (Dvorak et al., 1961; Goodall, 1972) and hamsters (Litchford, 1963). *H. microstoma* has an indirect life cycle in which arthropods such as flour beetles (e.g. *Tribolium confusum*) serve as intermediate hosts and autoinfection through eggs is not possible (Dvorak *et al*.,

1961). However, experimental direct infections in congenitally athymic mice have been demonstrated (Andreassen *et al*., 2004). Accidental ingestion of the arthropod host releases dormant cysticercoids which excyst in the duodenum and migrate to the bile duct within 4-5 days where they mature and start producing eggs 15-16 days post infection (De Rycke, 1966; Moss, 1971).

The ease of maintaining this species in the laboratory and its practical advantage over *H. nana* makes it a preferable model even to *H. diminuta* which require costlier maintenance due to its host being the rat. Moreover, it is one of the four tapeworm species to have its complete genome characterised (Cunningham and Olson, 2010) . Despite all the advantages, it is somewhat challenging and intriguing to learn that with respect to anthelmintic susceptibility, *H. microstoma* has proven to be a resilient species. From juvenile cysticercoids in the flour beetle intermediate host (Evans et al., 1979) to bile duct dwelling adult worms, all have shown resilience to benzimidazole based preparations (McCracken *et al*., 1992) compared to the highly susceptible *H. diminuta* (Coles, 1983) . Exact explanations remain elusive but one possibility may be that a large portion of the worm is protected from direct contact with drugs passing down the lumen of the intestine, because of its location higher up in the bile duct (McCracken *et al*., 1992).

Table 5.2. Growth of *H.microstoma* in *M. musculus* *

Days p.i.	Av. length(mm)	Development and position in gut
1-2	0.25-0.5	no external segmentation or genital system; worms localized in the first 10-20 cm of the intestine
3	1.58	some internal segmentation; appearance of genital system; worms localized in the first 10 cm of intestine
4-5	3.4-3.85	external segmentation and male & female genital system discernable; worms localized in the bile duct
6	5.85	testes in few segments
7	9.15	testes mature
8	13.5	early-mature to mature proglottids
9-10	17-20.5	all proglottides mature
11	27	disappearance of female glands; few pre-oncospheres
12	36	pre-oncospheres, no hooks
13	46.5	semi-gravid proglottides

14	62.5	near gravid proglottides
15-16	94-129	gravid proglottides

*adapted from (Cunningham and Olson 2010)

At the moment, crude coprological methods such as the Macmaster and Kato-Katz are still the mainstay in most treatment efficacy monitoring. While these techniques have been shown to be robust and accurate in gastrointestinal nematodes (Levecke *et al*., 2011) it has continued to be a problem in monitoring treatment efficacy in cestodes (Abbott and Barrett, 2008). The low sensitivity is thought to be due to the sporadic dispersion of gravid proglottides as well as the unequal distribution of eggs in the faecal mass (Nilsson *et al*., 1995). Lately, advanced methods such as immunological based detection have been shown to be more sensitive in detecting the horse tapeworm *A. perfoliata* infection (Skotarek *et al*., 2010). Ultimately, autopsy for the recovery of worms will provide an indication of treatment efficacy. Apart from worm counts, other autopsy indices such as worm biomass will in essence strengthen overall conclusions. Worm biomass assessment can either be done immediately after isolating worms from the host intestine (wet biomass) and/or also after removing worm fluid content by drying the worms (dry biomass).

Having demonstrated in the results presented in Chapter 3 that *H.microstoma* is susceptible to CPs *in vitro*, here I present the results of experiments that aimed to assess whether CPs also have detectable activity against a natural cestode of mice in its normal host *in vivo*.

5.2 Experimental design and results

Effects of CPs on adult patent stage of *H. microstoma in vivo*

5.2.1 Experiment 1

A pilot assessment of the anthelmintic efficacy of crude papaya latex and papaya latex supernatant against *H.microstoma in vivo*

5.2.1.1 Experimental design

This experiment was designed to assess the effect of crude papaya latex (CPL) as used in earlier work (Stepek *et al*., 2005) and material from a new source (Enzymase, Brussels, Belgium) which is referred to as papaya latex supernatant (PLS; see Chapter 2, Materials and Methods for further details) as used

in all of my *in vitro* experiments. Fifteen mice were used in this experiment (see chapter 2). Mice were divided into 3 groups (n=5). Group 1 received CPL at 240nmols, group 2 received PLS at 240nmols while group 3 received water, all daily for 5 days from day 27 until day 31 post infection. On the day of treatment, mice were fasted for 4-5 hours as opposed to the original protocol described earlier for 6 hours (Stepek et al., 2006b, 2007b, c) which was based on a clinical trial in Brazil (Hansson *et al*., 1986). One mouse died from group 1 during the course of treatment and was excluded from analysis reducing group 1 sample size (n=4).The effectiveness of treatment was determined through faecal egg counts as well by autopsy and recovery of worms and assessment of their biomass.

5.2.1.2 Results

The faecal egg counts (Fig 5.1A) in neither the group receiving crude papaya latex nor the group receiving papaya latex supernatant showed any convincing reduction compared to the control group receiving water. Indeed arithmetically the mean values were higher than the control group following treatment. This difference in FEC between treatment groups was not statistically significant (rmGLM, between subjects test, $F_{2,11}$=2.541 P=0.124) and there was no evidence of a divergence between treatment groups with time (within subjects test, time*treatment interaction $F_{3.6,19.8}$=2.08 P=0.127).

However, there was a significant difference in worm burdens (Fig 5.1B) between the treatment groups (Kruskal-Wallis test, χ^2_2=7.65, P=0.022). As can be seen from Fig 5.1B, worm burdens appeared to be lower in the animals treated with PLS. *Post hoc*, comparison of worm burdens in group 2 (treated with PLS) with group 3(treated with water) indicated that there was a significant difference between the worm burdens in these two groups (Mann-Whitney U test, z=-2.495, P=0.013).

However, there were no significant differences observed in either of the two biomass assessments (Fig. 5.1C). The mean wet biomass (Fig 5.1C) of both treatment groups did not show a difference as compared to the control and this was not statistically significant (Kruskal-Wallis test, χ^2_2=0.771, P=0.68). The mean dry biomass (Fig 5.1C) of both treatment groups, however, did appear to differ numerically from the value of the control group although this was not statistically significant (Kruskal-Wallis test, χ^2_2=4.271, P=0.118).

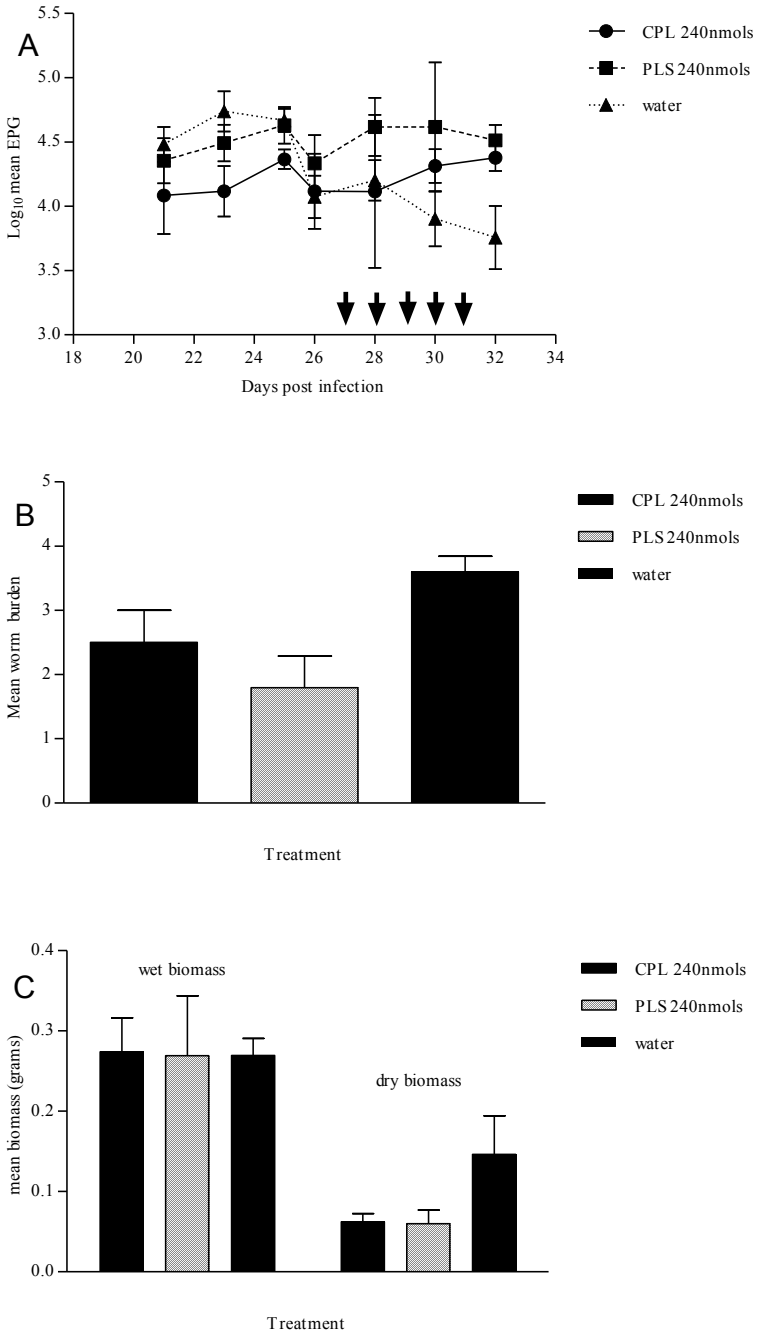


Fig 5.1 Efficacy of papaya latex against *H. microstoma in vivo*
Three indices used in assessing the effects of CPL and PLS on *H. microstoma in vivo*. (A) The log mean eggs per gram of faeces before (days 21-26) and during (days 27-31) treatment (arrows) of C3H mice infected with *H. microstoma* and treated with either CPL (*n*=4), PLS (*n*=5) or water (*n*=5). (B) The mean worm burden of C3H mice infected with *H. microstoma* and treated either with CPL 240 nmols (*n*=4), PLS 240nmols (*n*=5) or water (*n*=5). (C) The mean biomass of *H. microstoma* in C3H mice treated either with CPL 240 nmols (*n*=4), PLS 240nmols (*n*=5) or water (*n*=5). The amount of active CP expressed in nmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean.

5.2.2 Experiment 2

Assessment of the anthelmintic efficacy of PLS against *H.microstoma in vivo*

5.2.2.1 Experimental design

This experiment was designed as a follow up from the pilot experiment, Experiment 1. Eighteen mice were used in this experiment (see chapter 2). To increase the likelihood of detecting a significant effect, the number of animals per group was increased from 5 to 9. Mice were divided into 2 groups. Each mouse in treatment group 1 received 0.2 ml dose of PLS at 240 nmols while group 2 received 0.2 ml water. Unlike in experiment 1 where mice were starved prior to oral dosing, all animal experiments conducted afterwards involves no fasting as it was found that fasting had no effect on treatment outcome (Luoga *et al*., 2011). All groups received daily treatment for 6 days starting from day 27 until day 32 post infection. The effectiveness of treatment was determined through faecal egg counts which were performed every other day from day 22 till 32 post infection as well as by autopsy for recovery of worms and assessment of their biomass.

5.2.2.2 Results

The faecal egg counts (Fig 5.2A) in the group receiving PLS did not show any convincing reduction compared to the control group receiving water. Indeed, as in the first experiment, arithmetically the mean values were higher than the control group following treatment. This difference in FEC between treatment groups was not statistically significant (rmGLM, between subjects test, $F_{1,16}$=1.578 P=0.227) and there was no evidence of a divergence between treatment groups with time (within subjects test, time*treatment interaction $F_{3.702,59.232}$=0.763 P=0.544).

However, there were significant differences in worm burdens (Fig 5.2B) (Mann-Whitney U test, z=-2.789, P=0.005), wet biomass (Fig 5.2C) (Mann-Whitney U test, z=-2.518, P=0.012) and dry biomass (Fig 5.2C) (Mann-Whitney U test, z=-1.987, P=0.047) between the treated group and the control group.

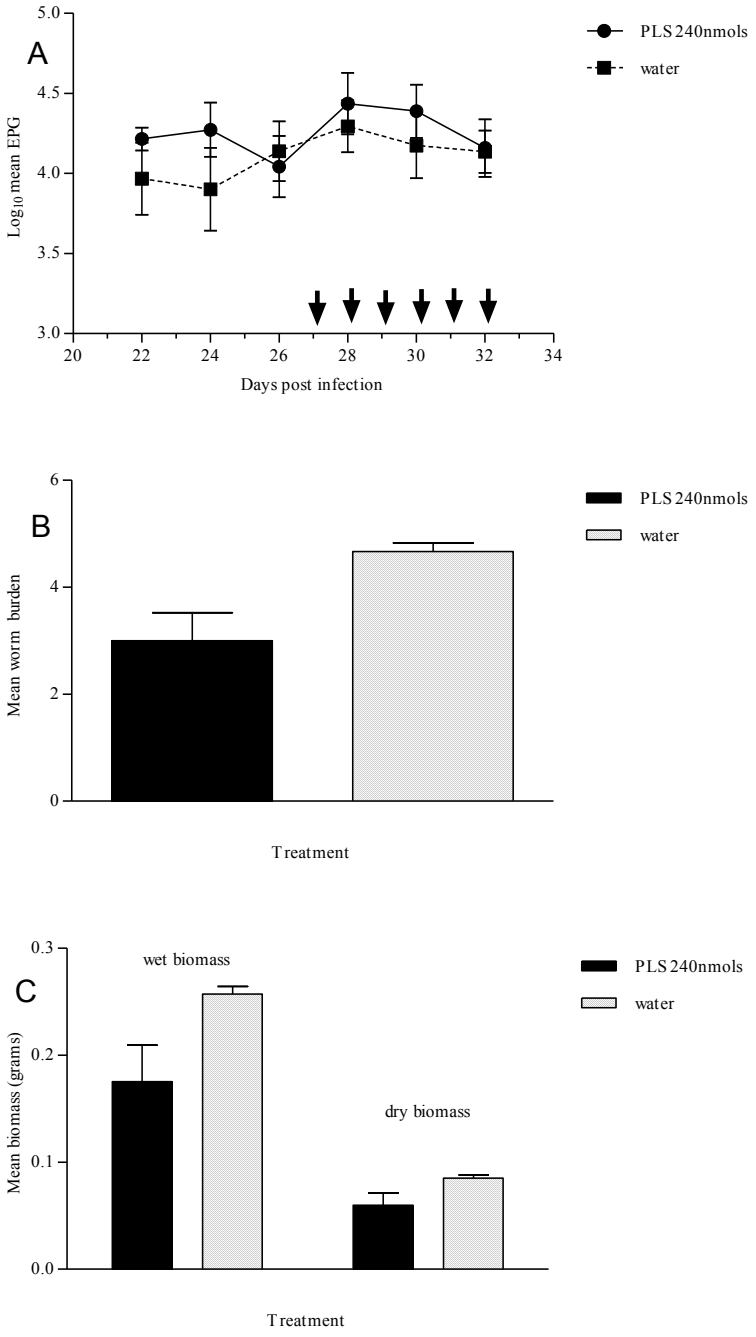


Fig 5.2 Efficacy of PLS against *H. microstoma in vivo*
The three indices used for assessing the effects of PLS on *H. microstoma in vivo*. (A) The log mean eggs per gram of faeces before (days 22-26) and during (days 28-32) treatment (arrows) of C3H mice infected with *H. microstoma* and treated with PLS (*n*=9) or water (*n*=9). (B) The mean worm burden of C3H mice infected with *H. microstoma* and treated with PLS 240nmols (*n*=9) or water (*n*=9). (C) The mean biomass of *H. microstoma* in C3H mice treated with PLS 240nmols (*n*=9) or water (*n*=9). The amount of active CP expressed in nmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean.

5.2.3 Experiment 3

H. microstoma egg viability experiment

5.2.3.1 Experimental design

This experiment was planned as a follow-up to Experiments 1&2 where *H. microstoma* fecundity appeared to have been unaffected by *in vivo* PLS treatment. Two mice were used in this experiment (see chapter 2). The two mice were treated differently; 1 received 2 ml dose of PLS at 240 nmols and the other received 2 ml dose of water. Unlike in experiments 1 & 2 where treatment was commenced much later (day 27 post infection) for better egg counts there was no need to wait egg production to reach it's peak hence treatment was started earlier (day 17) as *H. microstoma* would have reached the gravid proglottid stage by this time (Cunningham and Olson, 2010). Both mice received daily treatment for 6 days starting from day 17 until day 22 post infection. On the 6th day of treatment (day 22), both mice were autopsied and their worms removed. Three worms were isolated from each of the two mice. From each worm, the distal most proglottid segments of ~1cm (longitudinal length) were cut and fed to a group of 3 previously starved *T. confusum* beetles, hence nine beetles were exposed to cestode eggs from each of the two treatments of the mouse hosts. From the same mice, their colons were dissected to recover any detached proglottid segments. Any detached proglottid segment found in each animal were fed to 3 more previously starved (5 days) *T. confusum* beetles. After indications that the fed segments have been consumed, regular beetle diet, consisting of a mixture of white and wholemeal flour, was added for conventional maintenance until after 30 day when all the beetles were dissected in Hanks' saline to recover any developed cysticercoids.

5.2.3.2 Results

T. confusum beetles fed with the cut terminal proglottid segments from *H. microstoma* which had been treated *in vivo* with PLS were found to harbour intact cysticercoids (Table 5.3). In fact the mean number of cysticercoids recovered was comparable to that of the control group of beetles fed worms from mice which had been treated with water (Fig 2A). Beetles fed with detached proglottids segments recovered from the colon of treated mice also harboured intact cysticercoids (Table 2B) and the mean burden was comparable to that in the control beetle group fed with detached proglottids from mice treated with water (Fig 2B).

Table 5.3 *H. microstoma* egg viability test
The number of *H. microstoma* cysticercoids recovered from *Tribolium* beetles (*n*=9/ treatment group) fed with *H. microstoma* distal proglottid cut segments (from three *worms*) from each of two mice treated *in vivo* either with PLS or water

H. microstoma in vivo treatment	Proglottid segment origin	No of cysticercoids recovered		
		Beetle1	Beetle 2	Beetle 3
PLS	Worm 1	0	1	0
	Worm 2	14	4	39
	Worm 3	1	30	10
Water	Worm 1	24	26	38
	Worm 2	0	0	0
	Worm 3	2	11	17

Table 5.4 *H. microstoma* egg viability test
The number of *H. microstoma* cysticercoids recovered from *Tribolium* beetles fed with detached *H. microstoma* proglottid segments from the colon of a mouse infected with *H. microstoma* and treated *in vivo* either with PLS (*n*=3) or water (*n*=3).

H. microstoma in vivo treatment	No of cysticercoids recovered		
	Beetle 1	Beetle 2	Beetle 3
PLS	6	7	0
water	5	5	2

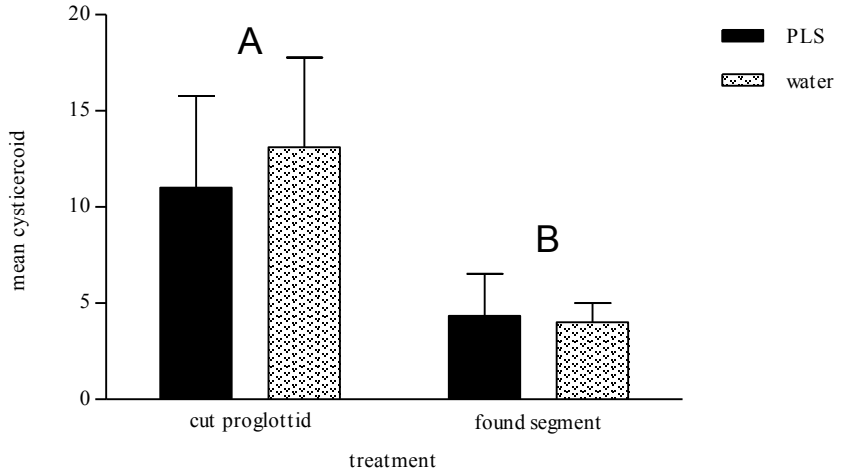


Fig 5.3*H. microstoma* egg viability experiment
(A) Mean number of cysticercoids recovered from *Tribolium* beetles (*n=9*) fed with distal cut proglottid segments (*n=3*) from *H. microstoma* from C3H mice (*n=2*) treated *in vivo* either with PLS 240nmols or water. (B) Mean number of cysticercoids recovered from *Tribolium* beetles (*n=3*) fed with colon found detached segments (*n=3*) from C3H mice (*n=2*) treated *in vivo* either with PLS 240nmols or water. The amount of active CP expressed in nmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean.

5.2.4 Experiment 4

Confirmation of the effects of PLS against *H.microstoma in vivo*

5.2.4.1 Experimental design

This experiment was a follow-up from Experiment 2 to confirm the effects demonstrated in the reduction in worm burden and biomass. Twenty mice were used in this experiment (see chapter 2). Although the previous Experiment 2 incorporated the use of a larger sample size *n=9*, this experiment was attempted using a smaller sample size *n=5* because more groups were needed to factorially confirm the effect of PLS. Mice were divided into 4 groups. Each mouse in treatment group 1 received 0.2mls dose of PLS at 240 nmols. Group 2 received 0.2 ml dose of PLS at 240nmols + E-64 at 280 nmols. Group 3 received 0.2 ml dose of E-64 at 280 nmols and group 4 received 2 ml water. All groups received daily treatment for 6 days starting from day 17 until day 22 post infection. In this experiment, assessment of efficacy of treatment through faecal egg counts was not carried out due to the earlier results which had shown no differences between treated and control groups (Experiments 1 & 2). Therefore, worms were subjected to earlier treatment (day 17 post infection for the first dose of PLS, rather than day 27 post infection). Treatment effects were only assessed at autopsy through recovery of worms and assessment of their biomass.

5.2.4.2 Results

Arithmetically, there was only a 10-15% reduction in worm burdens when comparing mean values between the treated group and other control groups (Fig 3A). However, this was not statistically significant (Kruskal-Wallis test, χ^2_2=6.97, *P*=0.73). Unexpectedly and surprisingly, in the wet biomass assessment, the mean values of the group treated with PLS was higher when compared to other control groups except for the group receiving PLS + E-64 which had the highest mean biomass (Fig 3B). This was also not statistically significant (Kruskal-Wallis test, χ^2_2=3.263, *P*=0.353). In dry biomass assessment, there was a 15-40% reduction when comparing the mean values between the treated group and other control groups (Fig 3C). However, this was not statistically significant (Kruskal-Wallis test, χ^2_2=4.077, *P*=0.253). The data were analyzed (Figures 3A, 3B and 3C) by 2-way ANOVA (treatment with PLS and/or E64) and the effect of PLS was not significant on worm burden (*P= 0.384*), wet biomass (*P=0.202*) or dry biomass (*P=0.668*). Interestingly, however, the main effect of E-64 was significant on worm burdens $F_{(1,4)}$=7.2 *P*=0.016;(Mean for E-64 treated mice= 5 Mean for not treated=4.4) and dry biomass(*P*=0.049;Mean for E-64 treated mice= 0.10793 Mean for not treated=0.012276) but not on wet biomass (*P*=0.376).

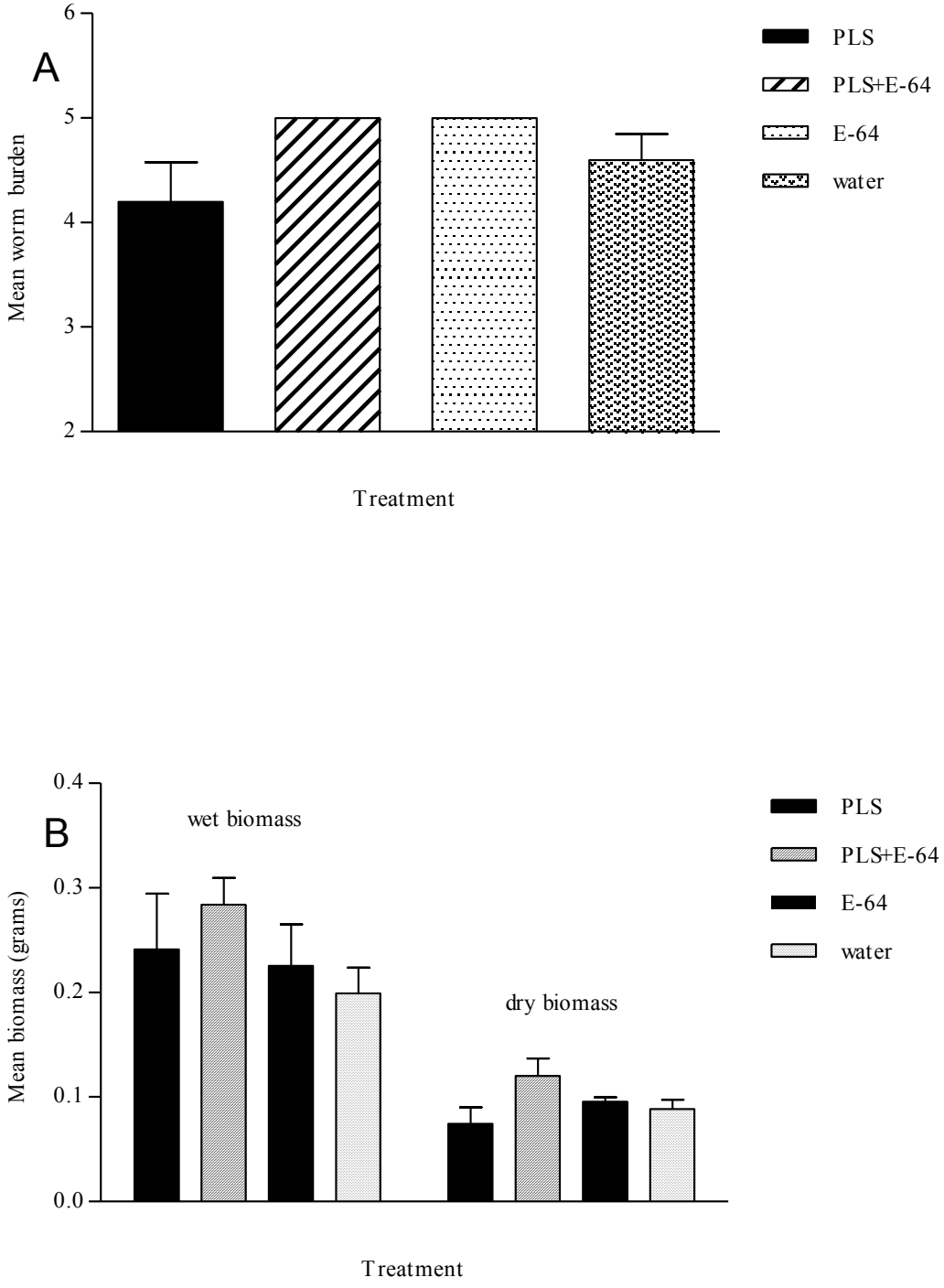


Fig 5.4 Confirmation of the effects of PLS against *H. microstoma in vivo*
(A) Mean worm burden (*n=5*) of C3H mice infected with *H. microstoma* and treated either with PLS 240nmols, PLS 240nmols pre-treated with 280nmols E-64, E-64 280nmols or water. (B) Mean biomass (wet & dry) (*n=5*) of C3H mice infected with *H. microstoma* and treated either with PLS 240nmols, PLS 240nmols pre-treated with 280nmols E-64, E-64 280nmols or water. The amount of active CP expressed in nmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean.

5.2.5 Experiment 5

Observation of PLS mediated damage against *H. microstoma in vivo*.

5.2.5.1 Experimental design

This experiment was simply a further extension of Experiment 4 to graphically document the effects of PLS inflicted on *H. microstoma in vivo*. Four mice were used in this experiment (see chapter 2). Due to the non-analytical nature of the experiment only 1 mouse (*n=1*) per group was used to represent each of the 4 different treatment combinations. Mice were divided into 4 groups. Group 1 received 0.2mls dose of PLS at 240nmols. Group 2 received 0.2mls dose of PLS at 240nmols + E-64 at 280nmols. Group 3 received 0.2mls dose of E-64 at 280nmols and group 4 received 0.2mls water. All the mice received daily treatment for 6 days starting from day 17 until day 22 post infection. The efficacy of treatment was assessed through the recovery of detached proglottids found in the large intestine downwards which were immediately fixed in glutaraldehyde and later dehydrated in ethanol, critical point dried and gold coated before finally being viewed under scanning electron microscopy for signs of any damage.

5.2.13 Results

The detached segment recovered from the PLS treated mouse appears to sustain treatment related damage albeit not extensive. On closer inspection (Fig 5.5A) there appears to be very superficial erosions which was not present in all the other segments (Figs 5.5B-D) found from other treatment combinations, all of which maintained their rather velvety appearance due to the intact brush border layer of the tegument.

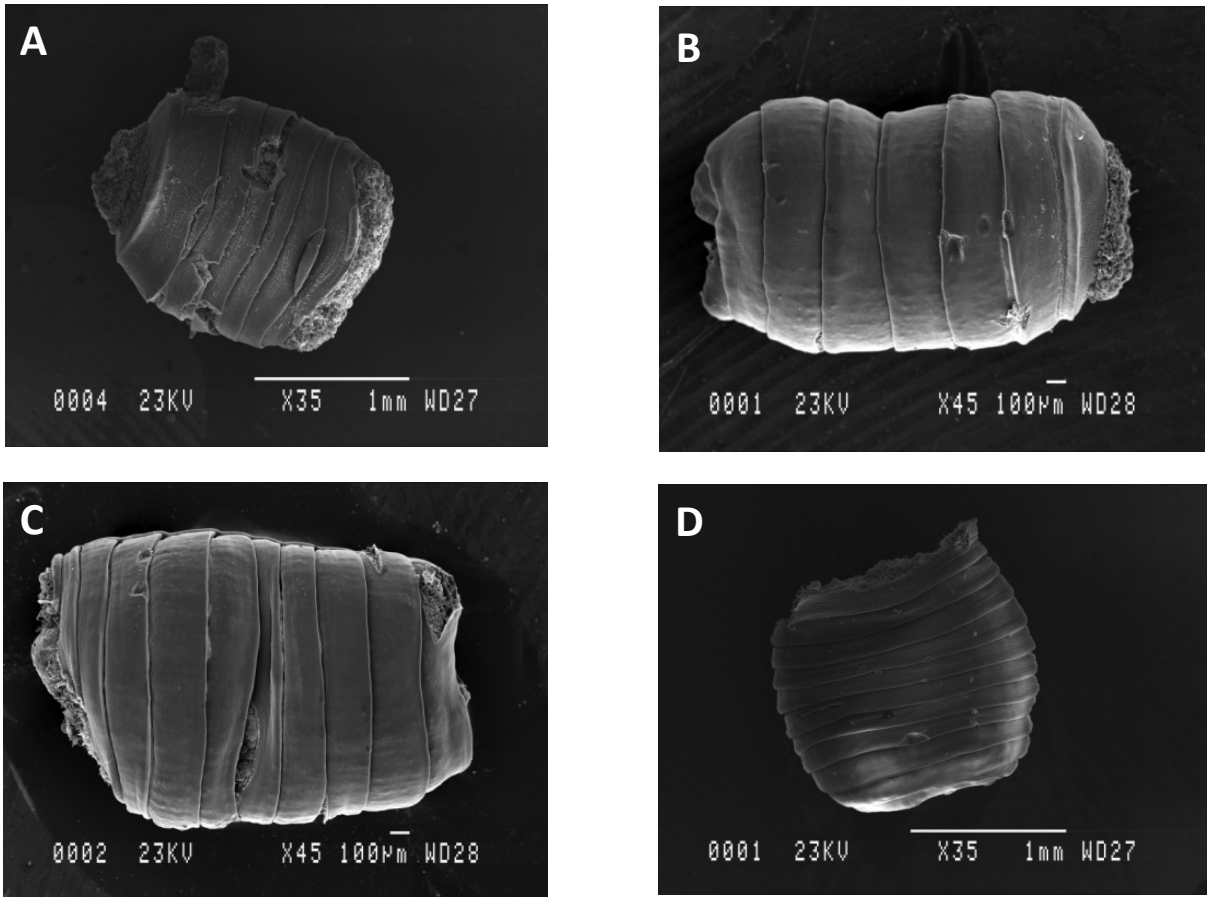


Fig 5.5 SEM images of the effects of PLS against *H. microstoma in vivo*
SEM images of detached proglottid segments of *H. microstoma* recovered from the colons of infected mice treated with (A) PLS 240 nmols (B) PLS 240 nmols pre-treated with 280 nmols E-64 (C) E-64 280 nmols (D) water. Note the superficial erosions in (A) and the otherwise velvety appearance in other segments. The amount of active CP expressed in nmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2).

5.2.6 Experiment 6

Assessment of dose-dependency of PLS treatment against H. microstoma in vivo

5.2.6.1 Experimental design

This experiment was carried out to assess whether or not the reduction in worm burdens and biomass effects demonstrated by PLS against *H. microstoma* in Experiment 2 was dose- dependant. Twenty mice were used in this experiment (see chapter 2). Compared to previous Experiments 1 & 2 which sought to observe significance between treatments, this experiment was a correlational study between PLS dose and worm autopsy indices. Therefore, a smaller sample size *n=3* was thought to be generally sufficient for each level of treatment. Mice were divided into 7 groups of different doses of PLS. This experiment was also executed simultaneously with Experiment 3 in which mice from the group receiving water were incorporated as group 1 (0 nmols dose) in this experiment *n*=5 and mice receiving 240 nmols of PLS *n*=5 were incorporated as group 5. Group 2 *n=3* received 30 nmols of PLS, group 3 *n*=3 received 120 nmols, group 4 *n*=3 received 180 nmols, group 6 *n=3* received 360 nmols and group 7 *n*=3 received 480 nmols. All groups received treatment daily for 6 days starting from day 17 until day 22 post infection. Treatment effects were assessed through worm count and biomass assessment at autopsy.

5.2.6.2 Results

Autopsy revealed a weak but significant negative correlation between PLS dose and worm burden (*R*=-0.397 *n*=25 P=0.049) (Fig 5.6A). There was also a weak negative correlation between PLS dose and dry biomass which was not significant (*R*=-0.265 *n*=25 *P*=0.201) (Fig 5.6B). The wet biomass in contrast showed a weak positive trend with increasing doses of PLS but this was not significant as well (*R*=0.206 *n*=25 *P*=0.323) (Fig 5.6B).

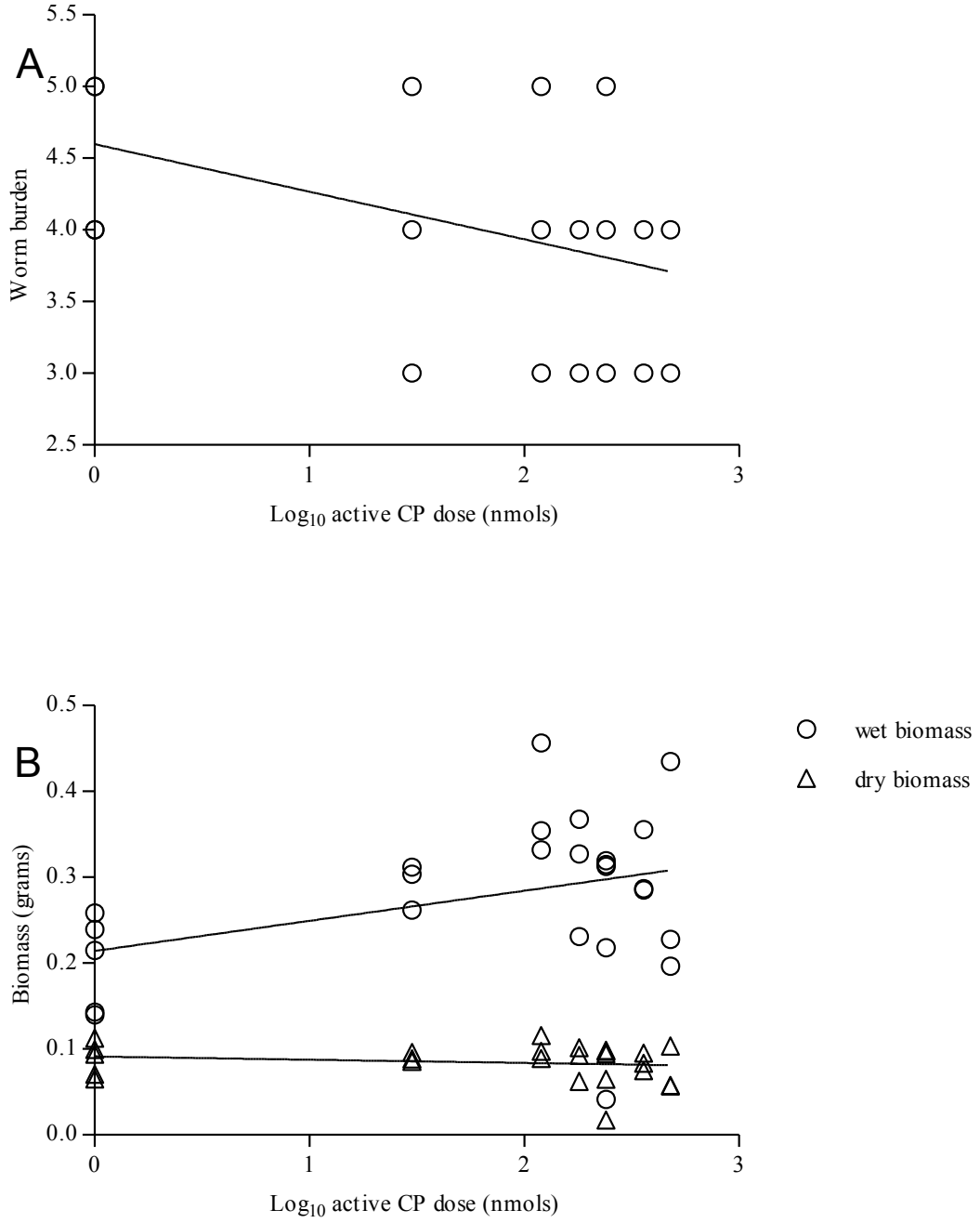


Fig 5.6 PLS dose dependency against *H. microstoma in vivo*
Three indices used to assess the relationship between PLS dose and *H. microstoma in vivo*. (A) The correlational trend between increasing PLS dose and worm burden.(B) The correlational trend between increasing PLS dose and biomass. All groups were of the same sample size (*n=3*) except groups 1 and 5 (*n=5*). For statistical analyses see text. The line of best fit is provided to guide the eye. The amount of active CP expressed in nmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2).

5.2.7 Experiment 7

Assessment of *H. microstoma* recovery *in vivo* post PLS treatment

5.2.7.1 Experimental design

This experiment was designed to assess whether or not *H. microstoma* have the ability to recover *in vivo* post PLS treatment since tapeworms have been shown to repair themselves quickly after sustaining damage (Befus and Threadgold, 1975). Twenty mice were used for this experiment (see chapter 2). The mice were divided into 4 groups with 5 mice per group. Group 1 received 0.2 ml PLS at 240 nmols, group 2 received 0.2 ml water, group 3 received 2 ml PLS at 240 nmols and group 4 received 0.2 mls water. All groups received daily treatment for 6 days from day 17 until day 22 post infection. Groups 1 & 2 were sacrificed immediately at the end of the treatment week (day 22 post infection) while groups 3 & 4 were left to continue without treatment for another week until day 28 of infection. The effectiveness of treatment was determined through autopsy for recovery of worms and assessment of their biomass on days 22 and 28 post infection.

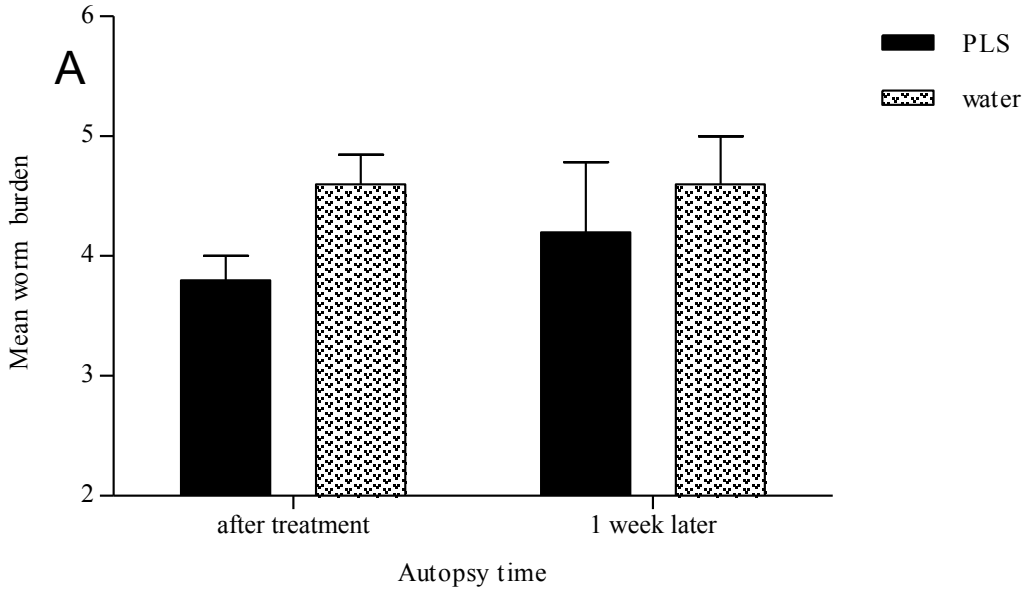
5.2.7.2 Results

Among all three indices studied there were obvious reductions among worms in treated mice compared with those among the controls irrespective of autopsy time. However, there were appreciative increases in worm burden and biomass when animals were autopsied later on day 28. Data were further explored using 2-way GLM to assess the main effects of time and treatment and also the interaction between the two variables.

The effect of treatment on worm burden was not significant $F_{1,16} = 2.4$ $P=0.141$ (although treated mice had 17.4% fewer worms than controls after treatment and 8.7% fewer worms than controls when autopsied 1 week later) and neither was the effect of time on worm burden $F_{1,16} = 0.267$ $P=0.613$. There was no significant interaction between time and treatment on worm burden $F_{1,16} = 0.267$ $P=0.613$.

The effect of treatment on wet biomass, however, was close to significance $F_{1,16} = 3.514$ $P=0.079$ and the percentage reduction in wet biomass was 24.2% post treatmnet and 16.6% when autopsied 1 week later. Surprisingly, the effect of time was also significant $F_{1,16} = 4.596$ $P=0.048$, wet biomass values being higher in both treated and control mice on day 28 compared with day 22. However, the interaction of treatment and time was not significant $F_{1,16} = 0.023$ $P=0.881$.

The effect of treatment on dry biomass was significant $F_{1,16} = 6.268$ $P=0.024$. The effect of time was also significant $F_{1,16} = 4.681$ $P=0.046$. Again, perhaps surprisingly, there was no significant interaction between time and treatment $F_{1,16} = 0.086$ $P=0.773$.



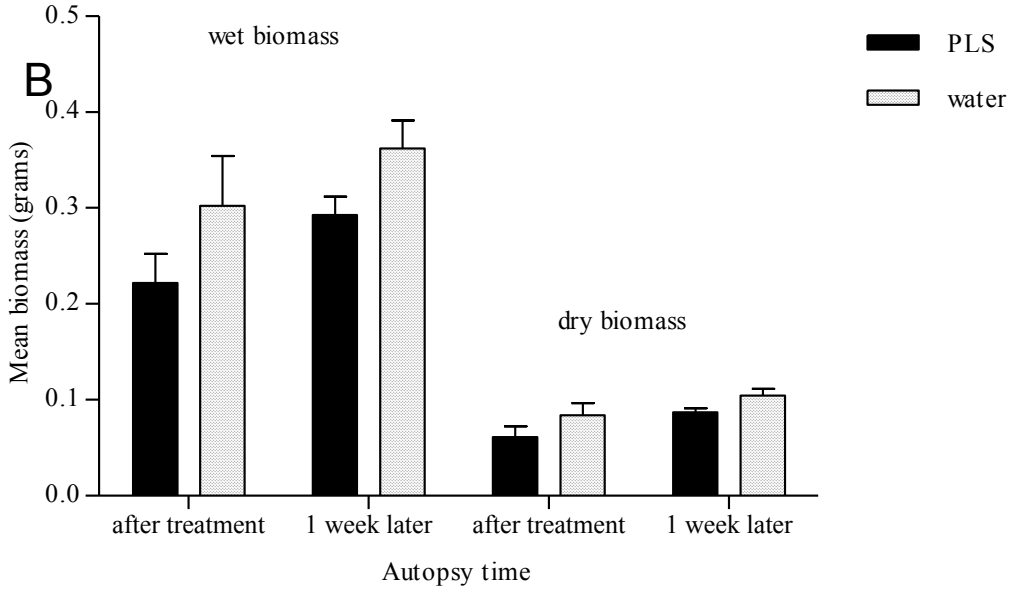


Fig 5.7 *H. microstoma* recovery post PLS treatment
(A) The mean worm burden of C3H mice infected with *H. microstoma* immediately after 6 days treatment of either 240nmols of PLS or water (*n*=5) and 1 week after withholding treatment (*n*=5). (B) The mean biomass of *H. microstoma* in C3H mice immediately after 6 days treatment of either 240nmols of PLS or water (*n*=5) and 1 week after withholding treatment (*n*=5). The amount of active CP expressed in nmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean.

Effects of CPs on prepatent stages of *H. microstoma in vivo*

5.2.8 Experiment 8

Assessment of the anthelmintic efficacy of PLS against *H. microstoma* during the migratory phase *in vivo*

5.2.8.1 Experimental design

This experiment was designed to test the hypotheses that young and still migrating *H. microstoma* which are present in the lumen of the intestine for 3-4 days before entering the bile duct, and therefore should be fully exposed to luminal CP after treatment, are more susceptible to CP than the adult worms as shown in earlier experiments. Eighteen mice were used in this experiment (see chapter 2). To increase the likelihood of a significant effect, the number of animals per group was increased from 5 to 9. Mice were divided into 2 groups. Each mouse in treatment group 1 received 0.2mls dose of PLS at 240 nmols daily for 5 days starting from day 2 until day 6 post infection, while mice in the control group 2 received 0.2mls water. Because 6 day old worms will be too small to recover, the experiment was extended until day 14 post infection by which time the worms have attained a reasonable and more easily quantifiable size. The effectiveness of treatment was determined through autopsy for recovery of worms and assessment of their biomass.

5.2.8.2 Results

Autopsy revealed significant effects of treatment on worm burden and biomass in the treated group as compared to the control group. There was a significant difference seen in worm burdens (Fig 5A) (Mann-Whitney *U* test, z=-2.147, P=0.032), in the wet biomass (Fig 5B) (Mann-Whitney *U* test, z=-3.311, P=0.001) as well as in the dry biomass (Fig 5C) (Mann-Whitney *U* test, z=-2.474, P=0.013) between the treated group and the control group.

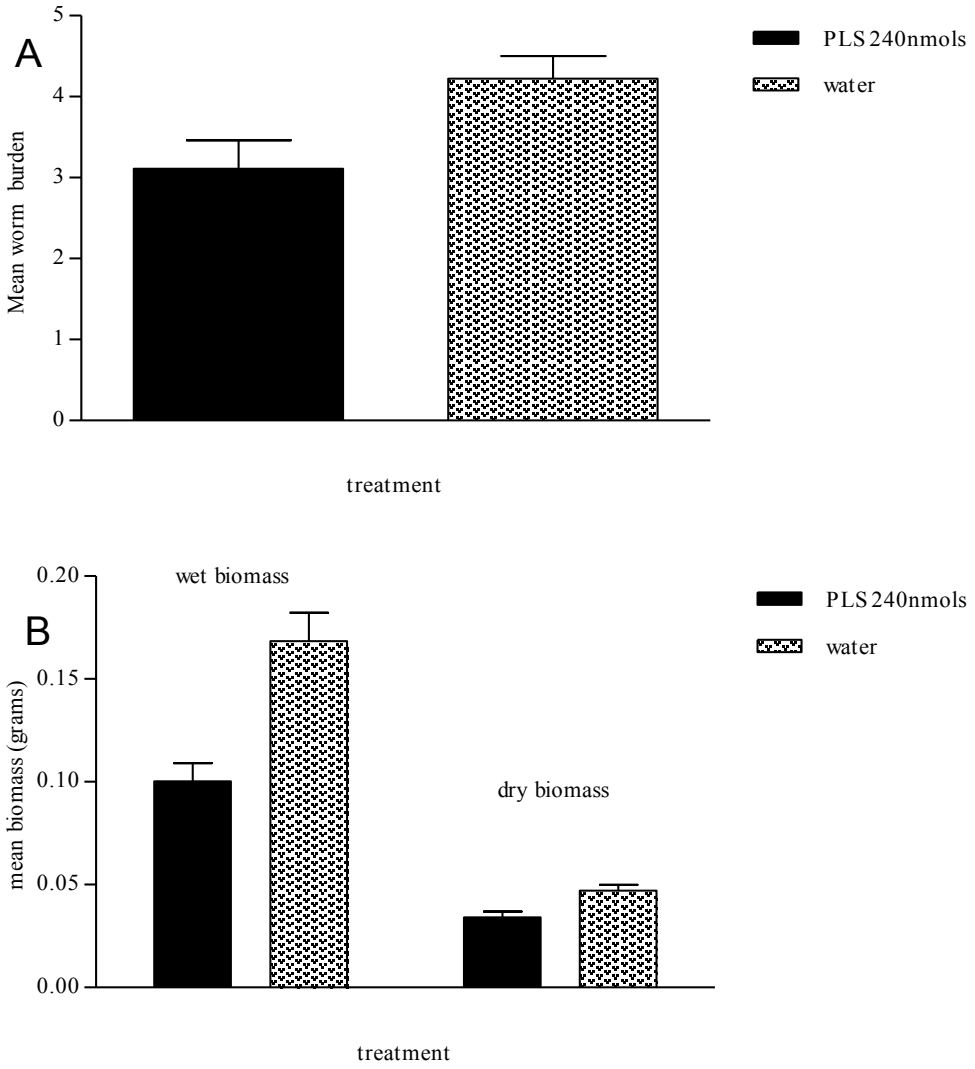


Fig 5.8 PLS effects on migratory stages of *H. microstoma*
The autopsy indices used to assess the effects of PLS on the migratory phase *H. microstoma in vivo*. (A) The mean worm burden of C3H mice infected with *H. microstoma* and treated with PLS 240 nmols (*n*=9) or water (*n*=9). (B) The mean biomass of *H. microstoma* in C3H mice treated with PLS 240 nmols (*n*=9) or water (*n*=9). The amount of active CP expressed in nmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean.

5.3 Discussion

The experiments described in this chapter are the very first to test the effects of papaya latex on the cestode *H. microstoma*, there being no reports in the literature on assessment of CP on this species in laboratory trials. In Experiments 1 and 2 in which faecal egg counts were employed as a measure for assessment of treatment efficacy, the results showed no reduction in mean EPG in treated groups as compared to the controls, despite the reductions in worm burden and biomass. This was somewhat surprising because as much as 90% reduction has been seen in faecal egg counts of nematodes such as *H. bakeri* treated with papaya latex (Stepek et al., 2007c). There are several possible explanations.

Firstly, it has to be emphasized that *H. microstoma* is a bile duct dwelling worm with only parts of its strobila exposed in the intestinal lumen. Since the administered CPs are primarily, going to be passing down the intestinal lumen, the scolex and neck regions are not likely to be exposed to high concentrations of the CPs. Hence the areas of major contact between CPs and the cestode are going to be in the terminal proglottids. In a short term administration protocol such as this, damage to these terminal proglottids, may well be reflected in loss of weight of these terminal, gravid proglottids, by their premature loss from the strobila, but also in liberation of the eggs that had formed in the developing and mature proglottids. The actual generation of eggs, however, may be protected from the effects of CP by their location in the bile duct.

Direct exposure of the surface of parasites to CPs appears to be a very important factor in this potential novel drug as CPs are poorly absorbed systemically. This was evident in the case of the sheep nematode *T. colubriformis* which tunnels deep inside the intestinal mucosa during developmental stage whose fecundity was not affected by papaya latex treatment whereas another sheep nematode *H. contortus* whose life cycle is entirely luminal succumbed (Buttle *et al*., 2011). Even if it appears that treatment with CPs did not affect FEC this does not conclusively establish that fecundity is not affected. Firstly, the faecal egg count is probably a poor indicator of the worm burdens in tapeworm infections as evident also by the wide error bars of the mean EPG values before treatment, suggesting high variability of eggs found in faeces (Figs 5.1A & 5.2A). These may be explained by the sporadic discharge of gravid segments as well as their unequal distribution in the faecal mass (Nilsson et al., 1995). This is especially the case with the horse tapeworm *A.perfoliata* where coprological detection of infection is unreliable due to low sensitivity (Abbott and Barrett, 2008) and in this system it has been reported that there is no correlation between tapeworm burden and egg detection (Meana *et al.*, 1998; Proudman and Edwards, 1992).

Nevertheless, even though there were many eggs in the faeces of infected mice post treatment, it was possible that the eggs had experienced more subtle harmful effects limiting their capacity to develop normally after exposure to CPs. It was then imperative to ascertain whether fecundity was affected despite no reduction in egg counts through an egg viability test (Experiment 3). Disappointingly, *H. microstoma* eggs were not affected at all because intact *H. microstoma* cysticercoids were recovered from *Tribolium* beetles fed with either cut terminal proglottid segments or detached proglottid segments recovered from the colons of mice infected with *H. microstoma* and treated with PLS. PLS did not appear to have had any effect on *H. microstoma* fecundity when administered to infected mice. A similar result, with unaffected fecundity, was also observed in PLS treated rats infected with *H. diminuta* (Chapter 7).

Structurally, cestode eggs have a thicker outer shell compared to trichostrongyloid nematode eggs such as those of *H. bakeri*, but not those of *Trichuris* and *Ascaris* species. This robust egg shell is required because of the need for the eggs to survive under harsh external environmental conditions before continuing its life cycle in an intermediate host.

While it can be concluded that the arithmetic mean worm counts were reduced numerically in all the experiments reported here (1, 2, 4,7&8), there was considerable variation in the degree of reduction, ranging from 9% (Experiment 3) to 50% (Experiment 1) and not all were statistically significant especially in experiments utilising smaller sample sizes. A confirmatory experiment with E-64 also failed to demonstrate any significant main effects of PLS treatment relative to the other control treatments (Experiment 4). Interestingly, the dose of PLS was found to be significantly negatively correlated with worm burden (Experiment 6), suggesting that fewer worms survived treatment when the dose was high. This dose-dependent reduction in worm numbers may suggest that despite the location of the scoleces in the bile duct, they were in fact not well protected from the effects of treatment with PLS. However, the recovery of tiny scoleces is never 100% accurate and there remains the possibility that as a result of the stress on the strobila from the CPs, the worms had destrobilated and their scoleces still persisted somewhere in the intestine without having been detected at autopsy and post-mortem examination. Indeed in Experiment 7 mice autopsied later, in fact a week after the completion of treatment harboured more and bigger worms compared to those autopsied immediately after treatment. However, this effect had no connection with treatment as the interaction of treatment and time was not significant. This suggests that the reductions seen in earlier experiments were temporary and that *H. microstoma* do re-grow post treatment. In comparison, *H. diminuta* in a similar experiment but receiving a higher dose of PLS (Chapter 7) were smaller.

When recovered worms were subjected to biomass assessments, there were notable inconsistencies especially in wet biomass assessments and instead of finding reductions, in some cases the arithmetic mean worm biomasses were higher than those of the control untreated groups of mice (Experiment 1 and 4). Even more counter intuitively, in one instance the PLS dose was found to be positively correlated with wet biomass (Experiment 6). This could be due to several factors. Firstly, the process of removing excess fluid that surrounds and coats the worms is never 100% reliable using filter paper. This is why a second biomass assessment is made when worms are completely dry after removal of all fluid content by drying the worms in an oven. Secondly, cestodes being different in structure from nematodes and lacking a tough cuticle might be susceptible to fluid influx due to dysfunctional membrane transport systems from damaged or dead proglottids, thereby resulting in unexpected biomass values. This possibility is further strengthened when it is considered that all the mean dry biomass assessments showed an arithmetic reduction compared to the control groups, with the percentage reductions ranging from 16%(Experiment 4) to 59%(Experiment 1). Despite this consistent reduction trend, statistical analyses were inconsistent and could not be confirmed with E-64 treatment (Experiment 4) especially in experiments using small sample sizes (Experiment 1 and 4). This goes in tandem with the relationship with PLS dose which showed a negative correlation with dry biomass but was not statistically significant. Therefore, despite consistent arithmetic reductions of treatment group mean values and negative correlations, the wide variation in data distribution and hence non-significant outcomes of statistical analyses can only mean that papaya latex is only minimally efficacious against *H. microstoma in vivo*. This was also depicted graphically in Experiment 5 where the detached proglottid segments found from the mouse treated with PLS showed only a minimal effect on the tegument compared to the more profound damage observed in *H. diminuta* (Chapter 7).

One proposed explanation for the relatively weak effect of PLS on this worm is that the *H. microstoma* scolex which resides high in the bile duct is largely protected against the effects of CP (McCracken *et al.*, 1992). This prompted Experiment 8 in which young and still migrating worms, fully exposed in the intestinal lumen, would be subjected to CP treatment. However, if this was true, one might have expected a marked reduction in all autopsy indices but in actual fact, the reductions seen were minimal (Experiment 8). Secondly, the IC_{50} value needed for *in vitro* experiments (Chapter 4) were comparatively much higher than in nematodes (Stepek *et al*., 2005) immediately imposing limitation on the dose that can be ethically given to the treated animals. Thirdly, CPs become inactivated to a large extent when they pass through the stomach leaving less active enzyme available to affect worms in the intestine (Huet et al., 2006). This has been shown before when mice infected with the stomach dwelling *P. muricola* experienced loss of worms during treatment with CPs only when the acid environment was neutralised by antacids (Stepek et al., 2007b). Finally, it may be possible that *H. microstoma,* having evolved to withstand the constant insult from pancreatic enzymes, may well have developed enzymic inhibitors that may non-specifically inhibit CPs resulting in temporary inhibition.

This result however, is in agreement with previous studies in which *H. microstoma* was found to be resilient to anthelmintics such as benzimidazoles when compared to the considerably more susceptible *H. diminuta* (McCracken *et al*., 1992). Even at the larval stage, *H. microstoma* was resistant to benzimidazole based preparations (Evans *et al.*, 1979; Evans *et al.,* 1980). To the best of my knowledge, only praziquantel has been shown to be efficacious against *H. microstoma* (Thomas and Gonnert, 1977). This is because, praziquantel affects worms by inducing contractions though increasing the influx of calcium (Mehlhorn et al., 1981), and thereby rendering paralysis in the worms and making them vulnerable to removal from the gut by peristalsis.

A more intriguing observation was when worms were treated with CPs blocked with E-64 (Experiment 3), because unexpectedly they were even larger than the controls. This might be due to exaggerated proglottid regeneration after an initial insult. The null hypothesis to this experiment was there would be no difference between worms treated with E-64 deactivated papaya latex and controls. It was not expected that worms receiving deactivated papaya latex would be bigger than the untreated controls. One possibility is that there may be a role of other components of papaya latex such as mucolytic enzymes and alkaloids in this effect (Mezhlumyan *et al*., 2003) . The cestode tegument, unlike the robust nematode cuticle is simply a surface extension of parenchymal cells comprising an epithelium (Smyth, 1998) that is both a barrier against the environment as well as a dynamic absorptive surface where various nutrients are absorbed through ionic pumps and endocytosis. It is plausible that other components of papaya latex may play a role in blocking or competitively binding themselves to transport molecules and ionic channel gates allowing osmotic diffusion of external contents into the worm.

While none of the experiments described in this chapter saw treatment related animal casualties nor poor weight gain (results not shown) nevertheless it has been observed that some mice (during pilot experiments) do develop toxicity to PLS doses much higher than the ones described in this thesis and hence I have limited the maximum dose to be 240nmols per day. Symptoms include poor appetite and

weight loss perhaps secondary to events happening in the gut. Autopsy performed on these mice revealed abdominal distension and gaseous intestinal swelling. While the exact pathophysiology is not clearly understood it needs to be emphasized that none of these symptoms were seen in rats receiving 10x the dose (2.4µmols) albeit proportionate to the dose per kilo body weight ratio. Also worth mentionaing that oral dosing of PLS in sheeps was similarly sound and safe (Buttle et al., 2011). Hence these possible side effects although needs to be kept in mind but should never hinder the progress of developing CPs further into useful novel anthelmintics.

In summary, CPs do indeed affect *H. microstoma in vivo* albeit minimally and temporarily. Given the high efficacy of PLS observed against nematode models and the better efficacy against *H. diminuta*, *H. microstoma* is not a sensitive enough model system for screening novel compounds for potential anthelmintic activity. Hence there is a need to explore other model systems. Had it been possible to administer higher doses of PLS to mice the effects might have been comparable to those seen in *H. diminuta* (Chapter 8). Nonetheless, the results indicate that perhaps with better formulation and improvement in delivery of CPs, these natural plant products may be developed further into useful anthelmintics for humans and our livestock.

CHAPTER 6

Assessment of the anthelmintic efficacy of cysteine proteinases against *H.diminuta* in mice *in vivo*

6.0 Summary

H. diminuta is naturally expelled from the mouse host about 10-12 days post infection but has been shown to survive the immunologically mediated response when treated with immunosuppressant drugs. Apart from utilising model systems in their permissive host to which the parasites are well adjusted, utilisation of unstable systems, such as *H.diminuta* in the non-permissive mouse host before the worms are expelled, may enable identification of anthelmintic activity more readily by tipping the balance in favour of worm loss. Moreover, this less stable system (compared to *H. diminuta* in its normal rat host) could be further exploited by blocking immunologically mediated expulsion from the mouse host by treatment with steroid immunosuppressants. The experiments described in this chapter indicate that treatment of mice infected with *H. diminuta* with papaya latex is efficacious, resulting in marked reductions in the worm burden and biomass, far more potent effects than those seen in permissive hosts. However, these effects were only minimally dose dependant and specific inhibition by E-64 only confirmed the role of cysteine proteinase as the active principles in papaya latex affecting worm growth but not worm elimination. The role of the immune system in contributing towards the potent effect is highly plausible when immunosuppression failed to achieve comparable potency.

6.1 Introduction

H. diminuta is a natural parasite of the rat but this cestode can establish in mice and can grow for a limited period of time before being immunologically expelled about 10-12 days post infection (Hopkins et al., 1972a). However, expulsion is greatly delayed in young hosts with immature immunity in which the worms can also grow much bigger than in adult mice (Befus and Featherston, 1974; Pike and Chappell, 1981) or during pregnancy and lactation when the host undergoes natural immunosuppression (Selby and Wakelin, 1975). Concurrent infections with nematodes (Courtney and Forrester, 1973) and protozoans (Machnicka and Choromanski, 1980) have been shown also to delay expulsion because of interference by the co-infecting species with host immunity, thereby facilitating longer survival of *H. diminuta*. This led to attempts of artificial induction of immunosuppression through corticosteroids, graft rejection agents like antithymocyte serum as well as cytotoxic drugs such as methotrexate (Hopkins *et al*., 1972b), all of which were found to prolong *H. diminuta* infections in mice. Conversely, if the murine host has experienced infection earlier, and has acquired immunity, the worms are rejected earlier and do not grow as large as in primary infections (Hopkins *et al*., 1972a). While the key kinetics underlying worm expulsion have been described (Webb *et al*., 2007), the complexity of the immune response is still not fully understood (McKay, 2010). Immunologically mediated tegumental damage was also evident with the observation of dark areas (DA) on the tegument of *H. diminuta* grown in mice by Befus and Threadgold (1975) and the demonstration of leucocyte

adherence to the tegument with subsequent macrophage mediated phagocytosis of microtriches (Andreassen *et al*., 1990), of which both workers suggested complement mediated damage.

The main reason behind utilising the same cestode species (*H. diminuta*) but in a non-permissive host is to assess the suitability of this particular combination as a screen for anthelmintic activity, with the objective of finding a more sensitive *in vivo* assay system for detecting anthelmintic activity than that reflected in the permissive host. Cestodes such as *H. diminuta* are well adapted to their primary definitive hosts in which they can survive for years. One hypothesis explaining how they survive is that they can repair damage to their surface quickly, much more readily than nematodes for example. In a non-permissive host the parasites survive initially and are able to repair damage to their surface (Andreassen and Hoole, 1989) but as the host response intensifies the balance swings in favour of the host, and the parasites are eventually expelled. It is possible that cestodes repair surface damage induced by CP and other agents that target their surface. Therefore, in this chapter I explore the suitability of using the *H. diminuta*-mouse model to detect CP mediated anthelmintic activity with the expectation that *H. diminuta* should be easier to expel from this non-permissive host, than from its primary definitive host, in which its ability to repair damage is likely to be more efficient. My hypothesis is that because this is a non-permissive system, and relatively unstable, it should be easier to contribute to the immunological/physiological stresses that the worms are experiencing when growing in mice, and CPs should therefore be more effective than in the rat host, tipping the balance in favour of reduced growth and earlier expulsion of worms. In any case the results will provide an interesting comparison with the results seen in the permissive host (*H. diminuta* in rats) and in the other permissive model-system (*H. microstoma* in mice) that I describe in Chapters 5 and 7. This will show how robust the efficacy of CPs is in affecting cestodes in different host combinations and under different circumstances. In turn, the results will enable assessment of the usefulness of CPs as a possible novel anthelmintic, because the broader the spectrum of activity the greater their appeal and likely acceptance by potential consumers in the farming, veterinary and medical professions (Behnke *et al*., 2008).

6.2 Experimental design and results

6.2.1 Experiment 1

Assessment of *H.diminuta* survival in the non-permissive mouse host

6.2.1.1 Experimental design

Thirty mice were used in this experiment (see chapter 2). Mice were allocated randomly to 6 groups with 5 mice in each group. 1 group (5 mice) was sacrificed each day starting from day 7 till day 12 post infection to ascertain worm burdens.

6.2.1.2 Results

In the experiment, the mean worm burden ranged steadily between 2.8 and 3 in mice killed on days 7 to 9 post infection before plummeting to 0.4 and 0.8 from days 10 to 11 post infection. There were no worms found on day 12 post infection (Fig 6.1).

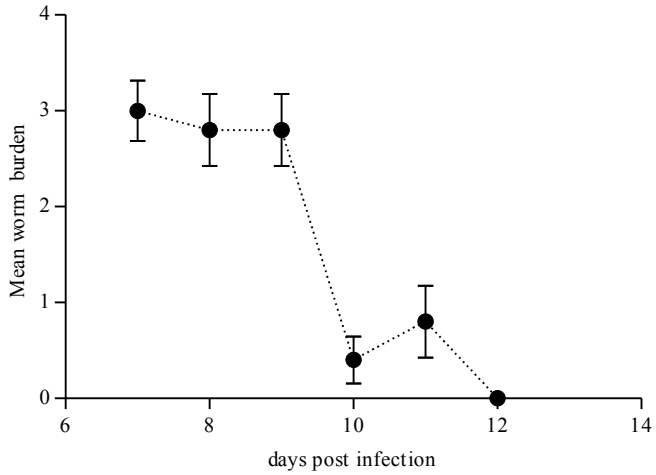


Fig 6.1 *H. diminuta* in the non-permissive host lifespan
The lifespan of *H.diminuta* in the non-permissive C3H mouse host expressed as mean worm burden per day post infection (*n*=5). Each point represents 5 mice killed on the same day. The mean worm burden was drastically reduced between days 10-12 post infection. Error bars represent the standard error of the mean.

6.2.2 Experiment 2

Assessement of the anthelmintic efficacy of PLS against *H. diminuta* in the non-permissive mice host *in vivo*.

6.2.2.1 Experimental design

This experiment was designed to test the hypothesis that *H. diminuta* in the non-permissive host is susceptible to the effects of CPs. Twenty mice were used in this experiment (see chapter 2). Mice were divided into 2 groups of *n=10*. Following the result from experiment 1, the duration and timing for autopsy in this experiment was chosen so as to not coincide with the phase of spontaneous immunologically mediated expulsion. Each mouse in treatment group 1 received 240nmol PLS (0.2mls) daily for 6 days starting from day 4 until day 9 post infection while the control group (group 2) received the same volume in water. Unlike experiments using mature worms that include assessment of fecundity through faecal egg counts, the effectiveness of treatment was determined through autopsy for recovery of worms and assessment of their biomass on day 9 post infection.

6.2.2.2 Results

Autopsy revealed a dramatic reduction of almost 80% in worm burden and biomass of the treated group as compared to controls (Fig 6.2A-B). These were all highly significant differences seen in worm burden (Fig 6.2A) (Mann-Whitney *U* test, z=-3.872, P<0.001), wet biomass (Mann-Whitney *U* test, z=-3.794, P<0.001) as well as dry biomass (Fig 6.2B) (Mann-Whitney *U* test, z=-3.492, P<0.001) as compared to the controls.

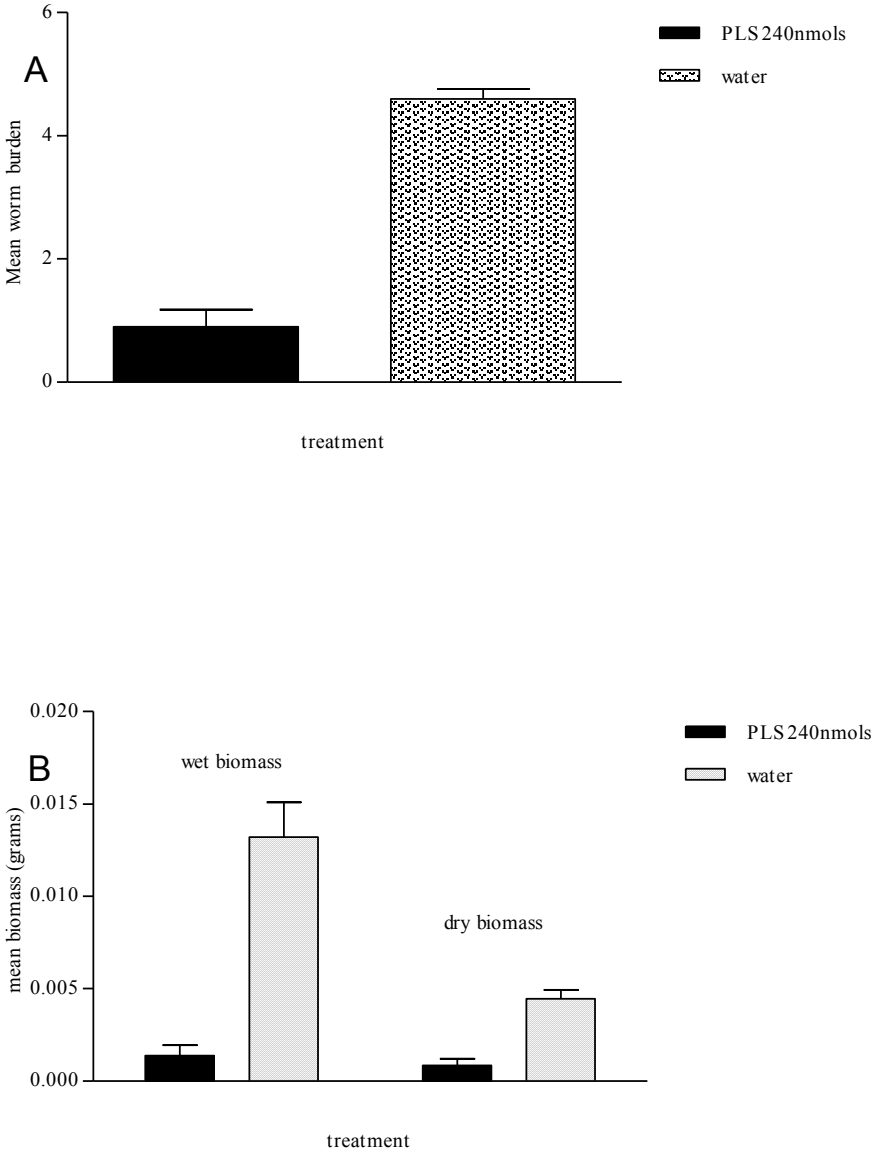


Fig 6.2 PLS efficacy against *H. diminuta* in the non-permissive host *in vivo*
(A) The mean worm burden of C3H mice infected with *H. diminuta* and treated with PLS 240nmols (n=10) or water (n=10). (B) The mean worm biomass of C3H mice infected with *H. diminuta* and treated with PLS 240nmols (n=10) or water (n=10). There was a highly significant difference observed between all autopsy indices as compared to the control group. For statistical analyses see text. The

amount of active CP expressed in nmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean.

6.2.3 Experiment 3

Confirmation of the effects of PLS against *H. diminuta* in the non-permissive host *in vivo*

6.2.3.1 Experimental design

This experiment was a follow-up from experiment 2 to confirm the effects demonstrated in the reduction in worm burden and biomass. Twenty mice were used in this experiment (see chapter 2). Although the previous Experiment 2 incorporated the use of a larger sample size *n=10*, this experiment was attempted using a smaller sample size *n=5* because more groups were needed to factorially confirm the effect of PLS. Mice were divided into 4 groups. Each mouse in treatment group 1 received 0.2mls dose of PLS at 240 nmol. Group 2 received 0.2 ml dose of PLS at 240 nmols + E-64 at 280 nmols. Group 3 received 0.2mls dose of E-64 at 280 nmols and group 4 received 0.2 ml water. All groups received daily treatment for 6 days starting from day 4 until day 9 post infection. The effectiveness of treatment was determined through autopsy for recovery of worms and assessment of their biomass on day 9 post infection.

6.2.3.2 Results

Arithmetically, there was only minimal reduction (18%) in worm burden when comparing mean values between the treated group receiving PLS (mean=3.6) and the control group receiving water (mean=4.4) (Fig 6.3A). In fact, the group receiving E-64 only (mean=3.4) had the fewest worms (Fig 6.3A). However, this was not statistically significant (Kruskal-Wallis test, χ^2_2=2.427, *P*=0.489). Surprisingly, in the wet biomass assessment, the treated group (mean=0.00444) had a 60% reduction of mean biomass compared to the control group (mean=0.01112) (Fig 6.3B). Also unexpectedly were the 61% and 43% reductions in mean biomass in the groups treated with PLS+E-64 (mean=0.00432) and E-64 only (mean= 0.00636) respectively when compared to the control group, although statistically there was no significance, P being just marginally the wrong side of the cut off (Kruskal-Wallis test, χ^2_2=7.15, *P*=0.067). Similarly, the dry biomass assessment, saw a more pronounced reduction of 78% between the treated group and the group receiving water (Fig 6.3B) which was statistically significant (Kruskal-Wallis test, χ^2_2=9.11, *P*=0.028). In assessing the main effects of PLS, data were analyzed by 2-way ANOVA (treatment with PLS and/or E64) and the effect of PLS was significant on both biomass assessments (wet biomass *P*=0.014 dry biomass *P*=0.003) but not on worm burden *P*=0.682. Despite the reduction seen in biomass as compared to the group receiving water, the effects of E-64 or PLS pre-treated with E-64 were not significant in any of the indices.

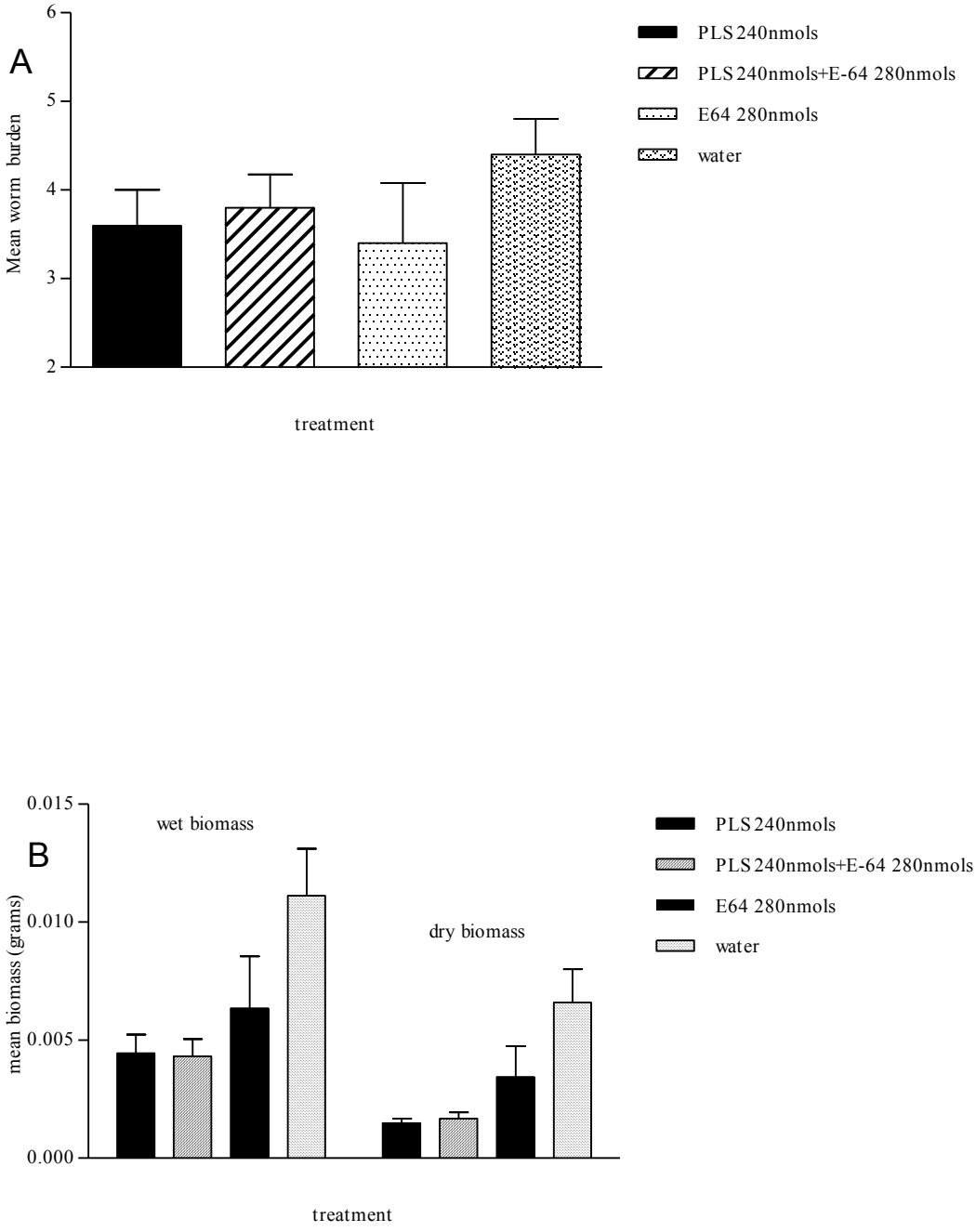


Fig 6.3 Confirmation of the effects of PLS against *H. diminuta* in the non-permissive host *in vivo*
The various autopsy indices used to measure the effects of PLS against *H. diminuta* in the non-permissive mouse host. (A) The mean worm burden of C3H mice infected with *H. diminuta* and treated either with PLS 240nmols (n=5), PLS 240nmols pre-treated with E-64 280nmols (n=5), E-64 280nmols (n=5) or water (n=5). (B) The mean biomass of *H. diminuta* in C3H mice treated either with PLS 240nmols (n=5), PLS 240nmols pre-treated with E-64 280nmols (n=5), E-64 280 nmols (n=5) or water. PLS appears to only minimally affect worm burden but other treatments seemed to be equally affected if not more. PLS appear s to have a substantial effect on worm biomass but this was also true with the group receiving PLS pre-treated with E64. The group receiving E-64 only also appears to be affected as compared to the controls. For statistical analyses see text. The amount of active CP

expressed in nmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean.

6.2.4 Experiment 4

Observation of PLS mediated damage against *H. diminuta* in the non-permissive host *in vivo.*

6.2.4.1 Experimental design

This experiment was simply a further extension of Experiment 3 to graphically document the effects of PLS inflicted on *H. diminuta* in the non-permissive mouse *in vivo*. Four mice were used in this experiment (see chapter 2). Due to the non- analytical nature of the experiment only 1 mouse (*n=1*) per group was used to represent each of the 4 different treatment combinations. Mice were divided into 4 groups. Group 1 received 0.2mls dose of PLS at 240nmols. Group 2 received 0.2mls dose of PLS at 240nmols + E-64 at 280nmols. Group 3 received 0.2mls dose of E-64 at 280nmols and group 4 received 0.2mls water. All mice received daily treatment for 6 days starting from day 17 until day 22 post infection. The efficacy of treatment was assessed through the recovery of detached proglottid segments found in the large intestine downwards which were immediately fixed in glutaraldehyde and later dehydrated, critical point dried and gold coated before finally being viewed under scanning electron microscopy for signs of any damage.

6.2.4.2 Results

Generally the detached proglottid segments recovered were longer in terms of number of proglottids per detached segment as compared to similar experiments using *H. diminuta* in the rat host or even *H. microstoma* in mouse. All segments recovered irrespective of the treatment received *in vivo* appears to have developed widespread wrinkling which was not seen in similar experiments with *H. diminuta* in the rat host or even *H. microstoma* in mice. On top of this unusal wrinkling the proglottid segment recovered from the mouse treated with PLS 240nmols appears to also sustain extensive tegumental damage in the form of tegumental erosions and digestion.

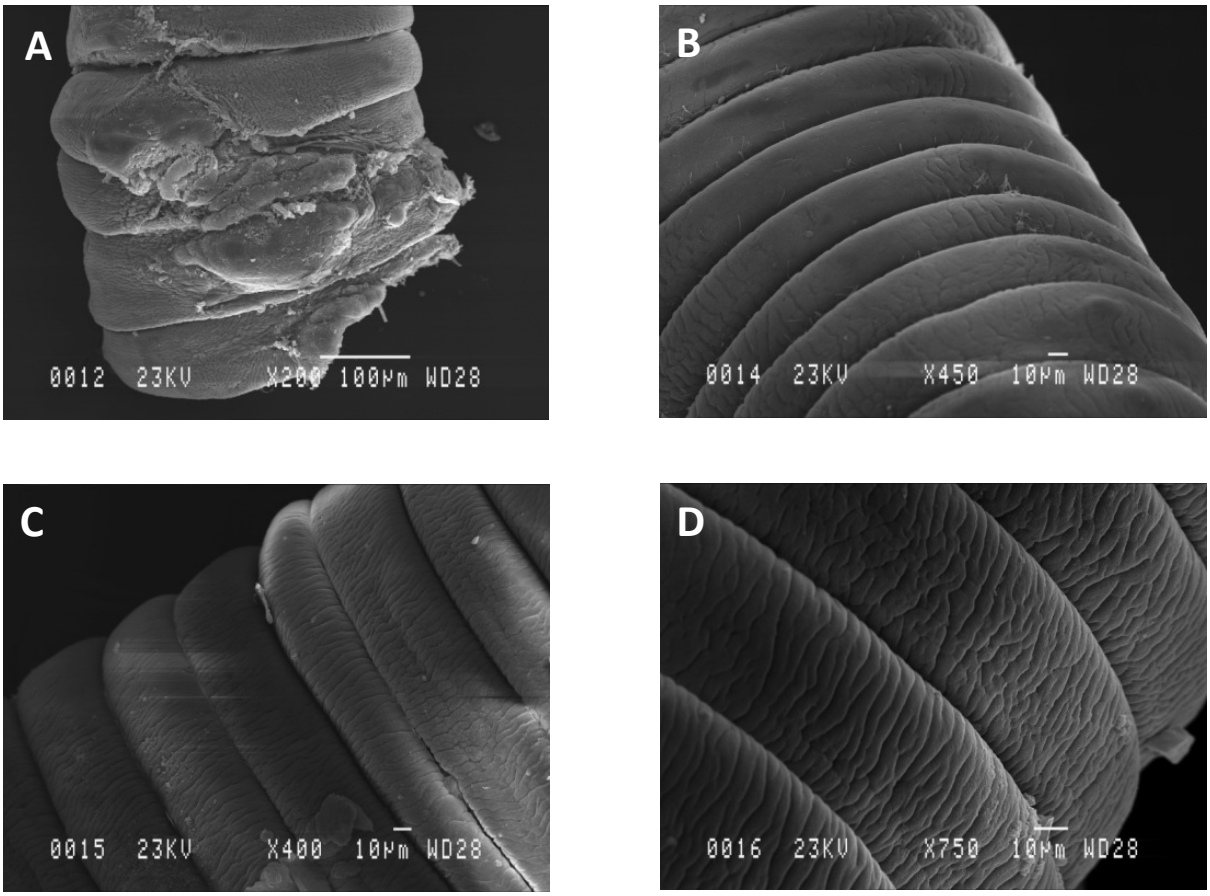


Fig 6.4 SEM images of the effects of PLS against *H. diminuta* in the non-permissive host *in vivo*
SEM images of detached proglottid segments of *H. diminuta* recovered from the colons of infected C3H mice treated with (A) PLS 240 nmols (B) PLS 240 nmols pre-treated with 280nmols E-64 (C) E-64 280 nmols (D) water. Note the extensive treatment related damage in the *H. diminuta* proglottid segment recovered from the mouse treated with treated with PLS 240 nmols which was not present in the other control segments. Note also the widespread wrinkling in all *H. diminuta* proglottid segments recovered irrespective of their treatment *in vivo*. The amount of active CP expressed in nmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2).

6.2.5 Experiment 5

Assessment of dose-dependency of PLS treatment against *H. diminuta* in the non-permissive host *in vivo*

6.2.5.1 Experimental design

This experiment was carried out to assess whether or not the reduction in worm burdens and biomass effects demonstrated by PLS against *H. diminuta* in Experiment 2 is dose-dependant. Twenty two mice were used in this experiment (see chapter 2). Compared to previous Experiments 2 and 3 which sought to observe significance between treatments, this experiment was a correlation study between PLS dose

and worm autopsy indices. Therefore, a smaller sample size *n=3* was thought to be generally sufficient. Mice were divided into 6 groups of different doses of PLS. This experiment was also executed simultaneously with Experiment 3 in which mice from the group receiving water were incorporated as group 1 (0nmols dose) in this experiment (*n*=5) and mice receiving 240nmols of PLS (*n*=5*)* were incorporated as group 5. Group 2 (*n*=3) received 30nmols of PLS, group 3 (*n*=3) received 120nmols, group 4 (*n*=3) received 180nmols and group (6 *n*=3) received 360nmols. All groups received daily treatment for 6 days starting from day 4 until day 9 post infection. Treatment effects were assessed through worm counts and biomass assessments at autopsy on day 9 post infection.

6.2.5.2 Results

Autopsy revealed a weak negative correlation between PLS dose and worm burden (R^2=0.006, *n=22, P*=0.615) (Figure 6.5A). Although not statistically significant, there were indications of a weak negative relationship between dose of PLS and worm biomass (wet biomass R^2=0.0758, *n=22, P*=0.194) (Figure 6.5B) and PLS dose especially dry biomass which is just outside of significance (R^2=0.1468, *n=22, P*=0.063) (Figure 6.6C).

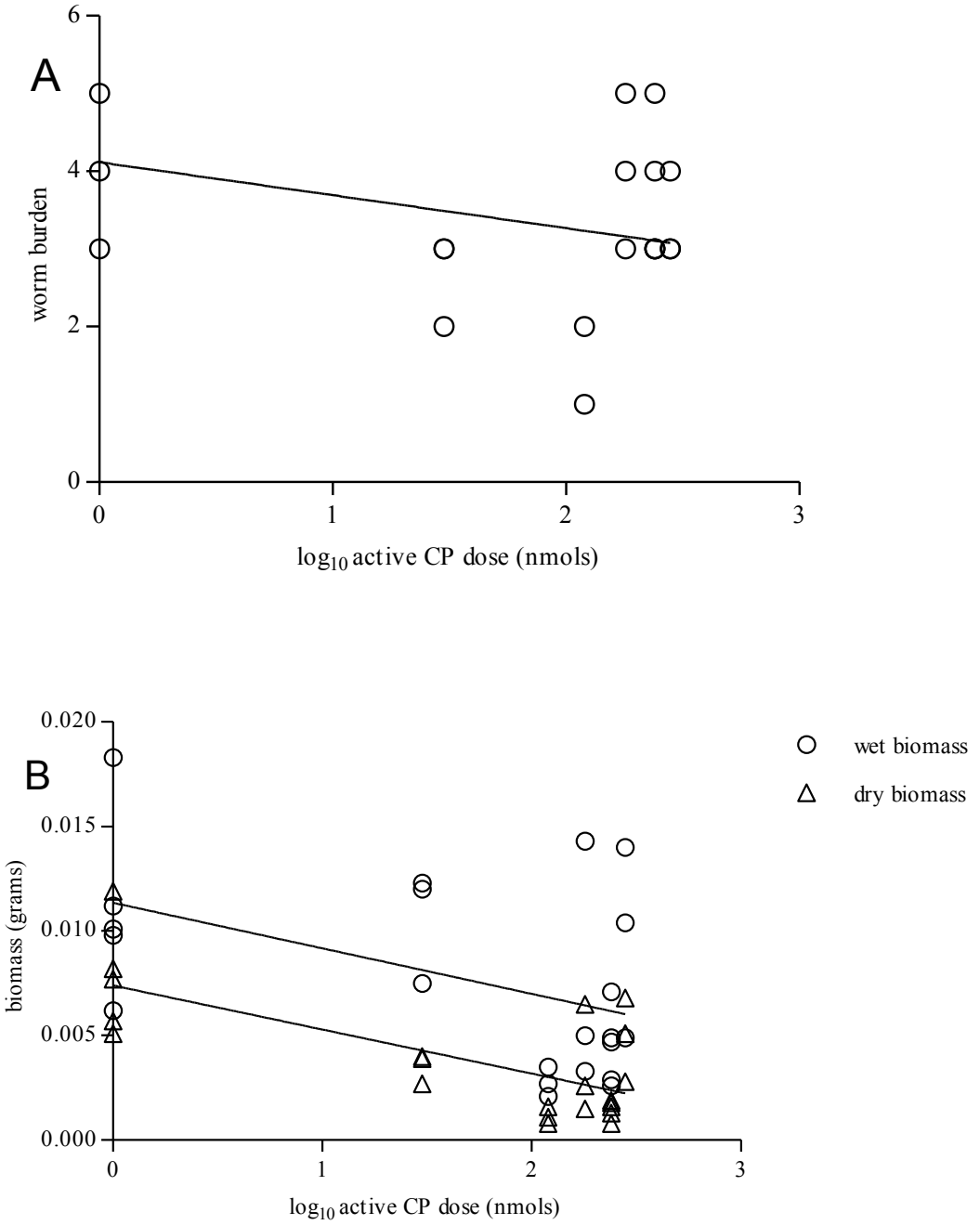


Fig 6.5 Dose dependency of PLS against *H. diminuta* in the non-permissive host *in vivo*
The various indices demonstrating the relationship between PLS dose and *H. diminuta* in the non-permissive mouse host. (A) The correlational trend between increasing PLS dose and worm burden.(B) The correlational trend between increasing PLS dose and worm biomass. All groups were of the same sample size (*n=3*) except groups 1 and 5 (*n=5*). For statistical analysis see text. The line of best fit is provided to guide the eye. The amount of active CP expressed in nmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2).

6.2.6 Experiment 6

Assessment of survival of *H. diminuta* in the immunosuppressed non-permissive mice host

6.2.6.1 Experimental design

Twenty mice were used in this experiment (see chapter 2). Mice were randomly divided into 5 groups of 5 mice in each group and the sixth group consisted of only 3 mice. Each mouse received 5 *H. diminuta* cysticercoids on day 0 of infection through oral gavage. Each mouse was injected with 0.1ml (1mg/ml) dexamethasone (Voren ^{TM} Boehringer-Ingelheim, UK) subcutaneously every other day starting from day 1 post infection until respective day of culling. All mice were given oxytetracycline hydrochloride (Terramycin, Pfizer Ltd) in their drinking water at a concentration of 3g/litre) for the duration of the experiment (Behnke et al., 1980). One group (5 mice) was sacrificed every other day starting from day 8 until day 12 post infection and then every 4 days until day 20 post infection to ascertain worm burdens. The sixth group (3 mice) was left until day 32 post infection before autopsy.

6.2.6.2 Results

Unlike in Experiment 1, the mean worm burden was almost preserved and ranged steadily between 4.4 and 5 until the end of the experiment at day 32 post infection (Fig 6.6A). The mean biomass climbed almost exponentially from day 8 before reaching a plateau from day 16 post infection (Fig 6.6B). However, at day 32, the mean worm biomass was reduced to almost 40% from their peak size despite retaining worm count (Fig 6.6B).

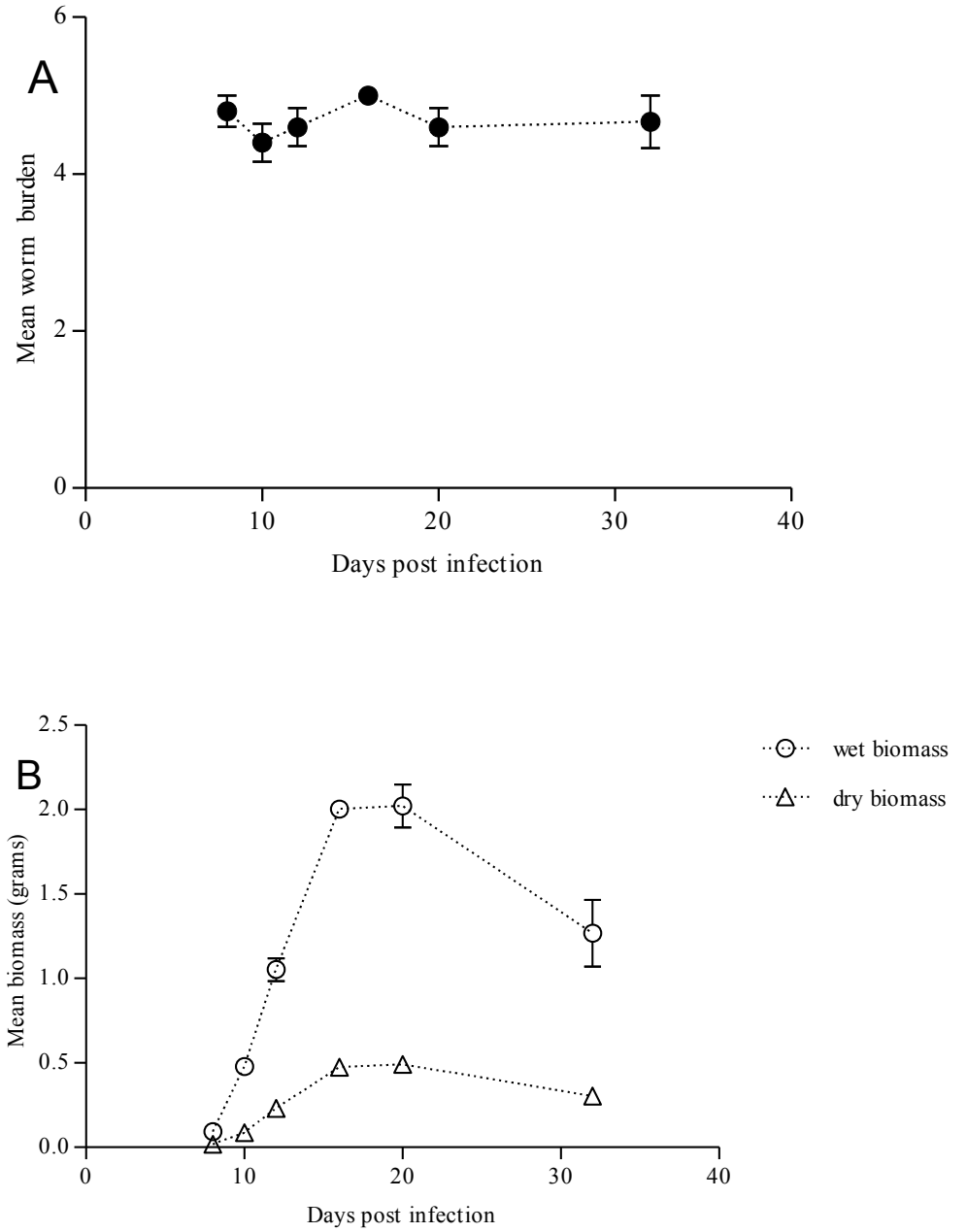


Fig 6.6 *H.diminuta* lifespan in the immunosuppressed non-permissive host
The lifespan of *H.diminuta* in the non-permissive immunosuppressed C3H mice host expressed as (A) mean worm burden per day post infection (*n*=5). (B) Mean worm biomass per day post infection (*n*=5). Each point represent 5 mice killed on same day except the last point which consisted of 3 mice. The mean worm burden was preserved until the end of experiment with only the mean worm biomass reduced from their peak mass at the end of the experiment. Error bars represent the standard error of the mean.

6.2.7 Experiment 7

Assessment of the efficacy of PLS against *H. diminuta* in the immunosuppressed non- permissive host

6.2.7.1 Experimental design

Following the success of Experiment 6 in which *H. diminuta* was able to be survive for longer than normal avoiding the usual immunologically mediated expulsion by immune suppression, this experiment was designed to determine whether the marked effects of PLS treatment on *H. diminuta* in the non-permissive host was partly immunologically mediated. The hypothesis is that when the immune system is suppressed, the PLS effects will not be as marked. Twenty mice were used in this experiment (see chapter 2). Each mouse was injected with 0.1ml (1mg/ml) dexamethasone (Voren ^{TM} Boehringer-Ingelheim, UK) subcutaneously every other day starting from day 1 post infection until the end of experiment on day 16 post infection. All mice were given oxytetracycline hydrochloride (Terramycin, Pfizer Ltd) in their drinking water at a concentration of 3g/litre) for the duration of the experiment (Behnke et al., 1980). Mice were divided into 2 groups (n=10). Each mouse in treatment group 1 received 0.2 ml doses of PLS at 240nmols daily for 6 days starting from day 11 until day 16 post infection while the control group (group 2) received 0.2mls dose of water. The effectiveness of treatment was determined through autopsy for recovery of worms and assessment of their biomass on day 16 post infection.

6.2.7.2 Results

Autopsy revealed a less dramatic reduction of a mere 23% in worm burden (Fig 6.7A) and about 45% reduction in biomass of the treated group as compared to controls (treated group mean wet biomass=0.91 grams, control group mean wet biomass=1.60 grams; treated group mean dry biomass=0.21 grams, control group mean dry biomass=0.39 grams) (Fig 6.7B). These were all highly significant differences seen in worm burden (Mann-Whitney U test, z=-2.746, P=0.006) and biomass (wet biomass Mann-Whitney U test, z=-3.25, P=0.001;dry biomass Mann-Whitney U test, z=-3.25, P=0.001) as compared to the controls.

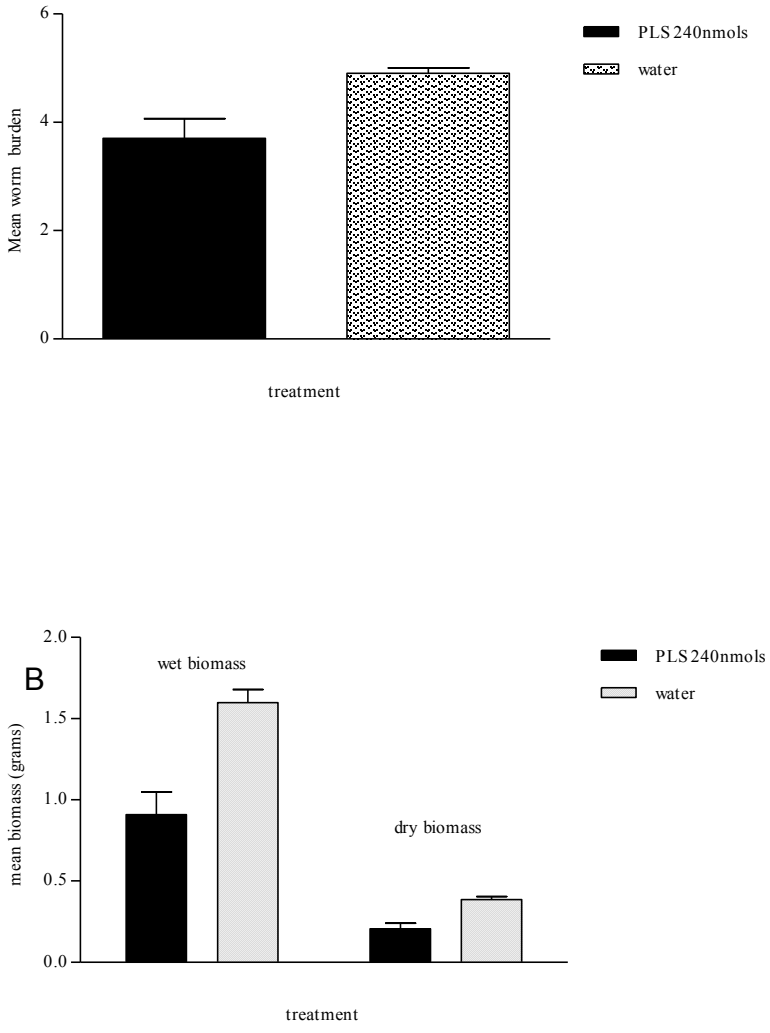


Fig 6.7 PLS efficacy against *H. diminuta* in the immunosuppressed non-permissive host *in vivo* (A) The mean worm burden of immunosuppressed C3H mice infected with *H. diminuta* and treated with PLS 240nmols (n=10) or water (n=10). (B) The mean biomass of *H. diminuta* in immunosuppressed C3H mice treated with PLS 240nmols (n=10) or water (n=10). There a highly significant difference observed between all autopsy indices as compared to the control group. For statistical analyses see text. The amount of active CP expressed in nmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean.

6.3 Discussion

Before embarking on definitive experiments in assessing PLS efficacy, explorative worm survival experiments were needed to ascertain the time course in which one can work with *H. diminuta* as it is known to be spontaneously expelled from the mouse host between days 10-12 post infection (Hopkins *et al.*, 1972a). Experiment 1 confirmed the observation of Hopkins *et al.* (1972a) which clearly limits anybody wishing to work with *H. diminuta* in mice to only 10 days post infection. However, the mean worm burden, before expulsion in this experiment (days 7 to 9 post infection) was somewhat lower than

expected (2.8-3) when considering that each mouse was infected with 5 cysticercoids. This was perhaps disappointing and it is not known why establishment of worms was so low in this experiment, since in *H. diminuta* infections in rodents given an inoculum of 5 cysticercoids normally yield 4-5 worms in each animal. Nevertheless, the results concurred with earlier work and are consistent with knowledge that *H. diminuta* in the non-permissive host undergoes expulsion from days 10-12 post infection until all the worms are eventually lost by the end of weeks 2-3 of infection (Hopkins *et al*., 1972a). Because the expulsion of *H. diminuta* in the non-permissive host is immunologically mediated (Hopkins *et al*., 1972a; Persaud *et al*., 2007), Hopkins *et al*. (1972b) were able to prolong worm survival by induction of immunosuppression. Experiment 5 was designed to confirm this phenomenon ahead of further trials. Preliminary experiments (results not shown) using identical equivalent doses of dexamethasone instead of the original cortisone used by Hopkins *et al*. (1972b) failed to preserve worms beyond the normal survival window before spontaneous expulsion. It was only after several experiments utilising higher (>2 X the original dose) equivalent doses of corticosteroids that I was able to preserve *H. diminuta* from being expelled. Surprisingly, the preservation of *H. diminuta* in the non- permissive host was achieved up to 32 days post infection which is 12 days longer than the original experiment by Hopkins *et al*. (1972b). It is also important to note that despite the acquired longevity worm biomass was reduced over time suggesting an adaptation to limited resources often referred to as the crowding effect (Read, 1951). However, this particular group, which was left to endure for longer, had a smaller sample size n=3 because the original aim of the experiment was just to repeat that of Hopkins *et al*. (1972b) and to terminate the experiment on day 20 post infection. However, there were no losses and the 3 additional mice that had been included to compensate for possible losses were killed eventually on day 32. Hopkins *et al*. (1972b) however were only able to preserve the worms at the expense of substantial host loss (about 20% from the original sample size) which may be due to the administration of steroids through intramuscular injections and not providing antibiotics during periods of immunosuppression, the latter factor being rectified in their subsequent work. Surprisingly, in this experiment, using a different corticosteroidal variant injected subcutaneously rather than intramuscularly and at a higher dose than used by Hopkins *et al*. (1972b), the treatment managed to achieve preservation without experiencing any host casualties. Perhaps, newer generations of corticosteroids have less adverse effects than the earlier versions (Degreef, 1999). Nevertheless, all this has enabled me to work with *H. diminuta* for longer beyond its spontaneous expulsion window.

Unlike *H. diminuta* in its definitive rat host which was moderately affected by PLS (Chapter 7) and even *H. microstoma* which was only minimally affected (Chapter 5), *H. diminuta* in the non-permissive host proved to be the most sensitive model for assessing the anthelmintic efficacy of PLS in cestodes. Here, there were dramatic reductions in worm burden and biomass of almost 80% when *H. diminuta* was grown in the non-permissive mouse host and treated with PLS (Experiment 2). This may be explained by a two pronged attack on the worm namely PLS and the immune system. Either PLS (Chapter 7) or the immune system (Befus and Threadgold, 1975) has been shown to affect *H. diminuta* on their own thereby making them an unforgiving menace when both work in tandem. However, these effects need to be factorially confirmed using E-64 as a specific inhibitor for CP so that the effects seen are indeed the main effects of PLS. Despite a much varied response in arithmetical reductions (worm

burden 18%, wet biomass 60% and dry biomass 78%) as compared to the controls, this experiment (Experiment 3) confirmed that PLS do indeed affect the ability of the worm to repair itself after PLS treatment as non-parametric testing revealed that worm biomass was close to (wet biomass) and significantly different (dry biomass) in the PLS treated group. Furthermore, 2-way ANOVA was significant for the effect of PLS but not for E-64 or when the interaction between both (pls see text for statistical analysis). PLS treatment however cannot be said to be responsible for worm loss as the worm burden was not significantly different when differences between groups were tested by non- parametric tests or by 2-way ANOVA. It is also important to note in all confirmatory experiments (experiment 3 here; also in chapters 5 & 7) a smaller sample size n=5 was used due to logistical and practical considerations. This might explain the variability seen between the confirmatory experiments and the definitive experiments using a larger sample size n=10. This result is indeed identical to that in Chapter 7 where PLS was confirmed to affect worm size but not worm counts. What is not identical though is that worms receiving PLS pre-treated with E-64 or worms receiving E-64 only were also reduced in size when compared to the group receiving water alone. However, despite their magnitude, these were not statistically significant differences. In Chapter 5 & 7 where perhaps an exaggerated response of repair was seen in groups receiving PLS pre-treated with E-64, the results suggested that because PLS was being competitively inhibited other components of PLS like mucolytic enzymes and alkaloids (Mezhlumyan *et al.*, 2003) might still be at large diffusing through the tegument. Unlike in natural permissive hosts where cestodes are able to repair damage quickly (Befus and Threadgold, 1975), *H. diminuta* in the non-permissive mouse host is constantly being attacked by the immune system and hence is most likely unable to repair itself as efficiently as it does in its normal rat host and therefore appears to be affected proportionately. This was also true when worms in the group receiving E-64 only also appeared to be affected compared to the controls receiving water. What is striking is that all 3 different treatments appeared to affect *H. diminuta* in a proportionate manner not seen in similar confirmatory experiment of *H. diminuta* in the rat host where all other treatment groups had values that were indistinguishable from those of the controls receiving water. SEM images of recovered detached proglottid segments (experiment 4) may perhaps consolidate these earlier observations and postulations. Firstly It was noted that all the detached proglottid segments were longer in terms of number proglottids per detached segment found compared to the detached segments found in similar experiments in chapter 5 (*H. microstoma* in mouse) & 7 (*H. diminuta* in rats). This may be explained by perhaps due to the attacking immunity the worms were releasing their proglottids at a faster rate and hence longer groups of proglottids detached. Secondly all proglottid segments irrespective of treatment received *in vivo* exhibit widespread wrinkling which was also never seen in similar experiments in chapters 5 & 7. This phenomenon is again perhaps as a result of immune system attack. Thirdly the proglottid segment recovered from the mouse treated with PLS appeared to have sustained extensive and substantial damage unlike in similar experiment of *H. diminuta* in the rat host where the damage sustained was less severe (chapter 7). This could perhaps be explained by the combinative onslaught from both the immune system and PLS attacking the tegument. Having confirmed the effects of PLS, it was then found through Experiment 5, that the effects of PLS on worm size and numbers were not dose dependant. The experiment revealed variable but consistently negative but insignificant correlations

between PLS dose and worm indices (Fig 6.5). This again is in keeping with the results seen when *H. diminuta* in its natural rat host. To finally conclude this hypothesis, PLS should be administered to this model system in a situation in which the immune system will not be able to play its role. Following the success of preservation of worms through immunosuppresion (Experiment 6), the final Experiment 7 revealed a less marked reduction profile ranging from 23%-47% (Fig 6.7) compared to Experiment 2 (80%-90%) but nevertheless still statistically significant reduction. This supports the idea that the earlier marked effects seen were the consequence of a combined assault from both PLS and a fully functional immune system. In summary, all the results are sufficient to support my hypothesis that in exploiting this non-permissive, unstable system, the anthelmintic efficacy of CPs was greatly enhanced.

Clearly the complex immunological mechanism that facilitates the expulsion of *H. diminuta* in the non-permissive host, has generated an environment in which additional stresses on the worms, such as those imposed by the enzyme activity of PLS attacking the worm's surface, tip the balance in favour of expulsion and prevent the growth of larger worms. Apart from worm expulsion kinetics (Webb *et al*., 2007), immunologically mediated tegumental damage has been observed (Andreassen *et al*., 1990; Befus and Threadgold, 1975) On the other hand, in the permissive host, where such immune reactions do not occur, or are expressed less vigorously, the effects of PLS on worm burdens and biomass, as a result of the anthelmintic activity of CPs, are less marked because the worms presumably can cope better with the ensuing damage to their surface and can repair it sufficiently to avoid expulsion. Cestodes are known to be able to repair damage to their tegument rapidly, within hours in some cases *in vitro* (Befus and Threadgold, 1975) but given the simultaneous onslaught of both the immune system and the administered PLS, they were clearly overwhelmed.

CHAPTER 7

Assessment of the anthelmintic efficacy of cysteine proteinases against *H.diminuta in vivo* in rats

7.0 Summary

H. diminuta is a natural parasite of the common brown rat *Rattus norvegicus*, and provides a convenient model system for the assessment of the anthelmintic activity of novel drugs against cestodes. In contrast to earlier reports, the experiments described in this chapter indicated that treatment of rats infected with *H. diminuta* with papaya latex was efficacious, albeit moderately, resulting in a significant, but relatively small, reduction in worm burden and biomass. However, these effects were only partially dose dependant although specific inhibition by E-64 confirmed the role of cysteine proteinase as the active principles in papaya latex affecting worm growth but not worm elimination. However, over a longer duration than explored in the earlier experiments worm loss was magnified but the effects on worm growth were not enhanced. Worm fecundity was not affected at all.

7.1 Introduction

The anthelmintic effects of cysteine proteinases (CPs) against nematodes *in vivo* have been successfully demonstrated in mice (Satrija et al., 1995; Stepek et al., 2006b, 2007b, c), pigs (Satrija et al., 1994), sheeps (Buttle *et al*., 2011), chickens (Mursof and He, 1991) and there is evidence from trials in humans infected with the three major intestinal species that CPs also work effectively against human infectious intestinal nematodes (Hansson *et al*., 1986).

In comparison, little is known about the effects of CPs on cestode infections. There is some evidence that CPs are detrimental to the survival of cestodes in *in vitro* maintained assays (He et al., 1992; Stepek et al., 2007e); Chapter 3 this thesis), but some published data also indicate that this is not reflected in a reduction of parasite burdens when CPs are administered to infected hosts (de Amorin et al., 1999; He et al., 1992). Using lattices from the fig species of *F. insipida* and *F. carica* at 3-4mls/kg/day for 3 days, De Amorin et al. 1999 failed to demonstrate a significant reduction of parasites in albino mice infected with *H. nana* (6.3-8.3%). However, the methodology described in this paper was unclear as worm burden was expressed as a percentage of the wet weight of proglottids found in faeces in relation to total faecal and intestinal proglottids on autopsy and no dry weight assessment was carried out. Moreover, the authors did not quantify active molar enzyme activity which raises the question of enzyme viability. Using young pineapple juice at doses between 0.4-50%, He *et al.* 1992 also found no reduction in worm burden in Swiss white mice infected with *H. nana*. One pertinent issue with respect to this model system is that *H. nana* is a rather unusual cestode in that it can develop directly in rodent hosts without the involvement of intermediate hosts and infections can be augmented by autoinfection (Smyth, 1998), thereby making assessment of parasite burdens difficult and unreliable in relation to the originally administered eggs or cysticercoids. Moreover, the exact methodology

applied in this Indonesian paper could not be ascertained as copies of the paper are unavailable through library resources in the UK, and cannot be downloaded via the internet. To date, few results based on well designed *in vivo* trials with tapeworms have been published and whether CPs have *in vivo* activity against cestodes is still debatable. Nevertheless, it is very important to establish whether they are effective against cestodes *in vivo*, if potential drugs are to be taken forward to clinical trials and eventually are to be developed into useful therapeutic agents.

Two rodent infective cestode model systems are maintained widely and have been used extensively to study the biology and host-parasite relationships of cestodes. The first of these, *H. microstoma*, was discussed in Chapter 5. The second model is the intestinal lumen dwelling rat tapeworm *H. diminuta*. Despite being a natural parasite of rats (Abu Madi *et al*., 2001), these tapeworms can also grow temporarily in mice before they are expelled usually 10-12 days post infection by host immunity (Hopkins et al., 1972a). However, they have been shown to survive rejection in the immunocompromised host (Hopkins et al., 1972b). *H. diminuta* has an indirect life cycle in which arthropods such as flour beetles (e.g. *Tribolium confusum*) serve as intermediate hosts (Fox, 2007). Accidental ingestion of the arthropod host releases dormant cysticercoids which excyst in the duodenum of the vertebrate host and migrate and mature in the small intestine. Unlike *H. microstoma* which possesses hooks as organs of attachment, *H. diminuta* is only equipped with an unarmed rostellum, so the parasite cannot embed deeply into the intestinal mucosa, but is attached only by its suckers. This attachment is temporary because the worms can detach easily enabling movement and reattachment at new sites within the intestine (Smyth, 1998). The weak attachment of this species, suggests that it may be a sensitive model in which to assess the host-protective effects of anthelmintics such as the CPs being evaluated in this project. In this context also, *H. diminuta* exhibits a complex pattern of migratory behaviour. There is a circadian pattern of migration of which there is anterior migration from 12am to 6am and a posterior migration during the period of 12pm to 6pm (Tanaka and MacInnis, 1975). Various stimuli were thought to influence migration including availability of ingesta in the stomach (Read and Kilejian, 1969), nutrition (Dunkley and Mettrick, 1977) and even pH of intestinal contents (Mettrick, 1971). Finally it was concluded that the actual stimulus affecting migration was intestinal contraction as the worms move anteriorly against peristalsis during active feeding to avoid expulsion (Sukhdeo, 1992b).

Having demonstrated in the results presented in Chapter 4 that *H. diminuta* is susceptible to CPs *in vitro*, here I present the results of experiments that aimed to assess whether CPs also have detectable activity against a natural cestode of rats in its normal host *in vivo*. The hypothesis tested was that *H. diminuta* is susceptible to the anthelmintic activity of CPs when developing in its normal rat host.

7.2 Experimental design and results

7.2.1 Experiment 1

Assessment of the anthelmintic efficacy of papaya latex supernatant (PLS) against *H.diminuta in vivo*.

7.2.1.1 Experimental design

This experiment was designed to assess the effect of PLS against *H. diminuta in vivo*. Twenty rats were used in this experiment (see chapter 2). Rats were divided into 2 groups. Group 1 received PLS at 2.4 µmols and group 2 received water. The effectiveness of treatment was determined through faecal egg counts as well as by autopsy for recovery of worms and assessment of their biomass. Each rat in group 1 received a 2 ml dose of PLS containing 2.4 µmols of active CPs while each rat in group 2 received a 2 ml dose of water. Faecal egg counts were carried out on days 21st, 23rd , 25th ,28th, 30th and 32nd post infection. Treatment was administered once daily starting from days 27th until 32nd. 1 rat from the treatment group died during the course of treatment and was not included in the results analysis.

7.2.1.2 Results

The faecal egg counts (Fig 7.1A) in the treatment group receiving PLS did not show any convincing reduction compared to the control group receiving water. Indeed arithmetically, the mean values were higher than the control group following treatment. This difference in FEC between treatment groups was not statistically significant (rmGLM, between subjects test, $F_{1,17}$=2.772 P=0.114) and there was no evidence of a divergence between treatment groups with time (within subjects test, time*treatment interaction $F_{4.415,75.056}$=2.371 P=0.054).

However, there were significant differences in worm burden (Fig 7.1B) (Mann-Whitney U test, z=-2.936, P=0.003) and biomass (Fig 7.1C) (wet biomass, Mann-Whitney U test, z=-2.351, P=0.011; dry biomass, Mann-Whitney U test, z=-2.694, P=0.007) assessments between the treated group and the control group.

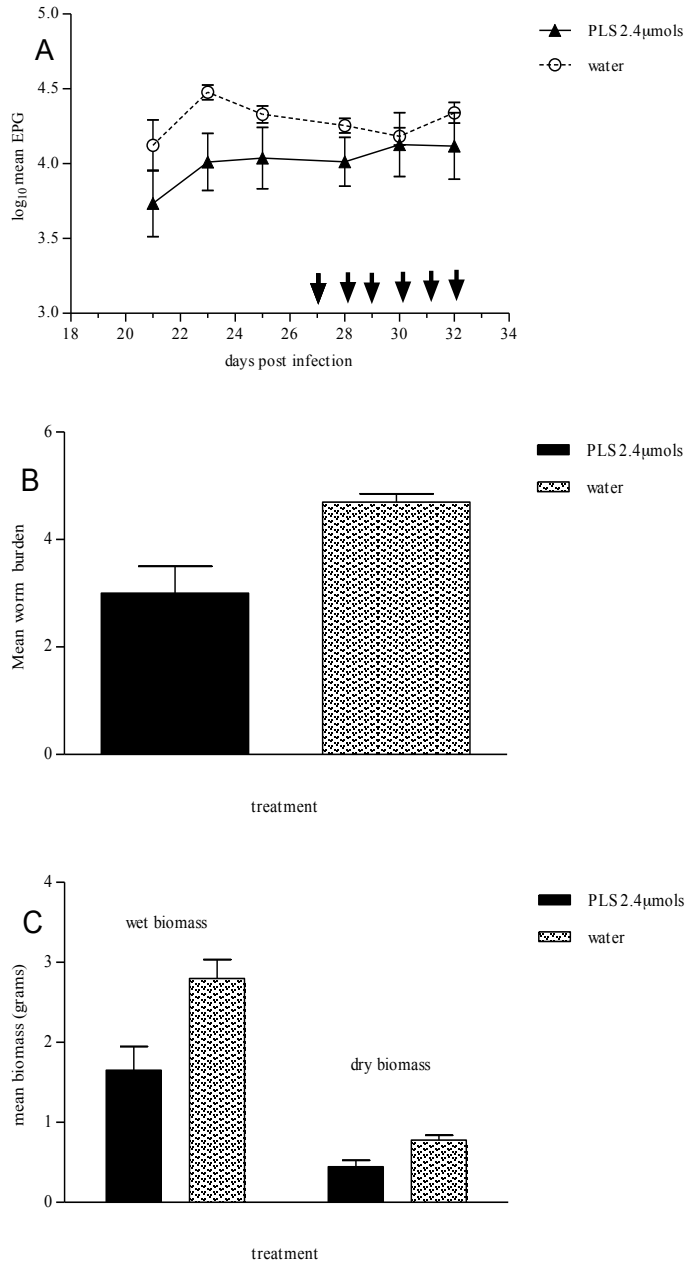


Fig. 7.1. Efficacy of PLS against *H. diminuta in vivo*
The various indices demonstrating the effects of PLS on *H. diminuta in vivo*. (A) The mean eggs per gram of faeces before (days 21-25) and during (days 27-32) treatment of Wistar rats infected with *H. diminuta* and treated with PLS 2.4 µmols (*n*=9) or water (*n*=10). There was no significant difference observed in faecal egg counts between treatment groups, nor a significant time*treatment interaction. For statistical analyses see text. Error bars represent the standard error of the log mean. (B) The mean worm burden of Wistar rats infected with *H. diminuta* and treated with PLS 2.4 µmols (*n*=9) or water (*n*=10). (C) The mean biomass of *H. diminuta* in Wistar rats treated with PLS 2.4 µmols (*n*=9) or water (*n*=10). There were significant differences observed in all three autopsy indices between the treated group as compared to the control group. For statistical analyses see text. The amount of active CP expressed in µmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean.

7.2.2 Experiment 2

H. diminuta egg viability experiment

7.2.2.1 Experimental design

This experiment was planned as a follow-up to Experiment 1 where *H. diminuta* fecundity appeared to have been unaffected *by in vivo* PLS treatment. Two rats were used in this experiment (see chapter 2). The two rats were treated differently; 1 received 2 ml dose of PLS at 2.4 µmols and the other received 2mls dose of water. Both rats received daily treatment for 6 days starting from day 17 until day 22 post infection. On the 6th day of treatment (day 22), both rats were autopsied and their worms removed. Three worms were isolated from each of the two rats. From each worm, the distal most proglottid segments of ~1cm (longitudinal length) were cut and fed to a group of 3 previously starved *T. confusum* beetles, hence nine beetles were exposed to cestode eggs from each of the two treatments of the rat hosts. From the same rats, their colons were dissected to recover any detached proglottid segments. Any detached proglottid segment found in each animal were fed to 3 more previously starved (5 days) *T. confusum* beetles. After indications that the fed segments have been consumed, regular beetle diet consisting of mixture of white and wholemeal flour were added for regular maintenance until after 30 days when all the beetles were dissected in Hanks' saline to recover any developed cysticercoids.

7.2.2.2 Results

T. confusum beetles fed with the cut terminal proglottid segments from *H. diminuta* which had been *treated in vivo* with PLS were found to harbour intact cysticercoids (Table 7.1A). In fact the mean number of cysticercoids recovered was comparable to that of the control group of beetles fed worms from rats which had been treated with water (Fig 7.2A). Beetles fed with detached proglottid segments recovered from the colon of treated rats also harboured intact cysticercoids (Table 7.1B) and the mean burden was comparable to that in the control beetle group fed with detached proglottid segments from rats treated with water (Fig 7.2B).

Table 7.1 *H. diminuta* egg viability test
Number of *H. diminuta* cysticercoids recovered from *Tribolium* beetles (*n*=9/ treatment group) fed with *H. diminuta* distal proglottid cut segments (from three *worms*) from each of two rats treated *in vivo* either with PLS or water

H. diminuta in vivo treatment	Proglottid segment origin	No of cysticercoids recovered		
		Beetle1	Beetle 2	Beetle 3
PLS	Worm 1	10	15	15
	Worm 2	14	11	13
	Worm 3	0	4	0
Water	Worm 1	4	6	7
	Worm 2	12	20	6
	Worm 3	9	14	18

Table 7.2 *H. diminuta* egg viability test
Number of *H. diminuta* cysticercoids recovered from *Tribolium* beetles fed with detached *H. diminuta* proglottid segments from the colon of Wistar rats infected with *H. diminuta* and treated *in vivo* either with PLS (*n*=3) or water (*n*=3).

H. diminuta in vivo treatment	No of cysticercoids recovered		
	Beetle 1	Beetle 2	Beetle 3
PLS	2	0	0
water	0	3	1

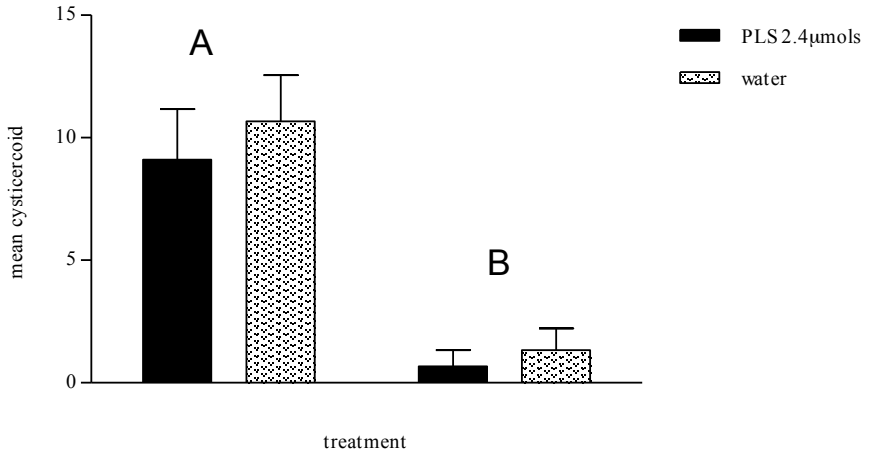


Fig 7.2 *H. diminuta* egg viability experiment
(A) Mean number of cysticercoids recovered from *Tribolium* beetles (*n=9*) fed with distal cut proglottid segments (*n=3*) from *H. diminuta* from rats (*n=2*) treated *in vivo* either with PLS 2.4 µmols or water. (B) Mean number of cysticercoids recovered from *Tribolium* beetles (*n=3*) fed with colon found detached segments (*n=3*) from Wistar rats (*n=2*) treated *in vivo* either with PLS 2.4 µmols or water. The amount of active CP expressed in µmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean.

7.2.3 Experiment 3

Confirmation of the effects of PLS against *H.diminuta in vivo*

7.2.3.1 Experimental design

This experiment was a follow-up from Experiment 1 to confirm the effects demonstrated in the reduction in worm burden and biomass. Twenty rats were used in this experiment (see chapter 2). Although the previous Experiment 1 incorporated the use of a larger sample size *n=10*, this experiment was attempted using a smaller sample size *n=5* because more groups were needed to factorially confirm the effect of PLS. Each rat was infected with 5 cysticercoids of *H. diminuta* on day 0 by oral gavage. Rats were divided into 4 groups. Each rat in treatment group 1 received 2 ml dose of PLS at 2.4 µmols. Group 2 received 2 ml dose of PLS at 2.4 µmols + E-64 at 2.8 µmols. Group 3 received 2 ml dose of E-64 at 2.8 µmols and group 4 received 2 ml water. All groups received daily treatment for 6 days starting from day 17 until day 22 post infection. In this experiment, assessment of efficacy of treatment through faecal egg counts was not carried out due to the earlier results which showed no differences between treated and control groups (Experiment 1). Therefore, worms were subjected to earlier treatment (day 17 post infection for the first dose of PLS, rather than day 27 post infection). Treatment effects were only assessed at autopsy through the recovery of worms and assessment of their biomass on day 22 post infection.

7.2.3.2 Results

Arithmetically, there was only minimal reduction (17%) in worm burden when comparing mean values between the treated group receiving PLS (mean=3.8) and the control group receiving water (mean=4.6) (Fig 7.3A). However, this was not statistically significant (Kruskal-Wallis test, χ^2_2=4.138, *P*=0.247). Biomass assessments however revealed more profound reductions. In the wet biomass assessment, the treated group (mean=1.5989grams) had a 38% reduction of mean biomass compared to the control group (mean=2.5857grams) (Fig 7.2B) which was statistically significant (Kruskal-Wallis test, χ^2_2=10.166, *P*=0.017). Also noted were increments rather than comparable mean values seen in the other control groups like the group receiving PLS+E64 (mean=2.8612grams) and the group receiving E64 only (mean=2.6917grams) which were 11% and 4% greater respectively as compared to the group receiving water (mean=2.5857grams)(Fig 7.3B). Similarly, the dry biomass assessment saw a comparable reduction of 41% between the treated group (mean=0.4452grams) and the group receiving water (mean=0.7529grams) (Fig 7.2B) which was also statistically significant (Kruskal-Wallis test, χ^2_2=8.806, *P*=0.032). As expected, a similar pattern of increment was also seen in the other control groups i.e. the group receiving PLS+E64 and the group receiving E-64 only which were 3.9% and 4.5% greater respectively. In assessing the main effects of PLS, data were analyzed by 2-way GLM (treatment with PLS and/or E-64) and the effect of PLS did not significantly affect worm burden (*P*=0.176) but was generally significant in affecting worm biomass (wet biomass *P*=0.068, dry biomass *P*=0.021). Surprisingly, the effects of E-64 was also found to significantly affect the increment rather than reduction in worm biomass (wet biomass *P*=0.005, dry biomass *P*=0.008) but not worm burden (*P*=0.176). Importantly the interaction between the main effects of PLS and E-64 was found significant in affecting worm biomass (wet biomass *P=0.014*, dry biomass *P=0.024*) but not worm burden (*P=0.*176). In other words E-64 had a different consequence depending on whether the rats were treated

with PLS or not. In PLS treated rats E-64 ablated the loss of weight completely but in non-PLS treated rats there was a margin increase in worm biomass.

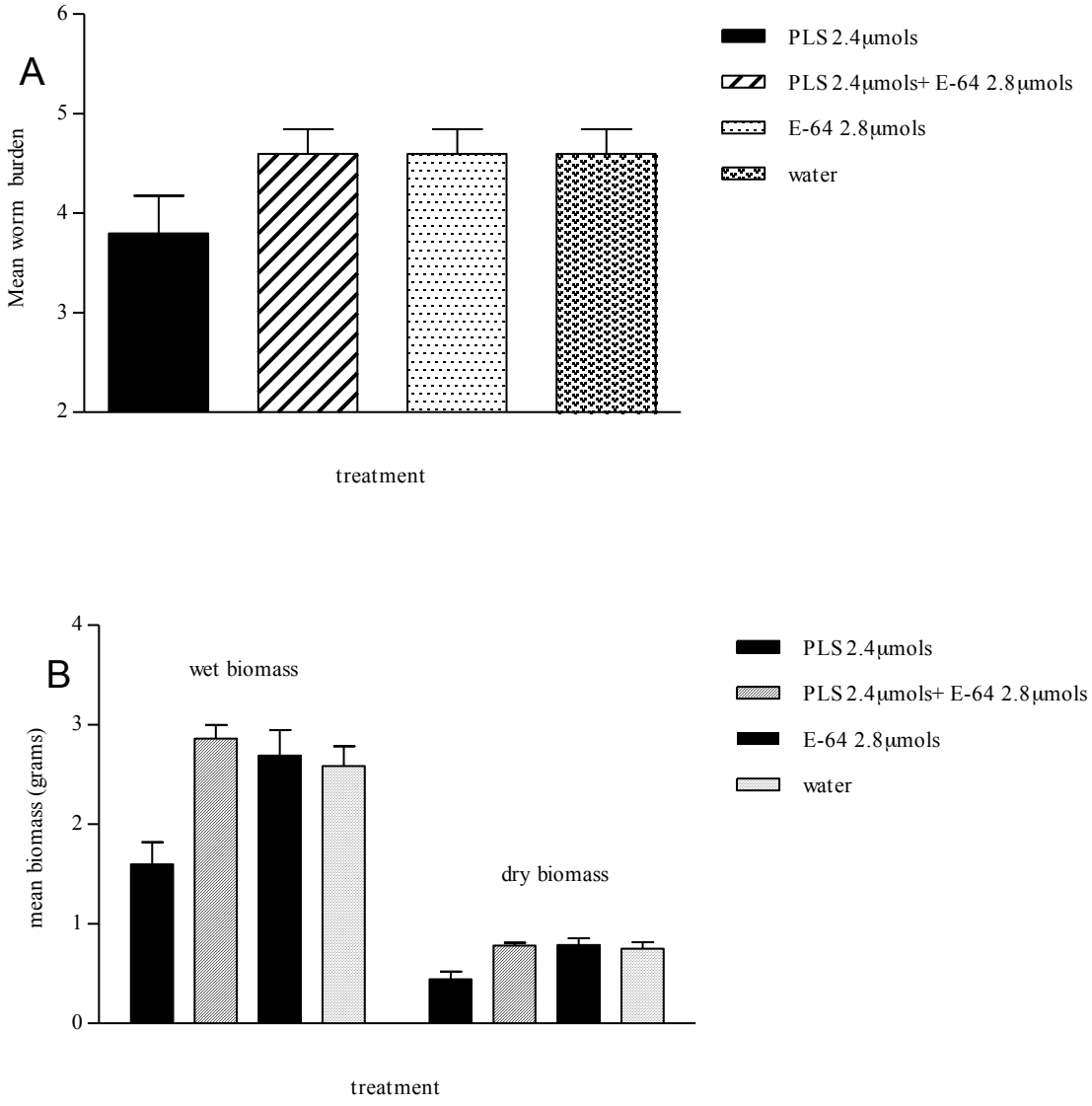


Fig 7.3 Confirmation of the effects of PLS against *H. diminuta in vivo*
The various autopsy indices used to measure the effects of PLS against *H. diminuta in vivo*. (A) The mean worm burden of Wistar rats infected with *H. diminuta* and treated either with PLS 2.4 µmols (*n*=5), PLS 2.4 µmols pre-treated with E-64 2.8 µmols (*n*=5), E-64 2.8 µmols (*n*=5) or water (*n*=5). (B) The mean biomass of *H. diminuta* in Wistar rats treated either with PLS 2.4µmols (*n*=5), PLS 2.4 µmols pre-incubated with E-64 2.8 µmols (*n*=5), E-64 2.8 µmols (*n*=5) or water. PLS appeared to only minimally affect worm burdens whilst moderately affecting worm biomass. Other treatments such as E-64 or when PLS was pre-treated with E-64 did not affect worm burden but surprisingly resulted in a slight increase in worm biomass when compared to the controls. For statistical analyses see text. The amount of active CP expressed in µmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean.

7.2.4 Experiment 4

Observation of PLS mediated damage against *H. diminuta in vivo.*

7.2.4.1 Experimental design

This experiment was simply a further extension of Experiment 3 to graphically document the effects of PLS inflicted on *H. diminuta in vivo*. Four rats were used in this experiment (see chapter 2). Due to the non- analytical nature of the experiment only 1 rat (*n=1*) per group was used to represent each of the 4 different treatment combinations. Rats were divided into 4 groups. Group 1 received 2mls dose of PLS at 2.4 µmols. Group 2 received 2mls dose of PLS at 2.4 µmols + E-64 at 2.8 µmols. Group 3 received 2 ml dose of E-64 at 2.8 µmols and group 4 received 2 ml water. All rats received daily treatment for 6 days starting from day 17 until day 22 post infection. The efficacy of treatment was assessed through the recovery of detached proglottid segments found in the large intestine downwards which were immediately fixed in glutaraldehyde and later dehydrated, critical point dried and gold coated before finally being viewed under scanning electron microscopy for signs of any damage.

7.2.4.2 Results

H. diminuta proglottid segments recovered from the rat treated with PLS 2.4µmols appeared to sustain treatment related damage as the damage observed was substantial, uneven and located not at the proglottid's anatomical point of attachment exposing *H. diminuta* eggs (Fig 7.4A). The segments recovered from control rats treated with either PLS 2.4µmols pre-treated with E-64 2.8µmols, E-64 2.8µmols or water did not show similar damage apart from the minimal, clean and sharp tegumental breaks or cracks which may be mechanical in origin (Figs 7.4 B-D).

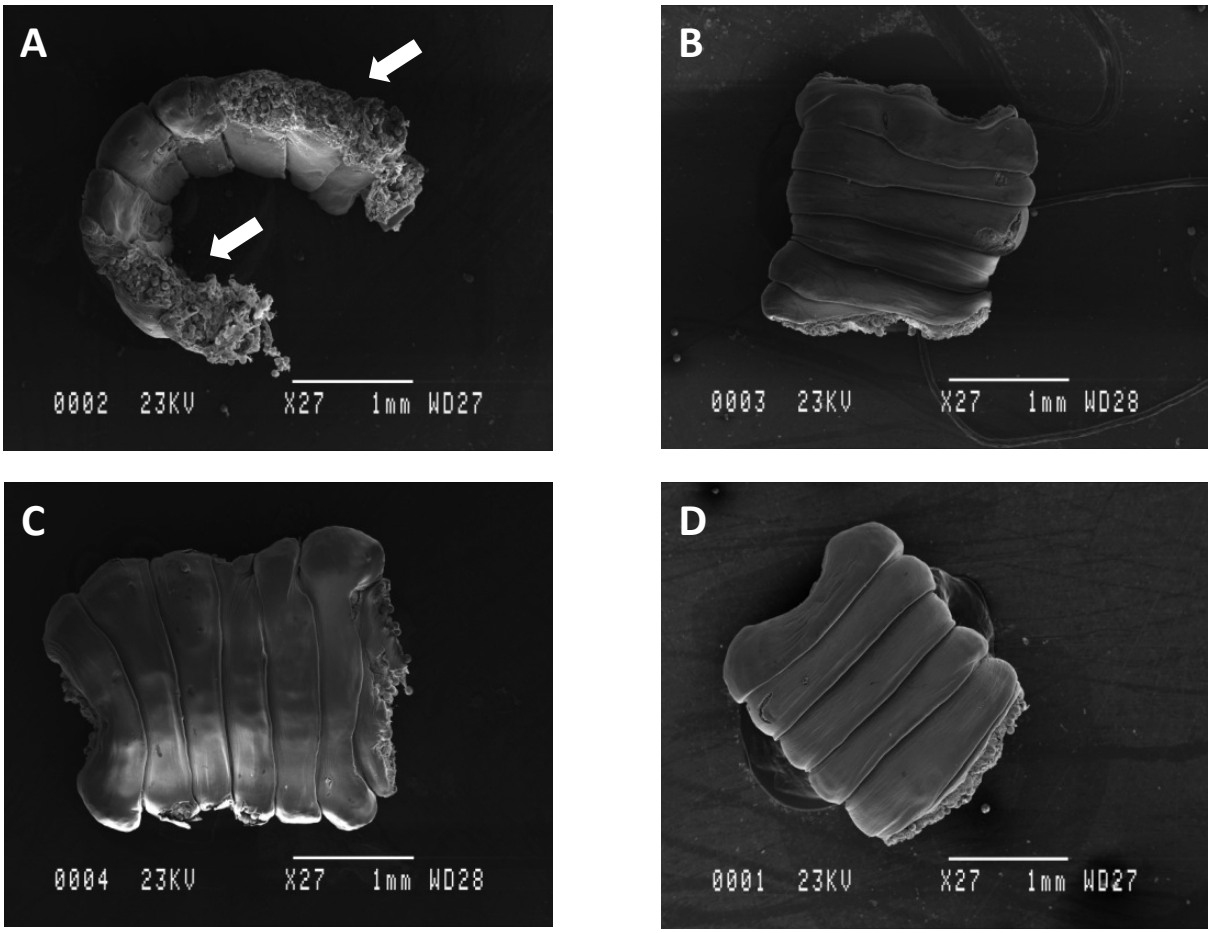


Fig 7.4 SEM images of the effects of PLS against *H. diminuta in vivo*
SEM images of detached proglottid segments of *H. diminuta* recovered from the colons of infected Wistar rats treated with (A) PLS 2.4 µmols (B) PLS 2.4 µmols pre-treated with 2.8 µmols E-64 (C) E-64 2.8 µmols (D) water. Note the extensive uneven damage (arrows) in (A) exposing *H. diminuta* eggs seen at the lateral aspect of the segment which is not the point of attachment. Note the otherwise minimal, clean and sharp breaks or cracks in other segments (B-D) which may be mechanical in origin. The amount of active CP expressed in µmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2).

7.2.5 Experiment 5

Assessment of the dose-dependency of *H. diminuta in vivo* in rats.

7.2.5.1 Experimental design

This experiment was carried out to assess whether or not the reduction in worm burdens and biomass observed following treatment of infected rats with PLS against *H. diminuta* in Experiment 1 is dose-dependant. Sixteen rats were used in this experiment (see chapter 2). This experiment was a correlational study between the dose of PLS and worm autopsy indices. Therefore, a smaller sample

size *n=3* was thought to be generally sufficient for each level of treatment. Rats were divided into 4 groups each receiving different doses of PLS. This experiment was also executed simultaneously with Experiment 3 in which rats from the group receiving water were incorporated as group 1 (0 nmols dose) in this experiment (*n*=5) and rats receiving 2400nmols of PLS *n=5* were incorporated as group 4. Group 2 (*n*=3) received 60 nmols of PLS, group 3 (*n*=3) received 240nmols. All groups received daily treatment for 6 days starting from day 17 until day 22 post infection. Treatment effects were only assessed through worm counts and biomass assessments at autopsy on day 22 post infection.

7.2.5.2 Results

Autopsy revealed no correlation between PLS dose and worm burden (R=0.001, *n=16, P*=1) (Figure 7.5A). Reassuringly, there was a significant negative correlation between worm biomass and PLS dose in assessment of dry biomass (R=-0.531, *n=16, P*=0.034) (Figure 5B). The wet biomass had a weak negative correlation with PLS dose but this was not significant (R=-0.186, *n=16, P*=0.49) (Figure 5B).

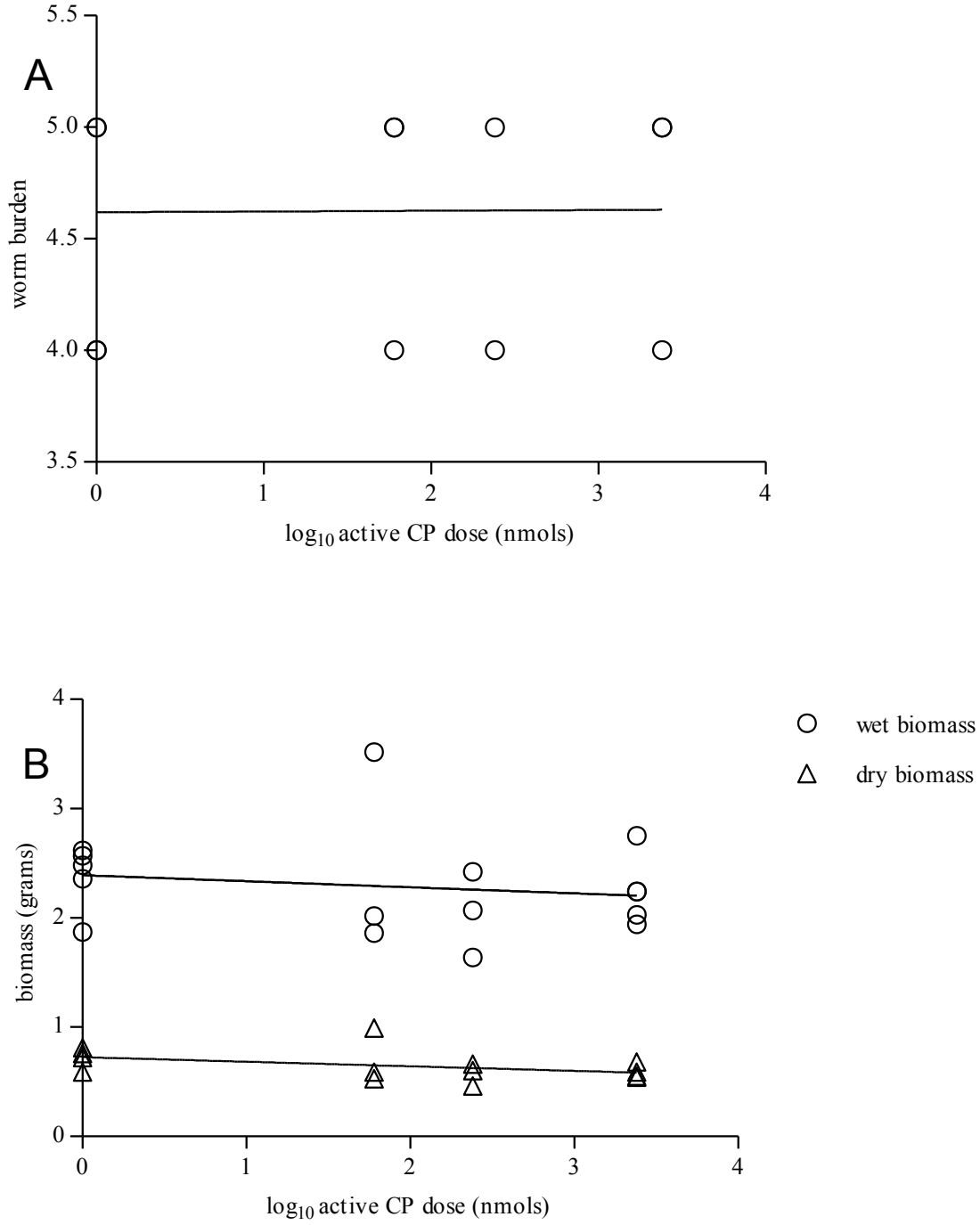


Fig 7.5 Dose-dependency of PLS against *H. diminuta in vivo*
The various indices demonstrating the relationship between PLS dose and *H. diminuta in vivo*. (A) The correlational trend between increasing PLS dose and worm burden. (B) The correlational trend between increasing PLS dose and worm biomass. All groups were of the same sample size (*n=3*) except groups 1 and 4 (*n=5*). For statistical analyses see text. The line of best fit is provided to guide the eye. The amount of active CP expressed in nmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2).

7.2.6 Experiment 6

Assessment of *H. diminuta* recovery *in vivo* post PLS treatment

7.2.6.1 Experimental design

This experiment was designed to assess whether or not *H. diminuta* have the ability to recover *in vivo* post PLS treatment since tapeworms have been shown to repair themselves quickly after sustaining damage (Befus and Threadgold, 1975). Twenty rats were used in this experiment (see chapter 2). The rats were divided into 4 groups with 5 rats per group (n=5). Group 1 received 2mls PLS at 2.4µmols, group 2 received 2mls water, group 3 received 2mls PLS at 2.4µmols and group 4 received 2mls water. All groups received daily treatment for 6 days from day 17 until day 22 post infection. Groups 1 & 2 were sacrificed immediately at the end of the treatment week (day 22 post infection) while groups 3 & 4 were left to continue without treatment for another week until day 28 of infection. 1 rat in group 3 died during the course of treatment and was excluded from the results reducing the number of sample in group 3 (n=4). The effectiveness of treatment was determined through autopsy for recovery of worms and assessment of their biomass on days 22 and 28 post infection.

7.2.6.2 Results

The first autopsy which was immediately after completing 6 days of treatment revealed a modest profile of reduction compared to the reductions seen in Experiment 1 and 3. In fact there was no arithmetical reduction seen in worm burden (Fig 7.6A) but there was 6-18% reduction seen in biomass (Fig 7.6B). Interestingly, the second autopsy which was conducted 1 week after withholding treatment revealed a more profound profile of reductions compared to 1st round of autopsy with all indices showing an increase in arithmetical reductions ranging from 12-22%. Data were further explored using 2-way GLM to assess the main effects of time and treatment and also the interaction between the two variables. The effect of treatment on worm burden was not significant $F_{1,15}$=3.151 P=0.096 and neither was the effect of time on worm burden $F_{1,15}$=0.014 P=0.907. There was no significant interaction between time and treatment on worm burden $F_{1,15}$=3.151 P=0.096. The effect of treatment on wet biomass, however, was close to significance $F_{1,15}$=3.386 P=0.086. Surprisingly, the effect of time was also significant $F_{1,15}$=6.663 P=0.021, wet biomass values being lower on day 28 compared with day 22 in both groups. However, surprisingly the interaction of treatment and time was not significant $F_{1,15}$=1.25 P=0.281 despite the greater discrepancy between mean values of treated and control rats on day 28 compared with day 22. The effect of treatment on dry biomass was significant $F_{1,15}$=7.682 P=0.014, the values in PLS treated rats being lower on both occasions, while the effect of time was just outside of significance $F_{1,15}$=4.143 P=0.06. Again, perhaps surprisingly, there was no significant interaction between time and treatment $F_{1,15}$=0.261 P=0.617.

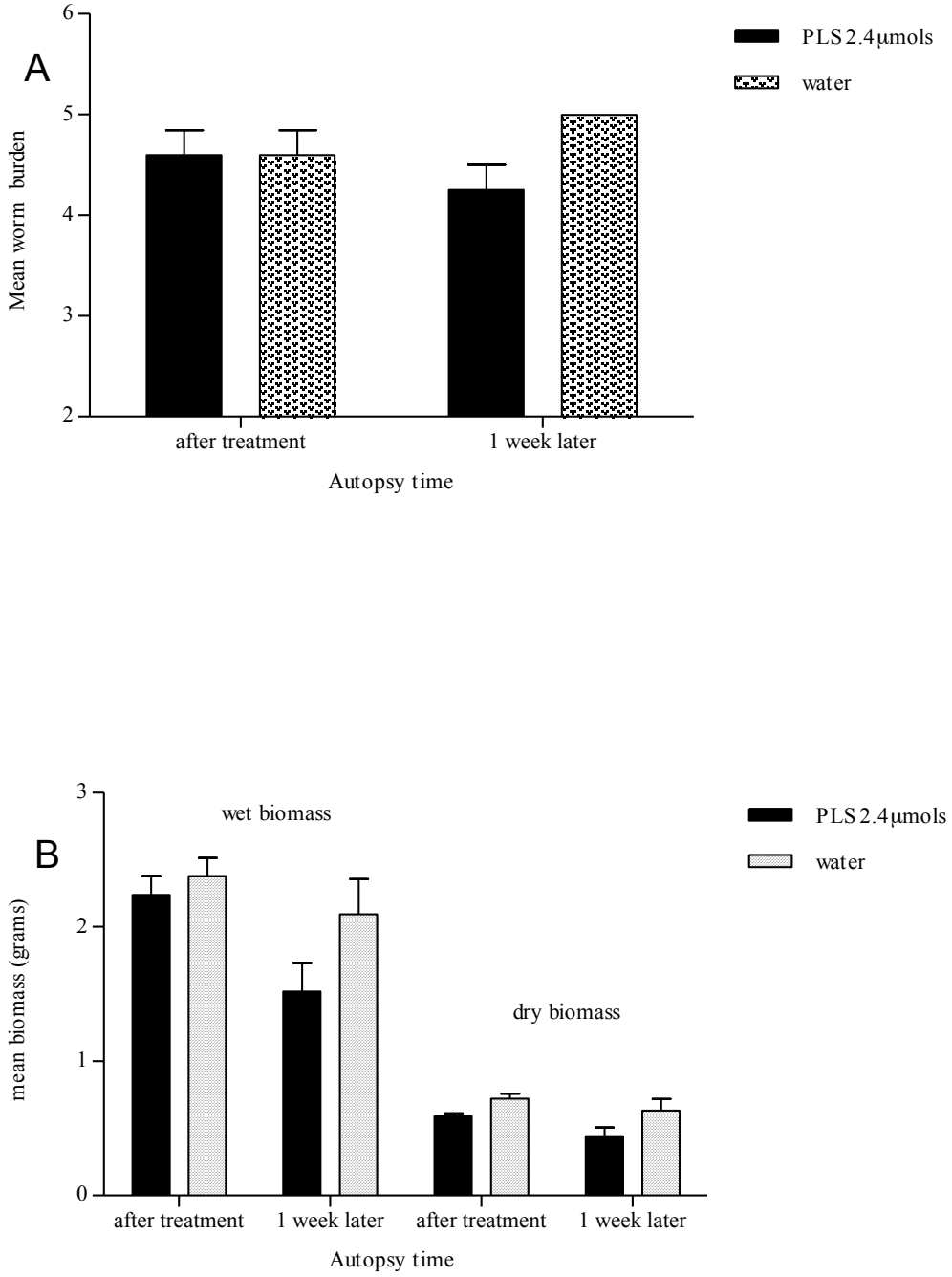


Fig 7.6 *H. diminuta* recovery post PLS treatment

(A) The mean worm burden of Wistar rats infected with *H. diminuta* immediately after 6 days treatment of either PLS 2.4 µmols (*n*=5) or water (*n*=5) and 1 week after withholding treatment of PLS 2.4 µmols (*n*=4) or water (*n*=5). (B) The mean biomass of *H. diminuta* in Wistar rats immediately after 6 days treatment of either PLS 2.4 µmols (*n*=5) or water (*n*=5) and 1 week after withholding treatment of PLS 2.4 µmols (*n*=4) or water (*n*=5). The amount of active CP expressed in µmols is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean.

7.3 Discussion

Following the disappointing results from using the *H. microstoma* model (Chapter 5) this chapter was designed to test the efficacy of CPs in a different model system, *H. diminuta*, in its natural host, the rat. With this model, I intended to explore the consequences of treatment with CPs for an entirely intestinal lumen dwelling tapeworm as opposed to *H. microstoma* which attaches in the bile duct and employs hooks to aid attachment to host tissues. The latter species, attaching in the common bile duct, might escape contact with CP especially in the sensitive neck region and the scolex itself.

Disappointingly, the faecal egg count results (Fig 7.1A) showed no reduction in the treated group as compared to the control. Despite the worms being fully exposed to the CPs because the worm is lumen dwelling, the dose still failed to show any reduction in faecal egg counts. Clearly, tapeworms cannot be compared directly to nematodes which once affected by CPs, usually die, exploding soon afterwards, resulting in the cessation of egg output which is then clearly reflected within 24h in a reduction of faecal egg counts.

It was then imperative to ascertain whether fecundity was still affected despite no reduction in egg counts by egg viability test (Experiment 2). Disappointingly, *H. diminuta* eggs were not affected at all because intact *H. diminuta* cysticercoids were recovered from *Tribolium* beetles fed with either cut terminal proglottid segments or detached proglottid segments recovered from the colon of rats infected with *H. diminuta* and treated with PLS. The difference in mean cysticercoids recovered between *Tribolium* beetles fed with cut terminal proglottid segments and recovered detached proglottid segments was perhaps due to their size where the cut proglottides were around 10mm in longitudinal length whereby the colon recovered detached segments were around 1-2mm.Unlike the benzimidazoles which were able to affect the development of *H. diminuta* larval stages (Evans and Novak, 1976) PLS does not appear to have any effect on *H. diminuta* fecundity when administered to infected rats. However, the experiment by (Evans and Novak, 1976) was an *in vitro* experiment in which infected *Tribolium* beetles were fed flour infused with benzimidazole drugs, whereas in the current work I tested the viability of eggs shed *in vivo* by infected rats. Structurally, cestode eggs have a thicker outer shell compared to trichostrongyloid nematode eggs such as those of *H. bakeri*, but not those of *Trichuris* and *Ascaris* species. This robust egg shell is required because of the need for the eggs to survive in harsh external environmental conditions before continuing its life cycle in an intermediate host. Perhaps, the egg shell does not contain similar protein targets found in the tegument and nematode cuticle which are susceptible to PLS attack. Similar findings of unaffected fecundity were also observed in *H. microstoma* (chapter 5).

Contrary to the unaffected fecundity, autopsy indices revealed a different perspective on the efficacy of PLS against *H. diminuta in vivo.* The pilot experiment (Experiment 1) revealed a moderate (46%-54%) but significant reduction profile. This came as a surprise since *H. microstoma* was only minimally affected (Chapter 5). In the non-permissive host however, a more marked effect was seen on *H. diminuta* (Chapter 6) possibly as a result of a compound attack from PLS as well as the immune system. However, these effects need to be confirmed using E-64 as a specific inhibitor for CP so that we can indeed be certain that of the cysteine proteinases in PLS are the active principles. Despite a

much varied response in arithmetical reductions (worm burden 17%, wet biomass 38% and dry biomass 41%) as compared to the controls this experiment (Experiment 3) confirmed that PLS cannot be said to be responsible for worm loss but does indeed affect the ability of the worm to repair itself after PLS treatment, as non-parametric testing revealed that worm biomass was significantly different (both wet & dry biomass) from the other control groups. Not surprisingly, both groups either receiving E-64 alone or PLS which had been pre-incubated with E-64, saw an increase in biomass (4-10%) compared to the group receiving water. This finding is comparable to similar observations from experiments conducted with *H. microstoma* which were thought to be due to an exaggerated response of repair following the perturbation of other components in PLS (since PLS being competitively inhibited) such as the mucolytic enzymes and alkaloids that PLS is known to contain (Mezhlumyan *et al.*, 2003). These might still be at large diffusing through the tegument. Even E-64 alone might still be diffusing through the tegument causing disturbance and the exaggerated repair. This postulation was strengthened by the fact that the main effects of E-64 was significant in a 2-way GLM. Therefore, PLS can possibly be said to affect the ability of *H. diminuta* to repair itself but not to the extent of expelling the damaged worms. SEM images of found detached proglottid segments from the colon following treatment perhaps provide further evidence of PLS efficacy. While the control segments were either intact or possibly sustained mechanical damage (Fig 7.4B-D), the segment found in the colon of the PLS treated animal appeared to have sustained more extensive, treatment-related damage (Fig 7.4A) which was uneven in appearance across the sampled proglottids. However, the possibly damaged appearance was not as extensive as that seen earlier in the *in vitro* experiment images (Chapter 4). One possible explanation could be due to the remarkable ability for repair in cestodes (Befus and Threadgold, 1975).

In evaluating these effects further it was also imperative to ascertain whether or not the effects were dose dependant (Experiment 5.). As expected, worm burden has no correlation with increasing PLS dose (Fig 7.5A). Worm dry biomass however showed the expected negative correlation (Fig 7.5B) with increasing PLS dose although wet biomass had only a weak correlation (Fig 7.5B) but nevertheless still in the expected direction. Having seen all the effects of PLS on *H. diminuta in vivo* after completing treatment it was also important to see whether or not these effects are permanent or temporary since cestodes have been shown to be able to repair damage very quickly (Befus and Threadgold, 1975). The reduction in worm burden especially required validation because there remained the possibility that what might have been interpreted initially as worm loss was in actuality generated by destrobilated scoleces persisting somewhere in the intestine and later regrowing to the original size as mature worms when the danger from local CPs had been swept away by peristalsis. Destrobilated scoleces have been shown to regrow even when transplanted into another host (Hopkins et al., 1972a). Experiment 6 revealed that arithmetically time does have an effect on worm loss because there was an overall 15% loss of worm burden when the assessment was conducted a week later than immediately after treatment. However, this proved not to be statistically significant indicating that actually worms are not lost over this short period of time in PLS treated animals. The interaction of time and treatment on worm burden was close to but not significant (P=0.096), and this reflected the slight drop in numbers in treated rats and the slight gain in mean worm burden in the control rats over time but the values are very small and whether they have a biological basis is not clear. It is possible that PLS is indeed responsible for some

loss of *H. diminuta* worms *in vivo* possibly through a synergistic interaction with the immune system whereby weak and vulnerable worms post PLS damage might not be able to continue to evade the host immunity as well as normal patent and healthy worm. Even in its natural rat host, *H. diminuta* has been shown to be rejected immunologically over a long time frame (Andreassen and Hopkins, 1980).

The effects on worm burdens differ from those on worm size as reflected in biomass where dry biomass was significantly reduced and mean wet biomass also fell with time although not significantly. Interestingly a fall in mean dry and wet biomass was also evident in control rats although not significantly in the case of dry biomass since the main effect of time and the interaction between time and treatment were not significant. In the case of wet biomass, however, the change over time (Fig 7.6B) alone was significant indicating that worms did get smaller over time in the experiment regardless of treatment and this might possibly be explained by interworm competition for resources as described by Read (1951) who coined the crowding effect.

Another observation worth mentioning is the occurrence casualties during experiment. In experiments 1 & 6, 1 rat from each experiment died during the course of treatment. Unlike some mice, rats were not affected at all by PLS treatment and this was reflected by no weight loss during a typical 6-days treatment course (results not shown). Casualties happened almost immediately during oral gavage as a result of slight inaccuracy in animal handling technique where the animal may have experienced gastro-oesophageal reflux and part of the PLS may find its way into the animal's lungs killing it instantaneously.

The results from this chapter provide the first convincing evidence showing that this tapeworm, *H. diminuta*, is affected by CPs *in vivo* albeit just moderately and this contrasts with previous reports based on experiments with other species of rodent cestodes (de Amorin et al., 1999; He et al., 1992). The results offer the possibility that with further refinement, CPs may be developed into broad spectrum anthelmintics that remove nematodes from their hosts and have significant effects also on any concurrently resident tapeworms.

CHAPTER 8

Assessment of the anthelmintic efficacy of PLS against the equine cestode *A. perfoliata in vitro.*

8.0 Summary

Papaya latex has been demonstrated to be efficacious against murine (Satrija et al., 1995; Satrija, 2001; Stepek et al., 2006b, 2007b, c), porcine (Satrija *et al*., 1994), ovine (Buttle *et al*., 2011), canine (Stepek *et al*., 2007e) and even helminths of chicken (Mursof and He, 1991). The experiments described in this chapter indicate that CPs are also efficacious against the equine cestode *A. perfoliata* by causing a significant reduction in motility leading to death of the worms. This is the first evidence for anthelmintic efficacy of CPs in an equine helminth, providing further evidence that the spectrum of activity of CP is not restricted to nematodes and supporting the idea that they can be developed into useful broad-spectrum anthelmintics..

8.1 Introduction

Horses are animals of economic importance (Huffman and Guidry, 1979), as working animals in some countries, for the provision of meat and especially in the racing and leisure industries in more affluent parts of the world. Maintaining their health is therefore crucial. Gastrointestinal helminths are a major cause of morbidity in the livestock industry including horses (Matthews et al., 2004; Nieuwhof and Bishop, 2005) in which nematodes such as *Parascaris*, the strongyloids and the cyasthostomins are central to the problem, with pathological effects that can be severe enough to cause marked distress to the animals. Infection with the equine cestode *A. perfoliata* has recently been recognized as a significant cause of morbidity in horses (Proudman and Trees, 1999). Intestinal problems may range from mild colic (Proudman et al., 1998) to necrosis (Rodriguez-Bertos et al., 1999) and there has even been a report of a fatal case of caecal rupture (Ryu et al., 2001).

Control of GI helminths is principally by chemotherapy (Behnke et al., 2008), and not surprisingly with the wide scale usage and over use of anthelmintics resistance has arisen among equine nematodes just as in those of cattle and sheep. Anthelmintic resistance among horse worms is a serious problem of some economic significance (Kaplan, 2002, 2004). Despite the lack of any reports of resistance among horse tapeworms, the issue should not be taken lightly especially in the wake of development of praziquantel resistance in other platyhelminths (Ismail *et al*., 1999)

Alternatives to conventional chemotherapy based on synthetic products should be explored in the wake of promising developments of plant based anthelmintics such as the CPs (Behnke *et al*., 2008). CPs have been shown to work against various helminths ranging from murine (Stepek et al., 2007c) to ovine (Buttle *et al*., 2011), and recent work shows that they are also effective against tapeworms (Stepek et al., 2007e) and See Chapter 4 and 6.

In earlier chapters, I explored the effects of CPs both in *in vitro* and *in vivo* against rodent tapeworms which are useful laboratory models, but are not of economic significance. In this chapter I present data assessing the efficacy of papaya latex against the equine cestode *A. perfoliata* which is an important pathogen of horses and which is not easy to treat with conventional anthelmintics (Matthews *et al.*, 2004).

8.2 Experimental design and results

8.2.1 Experiment 1

The efficacy of PLS against adult *A. perfoliata in vitro*.

8.2.1.1 Experimental design

Adult horse tapeworms, *A. perfoliata,* were sourced from Turner's abbatoir, Cheshire (see chapter 2). Worms were incubated in different combinations of treatments of either PLS with and without the activator cysteine (on a range of concentration of active CP), PLS pre-treated with E-64 and the control treatments of Hanks' saline with and without cysteine.

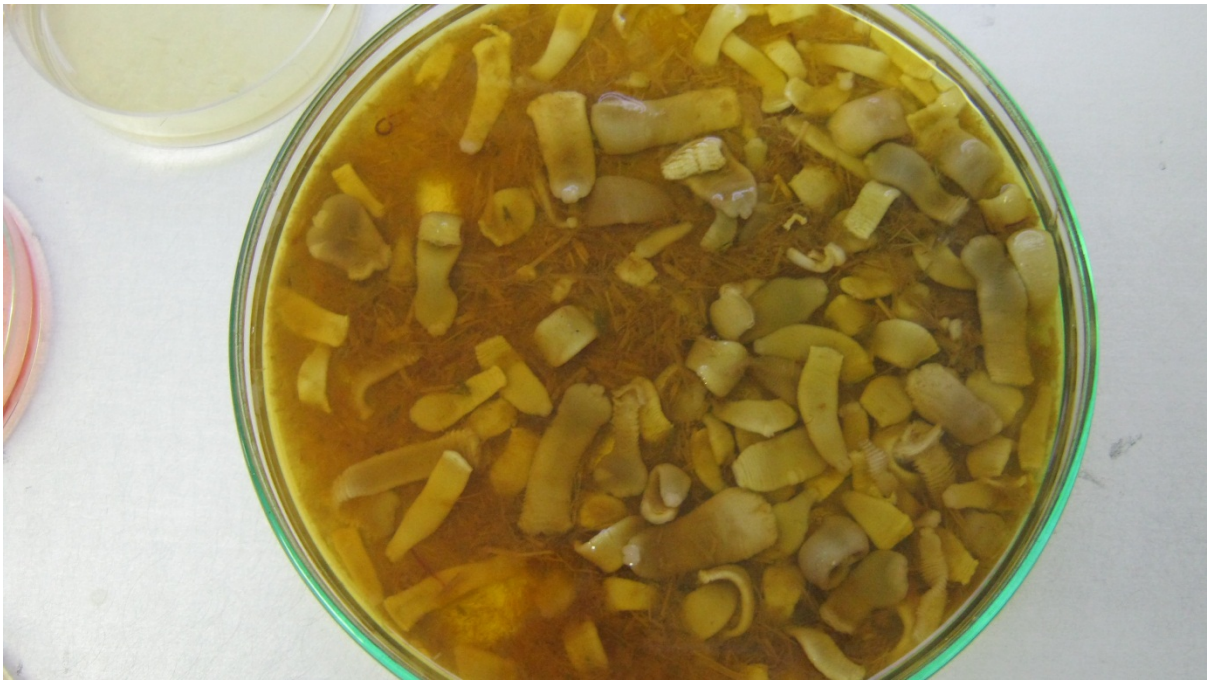


Fig 8.1 *A. perfoliata* in horse caecal fluid transferred from the flask.

8.2.1.2 Results

Arithmetically, it was evident that worm motility quickly decreased in the groups incubated in higher concentrations of PLS activity (1000µM- 3000µM). Motility decreased steadily in the groups incubated in intermediate concentrations of PLS activity (30µM- 300µM). On the contrary motility was relatively maintained until the end of experiment at 120min in worms incubated in low concentration of PLS activity (10µM), PLS priorly treated with E-64, Hanks' saline with or without 1mM cysteine. The motility reducing effects of PLS against *A. perfoliata* were both time ($F_{8,112}$=87.631 P<0.001) and concentration ($F_{6,14}$=245.924 P<0.001) dependant.

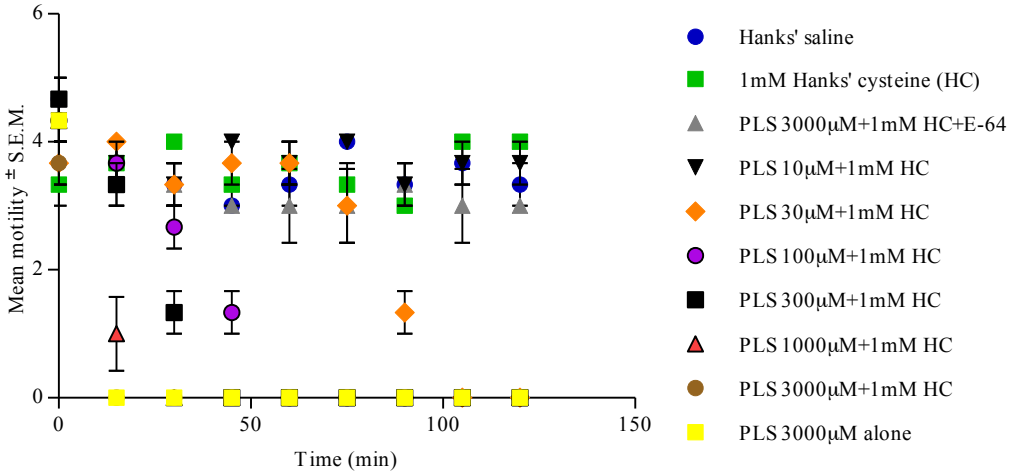


Fig 8.2 Motility of adult *A. perfoliata* in PLS *in vitro*
Motility of adult *A.perfoliata* incubated in varying concentrations of active PLS *in vitro*. The concentration of active CP expressed in µM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean n=3.

Table 8.1 IC_{50} values of PLS efficacy against various cestodes.

cestode	PLS $1C_{50}$ (µM)**
A.perfoliata	227
*H. diminuta**	818
*H. microstoma**	1107

*Data derived from chapter 4

** The concentration of active CP expressed in µM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2).

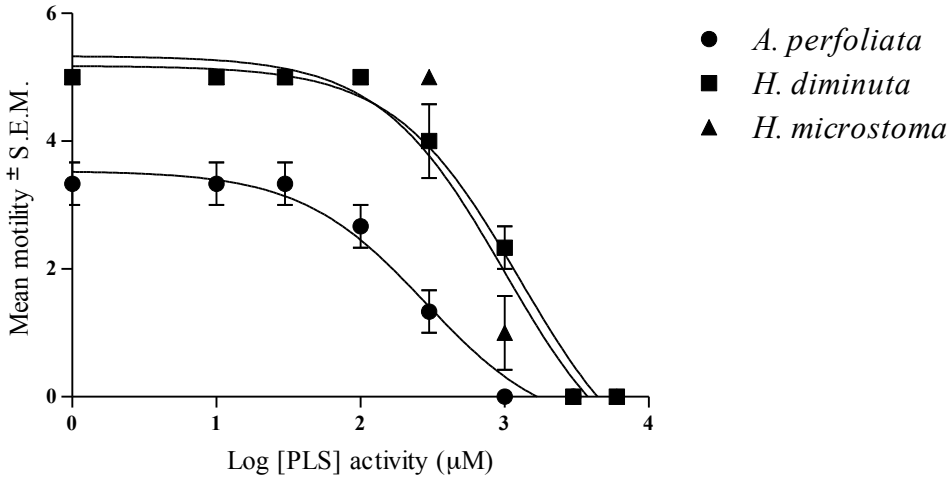


Fig 8.3 Concentration-inhibition curves of PLS activity against various cestodes at 30 minutes post incubation.
Note the greater efficacy of PLS against *A. perfoliata* with IC_{50} values of almost 4x less than murine cestodes. The concentration of active CP expressed in μM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 μl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean *n*=3.

8.2.2 Experiment 2

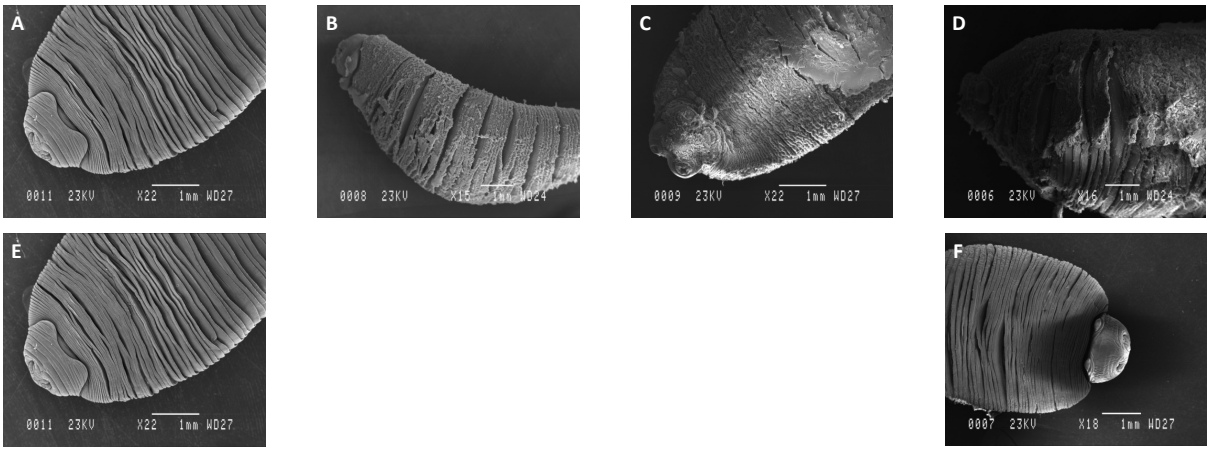
The observation of CP mediated damage against *A. perfoliata* under SEM

8.2.2.1 Experimental design

Experiment 2 was conducted simultaneouly alongside Experiment 1. Worms from the same stock used in Experiment 1 were incubated into either PLS 1000μM+1mM HC or Hanks' saline. At timely intervals of 10min, 20min and 60min, 1 worm was removed and fixed in glutaraldehyde, dehydrated in alcohol, critical point dried and gold coated before finally being viewed under SEM.

8.2.2.2 Results

Worms incubated in PLS showed marked generalised erosions as early as 10min (Fig 8.4B). After 20min of incubation in PLS and the progressive erosion was made more apparent and the tegument appears to undergo 'exfoliation' (Figs 8.4 C). After 60min of incubation in PLS the tegument was severely damaged and appears chapped and flaky.



0min 10min 20min 60min

Fig 8.4 SEM images of *A. perfoliata* in PLS
A. perfoliata incubated in PLS 1000 µM+1 mM HC *in vitro* (A) at 0 min (B) at 10 min (C) at 20 min (D) at 60 min. *A. perfoliata* incubated in Hanks' saline (E) at 0min (F) at 60min. Note the progressive damage to the tegument in worms incubated in PLS and intact worms incubated in Hanks' saline. The concentration of active CP expressed in µM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2).

8.2.3 Experiment 3

The efficacy of various CPs against *A. perfoliata in vitro*

8.2.3.1 Experimental design

Experiment 3 was designed as an extension of experiment 1 to observe the efficacy of other types of CPs against *A. perfoliata*. Adult *A. perfoliata,* were sourced from Lawrence J. Potter's abbatoir, Somerset (see chapter 2). Worms were incubated in different combinations of treatments of either CP with and without the activator cysteine (on a range of concentration of active CPs), CP pre-treated with E-64 and the control treatments of Hanks' saline with and without cysteine (3 replicates per treatment n=3).

8.2.3.2 Results

In all CPs tested the motility of adult *A. perfoliata* was rapidly reduced in the presence of higher concentrations of active CPs, but more gradually in the presence of moderate concentrations of active CPs (see Figs 8.5 A-C). Pineapple extract caused a significant reduction in motility which was both time ($F_{8,80} = 82.897$ $P<0.001$) and concentration ($F_{4,10} = 136.908$ $P<0.001$) dependant. Papain caused a significant reduction in motility which was both time ($F_{8,96} = 94.168$ $P<0.001$) and concentration ($F_{5,12} = 76.546$ $P<0.001$) dependant. Stem bromelain caused a significant reduction in

motility which was both time ($F_{8,96} = 55.798$ $P<0.001$) and concentration ($F_{5,12} = 32.816$ $P<0.001$) dependant. Motility however was relatively preserved when worms were incubated in the presence of low concentrations of active CPs or in the control solutions of Hanks' saline with or without cysteine and also in the presence of CP pre-treated with E-64 (see Fig 8.5 A-C). Although all CPs tested were efficacious, efficacy varied between the enzymes with pineapple extract being most efficacious at IC_{50} of 74µM and stem bromelain being the least efficacious with $1C_{50}$ of 834µM (tab 8.2).

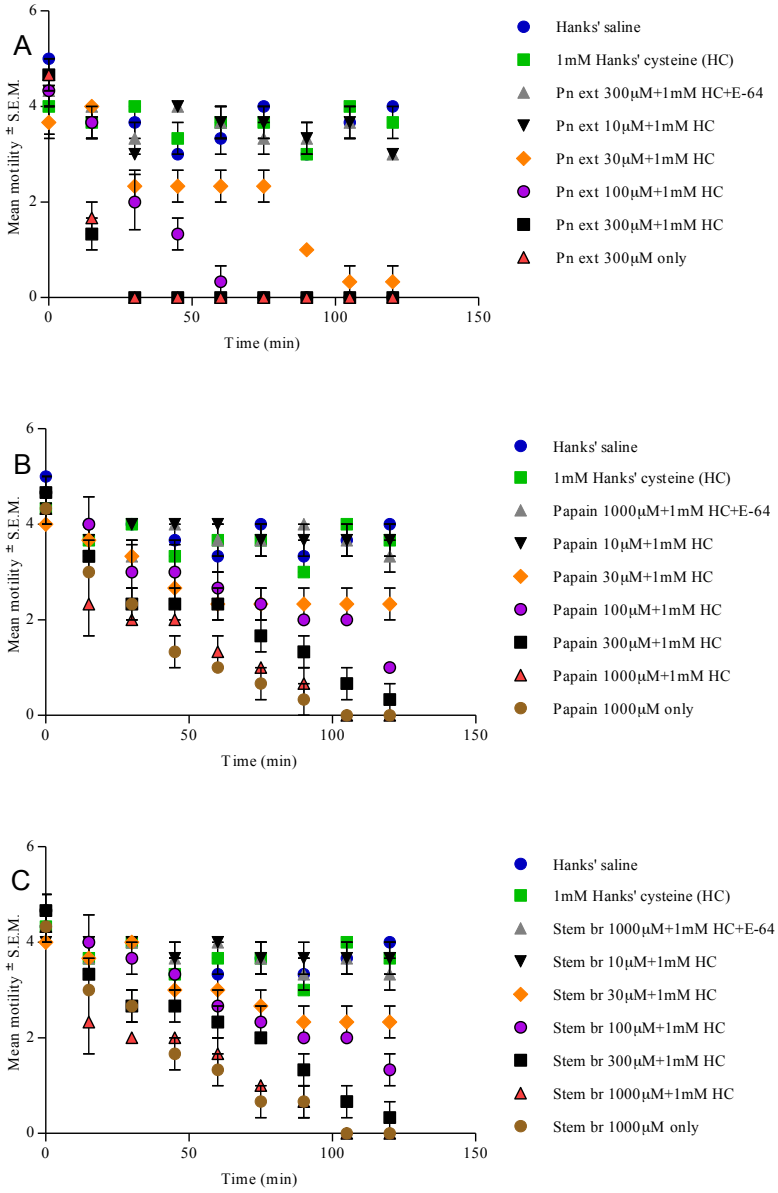


Fig 8.5 Motility of adult *A. perfoliata* in various CPs *in vitro*
Motility of adult *A. perfoliata* incubated in various CPs *in vitro* (A) pineapple extract (B) papain (C) stem bromelain. ▽, CP without cysteine (concentration equivalent to the highest concentration in particular experiment); △, 1000µM+1mM Hanks' cysteine; □, 300µM+1mM Hanks' cysteine; ○, 100µM+1mM Hanks' cysteine; ◆, 30µM+1mM Hanks' cysteine; ▼, 10µM+1mM Hanks' cysteine; ▲; CP pre-treated with E-64 (active CP concentration equivalent to the highest concentration in particular CP experiment); ■, 1mM Hanks' cysteine; ●, Hanks' saline. The concentration of active CP

expressed in µM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean *n*=3.

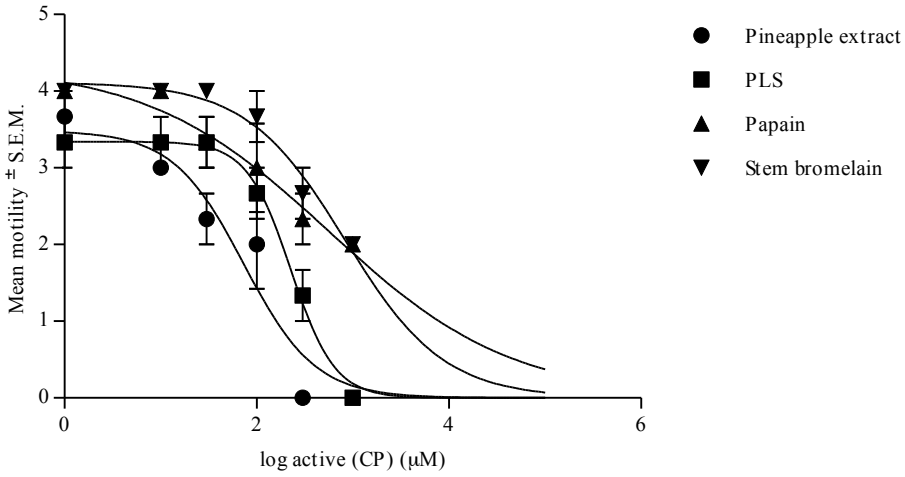


Fig 8.6 Concentration-inhibition curves of various CPs against *A. perfoliata in vitro* at 30 minutes post incubation.
The concentration of active CP expressed in µM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2). Error bars represent the standard error of the mean *n*=3.

Table 8.2 IC_{50} values of various CPs against *A. perfoliata in vitro*.

CP	$1C_{50}$ (µM)*
Pineapple extract	74
PLS	227
Papain	568
Stem bromelain	834

***** The concentration of active CP expressed in µM is the molar equivalent of the inhibitor E-64 used to block active CP in a 10 µl volume in a titration assay (see chapter 2).

8.3 Discussion

One of the important aspects in developing novel classes of anthelmintic is the need for them to show broad spectrum of activity against helminths and even parasites of other taxonomic groups, such as the protozoa, because the broader the spectrum against pathogens the better the chances of acceptance by users (Behnke *et al*., 2008). Papaya latex has been demonstrated to be efficacious against murine (Stepek *et al*., 2007c), porcine (Satrija *et al*., 1994), ovine (Buttle *et al.*, 2011) canine (Stepek *et al*., 2007e) and even helminths of chicken (Mursof and He, 1991). It has also been demonstrated to affect juvenile stages of plant parasitic nematodes (Stepek *et al*., 2007a). These observations suggest a

broad spectrum of activity and hence a promising prospect for CPs in conventional therapy. Moreover, in this thesis, evidence has been presented showing an even wider spectrum of activity than that already in the public domain, namely on rodent cestodes both *in vitro* and *in vivo*.

The experiments reported in this chapter showed a marked effect of CPs on an equine cestode, *A. perfoliata, in vitro* by significantly reducing its motility and finally causing death of the worms (see Figs 8.2 & 8.5). The reduction in motility was both time and dose dependant but independant of the presence of cysteine, similarly to the earlier reported observations on both rodent cestodes (see Chapter 4) but differing from the effects on nematodes which are dependant upon the presence of cysteine (Stepek et al., 2005). The most likely explanation of this is perhaps through its excretory process via the tegument that cestodes produce a reducing agent that in turn activates CPs.

Equally surprising was the finding that CPs appeared to be more efficacious against *A. perfoliata* than it was against other rodent cestodes (see Fig. 8.3) with IC_{50} values of 3-4 times lower than for rodent cestodes (see table 8.1). This greater sensitivity of *A. perfoliata* to CPs may be explained by the relative diminutive length of *A. perfoliata* size which ranged between 2-5cm compared to 14 day-old adult *H. diminuta* used earlier for *in vitro* experiments (chapter 4) which were are almost 15cm long. Thus exposed surface area may have been factor, but it may also be possible that the tegument itself is different from that of rodent cestodes in terms of its constituent proteins, as a result of the different diets and consequently physiological conditions in the intestines of horses compared to rodents. Efficacy also differed between different types of CPs (see Fig 8.6 and Table 8.2) with naturally occurring CPs like pineapple extract and PLS being more efficacious than singular purified CPs like papain and stem bromelain which is a similar observation in rodent cestodes (chapter 4).

It has been shown that CPs damage the tegument of murine cestodes possibly by hydrolysing particular proteins that make up the tegument resulting in generalised tegumental digestion. Consequently, at weakened points CPs eventually cause breaches of tegumental integrity and the subsequent release of internal contents. In this chapter, based on SEM observations, PLS has been shown to damage the tegument of *A. perfoliata* (see Fig. 8.4) in much the same manner, beginning with superficial generalised erosion of the surface of the tegument (Fig 8.4B) and with time, a much deeper and widespread generalised destruction (Fig 8.4D). Visually the images documenting damage to the surface of rodent cestodes (chapter 4) and those in this chapter to *A. perfoliata* were indistinguishable in terms of the sequence and extent of the changes that were observed. This is quite different from conventional cestocidals such as praziquantel which causes contraction in platyhelminths (Becker *et al*., 1980; Mehlhorn *et al*., 1981) or benzimidazoles which interferes with β-tubulin synthesis resulting in blebbing of the tegument (Schmidt, 1998a).

The results from the work described in this chapter constitute the first evidence of CP affecting an equine helminth and in turn provide an interesting possibility for the development of CP based anthelmintic for cestodes of horses, which are known to be a significant risk factor in equine health (Proudman et al., 1998). This prospect is particularly compelling for a number of reasons. Firstly, the greater efficacy of PLS against *A. perfoliata*, compared to rodent cestodes, demonstrated here, must be

a positive feature. Secondly, CPs have been shown to accumulate in the hindgut region, where *A. perfoliata* resides, for longer periods of time (see chapter 3) , than in more proximal regions and this is another positive feature since it should ensure prolonged and more intense exposure to CPs for tapeworms dwelling in this region of the gut. Lastly, it has also been demonstrated that large animals such as small ruminants, including sheep (Buttle et al., 2011), required less drug per kilo body weight than small animals like mice (Stepek et al., 2007c), thus minimising or perhaps even eliminating the problem of toxicity. More importantly, the results provided further evidence that the spectrum of activity of CP is not restricted to nematodes, supporting the idea that they can be developed into useful broad-spectrum anthelmintics.

CHAPTER 9

General discussion and conclusion

9.0 Summary

This chapter provides an overall view of all the work conducted during my research programme, highlighting key observations from the various chapters of this thesis. I refer to some of the challenges I experienced and identify areas for improvement and further work. Thus it is important throughout to refer to the individual chapters for additional, specific and detailed information on the experiments that were conducted and to which I refer here.

9.1 General discussion

Gastrointestinal (GI) helminths pose a significant threat to the livestock industry (Nieuwhof and Bishop, 2005) and are a recognized cause of global morbidity in humans (Chan, 1997b). Control relies principally on chemotherapy (Behnke et al., 2008) but in the case of nematodes is rapidly losing efficacy through widespread development and spread of resistance to conventional anthelmintics (Kaplan, 2004) and hence the urgent need for novel classes of anthelmintics. Extracts from papaya latex have been shown to be effective against nematodes from various hosts *in vitro* (Stepek et al., 2005; Stepek et al., 2006b, 2007b) and *in vivo* (Buttle et al., 2011; Stepek et al., 2006b, 2007b, d) and their effects were confirmed to be mediated by CPs (Stepek et al., 2005). Preliminary evidence has suggested that plant derived CPs may affect also other classes of helminths including cestodes (He et al., 1992; Stepek et al., 2007e). In developing novel classes of anthelmintics it is important to establish the breadth of their spectrum of activity because the broader the spectrum the better their chances of acceptance by the end users (Behnke et al., 2008). This project was initiated to establish systematically whether CPs are able to affect cestodes, causing damage sufficient to prevent the worms from surviving in their hosts.

One of the main problems in developing natural products and introducing them into conventional therapy is standardization of their active principles (Behnke et al., 2008). This is because variation may exist between different varieties or even between different stages of development of the same fruit (Vines and Oberbacher, 1963). Therefore, rather than using crude papaya latex which is unrefined and collected from multiple sources we chose to use a more standardized form of papaya latex from Enzymase (Brussels, Belgium) which was sourced from the a single plantation and was subjected to strict and standardized purification processes. Another key aspect in manipulating enzymes is preserving the amount of active enzymes because enzymes are incapable of exerting their biological effects on target molecules unless activated. PLS was the result of several further refinement processes including centrifugation and dialysis in which loss of enzyme activity was kept to a minimum.

Since PLS was used throughout the thesis, it was therefore important to address questions/concerns that might be raised by the insightful and critical reader/referee. An obvious concern is enzyme stability under the working conditions in the laboratory, i.e.- how stable is PLS during storage or during incubation of *in vitro* assays ?. In Chapter 3 we investigated the thermostability of PLS under various conditions within the context of the thesis. Under storage we observed that PLS was stable at lower temperatures where it remained active for up to 1 year. This was not unexpected, since proteins in general are known to undergo denaturation at higher temperatures (Somero, 1995). However, it was also noted that freezing affected the active component of PLSto some degree but this amounted to no more than 20% loss of enzyme activity and no further deterioration was observed. This loss of enzyme activity may be explained by cold denaturation. Unlike heat denaturation which is clearly correlated with temperature (Somero, 1995) the mechanism of protein inactivation by cold denaturation is multifaceted with much damage attributed to crystallisation of water through physical and chemical changes (Arakawa et al., 2001). Given that freezing affects PLS to some degree, one would naturally expect that repeated freeze-thawing will inactivate or at least reduce the amount of active enzymes even further as was shown by Cao et al. (2003). However, surprisingly, PLS was fairly stable throughout repeated freeze-thaw cycles of up to 5x. This may be due to the fact that PLS is not a singular purified enzyme but rather a partially refined enzyme complex which actually contains many components other than proteases some of which may protect the proteases physically and also contribute to the stability of CP as excipients.

The next important question to address was how stable is PLS under the typical *in vitro* motility assay conditions employed here and reassuringly, although perhaps initially counter intuitively given the temperature, PLS was observed to be stable during the whole 2hrs of incubation at 37°C under exactly typical *in vitro* motility assay conditions. Notwithstanding, papaya latex has been shown by others to resist temperatures of up to 60° (Chaiwut, 2007) before denaturation takes effect. Furthermore, the inclusion of cysteine in all CP assays should act as an excipient adding further stabilisation effects.

Apart from investigating PLS thermostability it was also important to investigate how PLS behaves in the animal model itself, because to date the anthelmintic properties of papaya latex have been reported only for oral treatment with the crude latex (Stepek *et al*., 2007b). It was observed that PLS activity declined by 75% within 60min in the stomach before steadily rising in the colon and the lower part of the small intestine. Similar observations were also made in the rat model confirming at least that active PLS was still detectable in the large intestines of both rodent model systems used in the experiments reported in this thesis, despite the published concerns about inactivation of orally administered CPs by stomach acid (Huet *et al*., 2006).

Having demonstrated that the CPs used were active, it was important next to address the key hypothesis on which this work depended, namely that in addition to being potent anthelmintics for nematode infections, CPs are effective also against cestodes. Although there have been preliminary reports of the *in vitro* effects of CPs against cestodes (He *et al*., 1992; Stepek *et al*., 2007e) these were either very brief one-off experiments or the effects were never confirmed in sufficient detail and to a

degree comparable to the work undertaken with nematodes (Stepek *et al*., 2005). I started by conducting preliminary experiments, based largely on the work of Stepek et al. (2007e) and noted similar observations. Extending these findings necessitated confirmation that the reduction in motility was indeed mediated by CPs. In Chapter 4 I demonstrated in a factorial experiment that worms incubated in PLS pre-treated with E-64 remained motile until the end of the experiment while worms incubated in the same amount of active CPs but not pre-treated with E-64 succumbed to the enzymes. This was confirmed statistically when the main effect of CP was shown to differ significantly from the other treatments that were included in these assays. Having confirmed that the motility reduction effects were mediated by CPs I proceeded to test a range of CPs on a range of concentrations. CPs, whether naturally occurring like PLS and pineapple extract or commercially available purified CPs like papain and stem bromelain, caused a significant reduction in motility leading to the death of the worms in both rodent cestode models. However, there was a striking difference between our observations on cestodes and earlier published reports on nematodes in that the reduction in motility mediated by CPs against cestodes were independent of the presence of the exogenous CP activator cysteine, whilst inclusion of cysteine was crucial in the case of all the nematodes studied to-date (Stepek *et al*., 2005). Cysteine acts as a reducing agent and the active site on CPs needs to be activated through reduction before the enzyme can digest its target molecules (Chapman *et al.*, 1997). Therefore, a possible explanation for this difference between cestodes and nematodes, the ability of CPs to still mediate damage to the tegument of cestodes independently of the presence of cysteine is perhaps because of the properties of the cestode tegument which differs fundamentally from the nematode cuticle in its structure and component composition. Perhaps through its excretory process through the tegument results in some form of reducing agent which in turn activates the CPs. The reduction in motility was also enzyme concentration dependant as has been reported for nematodes (Stepek *et al*., 2005).

Although CPs have been shown to affect various intestinal dwelling adult nematode species (Stepek *et al*., 2005; Stepek *et al*., 2006b, 2007b, e) they were not effective against the juvenile stages of *H. bakeri* (Behnke *et al*., 2008) but did affect some juvenile plant nematode species (Stepek *et al*., 2007a). So the question is raised as to whether the juvenile stages of cestodes are also susceptible to CPs. Using an artificial excystation protocol (Goodchild and Davis, 1972) I made similar observations on the juvenile artificially excysted scoleces of both rodent cestodes, to those seen on adult worms, in a range of CPs and in a range of concentrations of active CPs.

Another pertinent question was whether CPs from different plant sources differ to any significant degree in their efficacy against cestodes. It was observed that naturally occurring CPs such those found in pineapple extract and PLS were more efficacious than singular purified CPs. Commercially available purified enzymes are singular CPs while naturally occurring CPs contain a combination of more than one CP. For example, papaya latex contains papain, chymopapain, glycyl endopeptidase and caricain while pineapple extract contains fruit bromelain, ananain and comosain. It is possible that naturally occurring CPs work in synergy resulting in greater efficacy as compared to singular CPs working individually. Similar phenomenon have also been observed *in vivo* when crude papaya latex was found to be more efficacious than papain in reducing *H. bakeri* burdens in mice

(Stepek et al., 2007d). Kundu and Lyndem (2012) in screening the efficacy of several species of Cassia plant extracts against the poultry cestode *R. tetragona* observed that worms exposed to a combination of several species of cassia extracts resulted in greater efficacy and damage to the worms compared to singular species exposure.

It was interesting to note that juvenile artificially excysted scoleces were relatively more resistant to singular purified CPs than they were to naturally occurring CPs. This might suggest that the scolex which has not yet developed a strobila, where most damage occurs, is perhaps more resistant to CPs. This could be because of a lack of suitable target proteins in the tegument associated with the scolex, compared with that covering the strobila. This relative scolex resistance is also reminiscent of the scolex resistance towards immune system attack where destrobilated scolex is able to regrow. CP efficacy also did not differ substantiallybetween the 2 species of rodent cestodes albeit CP was slightly more efficacious against *H. diminuta*. This contradicted the literature since *H. microstoma* has been shown to be resistant to anthelmintics (McCracken *et al*., 1992). However, there was a difference in the efficacy of CPs between the rodent cestodes and the equine cestode *A. perfoliata* which was explored in Chapter 8. PLS was four times more efficacious against *A. perfoliata* compared with its effect against rodent cestodes. This was perhaps due to the apparent size discrepancy between the 2 species in which *A. perfoliata* is a mere 3-5 cm in length, while 14day old *H. diminutas* used throughout the thesis reach 15 cm.

While the motility assays provided strong evidence that CPs do indeed affect cestodes, the next important question to address was how? One way to answer this question was by careful observation of changes to the surface of the worms during incubation in CPs and quantification of any resulting damage. It was observed that in both rodent cestodes the appearance of damage was similar with generalised tegumental erosions mainly affecting the strobila. In lower concentrations the erosion was not marked, but rather superficial and at certain points there was tegumental discontinuity with the release of internal contents mainly eggs from gravid proglottid. In higher concentration of active CPs the damage was marked and extensive with deep erosions and an exfoliative appearance. However, despite being incubated in the same high concentration of active CP, the scoleces and the neck regions did not appear to sustain similarly extensive damage but rather lighter more superficial tegumental erosions. This might be suggestive again of a relative resistance of the scolex region as compared to the strobila, as proposed above, perhaps due to different CP protein target composition. This observation is quite different from the observations seen with praziquantel which has a predilection for the neck region while the strobila remains unaffected (Becker et al., 1981).

This mechanism of erosive damage is almost comparable to the events seen with nematodes (Behnke et al., 2008) differing only because nematodes are enclosed within a tough cuticle with high hydrostatic pressure. Thus, a slight breach in the cuticle literally results in an explosion of the internal contents of nematodes. Cestodes, however, are only limited by the thin plasma membrane like tegument, hence damage is rather progressive than sudden. This is a stark contrast to conventional therapies like praziquantel (Becker et al., 1981), niclosamide (Kumchoo et al., 2007) and even benzimidazoles (Schmidt, 1998b) whose effects appear to be mediated by a different mechanism

affecting the tegument. Rather than attacking the tegument from outside they appear to kill cestodes from the inside out, initially by diffusing within the tegument and then weakening the tegument from within (Mansour, 2002). The events observed in cross-sections of the tegument, however, are quite similar to those reported for other cestocidals. Most reports include an increase in the formation of vacuoles and vesicles and disruption in the glycocalyx outer layer (Becker et al., 1981; Mansour, 2002; Schmidt, 1998b). Schmidt (1998b) proposed that the vesicles may be transporting materials needed to synthesize and replenish the disrupted or lost glycocalyx layer, which tapeworms are known to be able to do most efficiently (Befus and Threadgold, 1975). So in part some of the resistance to CP attack observed in this work, may be explicable by rapid turnover and repair of damaged sections of the tegument. It may be that only in high concentrations of CPs is the balance between sustained damage and repair swung in favour of treatment. Hence the worms experience irreparable damage incapacitating them to such a degree that they cannot sustain their location in the hosts intestine and are driven out by peristalsis..

Since CPs were shown to affect cestodes *in vitro* and the effects were confirmed to be mediated by CPs, it was important next to answer the ultimate question as to whether CPs actually affect cestodes *in vivo*. In the past many natural compounds and products have been purported to possess anthelmintic efficacy as indicated by their effects on parasites *in vitro* but most have not lived up to their reputation when tested *in vivo* (Behnke et al., 2008). For example, He *et al.* (1992) found that young pineapple juice affected *H. nana in vitro* but not *in vivo*. Similarly fig latex has been shown to be ineffective at reducing *H. nana* worm burdens (de Amorin et al., 1999). My investigations, however ,surprisingly revealed that PLS was indeed efficacious against cestodes *in vivo*. Efficacy, however, varied between the cestode species tested and also depended on the index of efficacy gauged. In most of my experiments I used several indices to gauge CP treatment efficacy *in vivo* and this included a non invasive index such as faecal egg counts, as well as autopsy dependent indices including worm counts, worm biomass both wet and dry. I started with assessing whether or not CPs have any effect on worm fecundity by quantifying faecal egg counts. Disappointingly, in both rodent models faecal egg counts were not affected at all. The faecal egg count results showed no reduction in the treated group as compared to the control in either rodent cestodes. I initially started with *H. microstoma* (Chapter 5) before repeating a similar regimen on *H. diminuta* (Chapter 7). The initial impression following the disappointing results from *H. microstoma* was that the egg production centre in the neck region might have been spared from the exposure to CPs due to its location in the bile duct or perhaps also the dose administered may have been too small (240nmols in mice). However, this notion was proved wrong because *H. diminuta* despite being fully exposed to CP onslaught, because it is an entirely lumen dwelling cestode, and being administered with a dose 10x higher (2.4µmols) still showed no reduction in faecal egg counts (Chapter 7). Clearly, tapeworms cannot be compared directly to nematodes which once affected by CPs, usually die, exploding soon afterwards with resultant cessation of egg output that is then clearly reflected within 24h in a reduction of faecal egg counts.

Nevertheless, there was still a possibility that despite no overall reduction in egg counts the eggs exposed to CPs in the host's gut might not be viable anymore. It was then imperative to ascertain

whether fecundity was still affected despite no reduction in egg counts by an egg viability test. Disappointingly, again in both models eggs were not affected at all because intact cysticercoids were recovered from *Tribolium* beetles fed with either cut terminal proglottid segments or detached proglottid segments recovered from the colon of rodent hosts treated with PLS. Unlike the benzimidazoles which were able to affect the development of *H. diminuta* larval stages (Evans and Novak, 1976) PLS does not appear to have any effect on either rodent cestode's fecundity when administered to infected rats. Perhaps the eggs are protected within their thick outer shell which fail to express target proteins. Structurally, cestode eggs have a thicker outer shell compared to trichostrongyloid nematode eggs such as those of *H. bakeri*, but not those of *Trichuris* and *Ascaris* species, both of which have tough egg shells to enable the larvae within to develop to the infective stage within the eggs in the external environment, as in cestodes. This robust egg shell is required because of the need for the eggs to survive in the harsh external environmental conditions before continuing its life cycle in an intermediate host. On the basis of my results I can conclude that CPs do not affect egg viability or adult cestode fecundity in vivo.

Assessment of fecundity, however, is not a definitive marker of efficacy of treatment because the final indicator will always be the autopsy indices. Unlike in nematodes where autopsy indices can only be assessed through worm counts, in the case of cestodes treatment efficacy can also be assessed through worm biomass because a small worm compared to the controls is an indicator that it is under stress and might be expelled eventually either by the treatment or with the assistance of host immunity. Surprisingly, despite the unaffected fecundity both rodent models appeared to be affected by PLS albeit differing in the level of efficacy. PLS was only minimally efficacious against *H. microstoma* (Chapter 5). Depending on the exact circumstances of each experiment, there were varying levels of reduction in worm burdens and biomass and these were generally not significant, depending to some extent on the sample size utilised, with larger samples just touching a significant level of reduction. The reductions were also not dose dependant and could not be said to be mediated by CPs because the main effects of CPs were not significant in a factorial experiment. Although it is easy to explain this relative resistance of *H. microstoma,* compared to *H. diminuta* ,which was more susceptible by referring to the literature in which *H. microstoma* is generally accepted to be resistant also to conventional anthelmintics (Evans *et al*., 1979; McCracken *et al*., 1992), there are other factors that need to be considered as well. Firstly the efficacy of CPs against *H. microstoma in vitro* was quite comparable to that of *H. diminuta* (Chapter 4). Secondly, the dose that was being administered to mice was 10x lower than the dose administered to rats. Thirdly the mechanistic basis by which CPs inflict damage upon cestodes appeared to be different compared to other conventional anthelmintics (Chapter 3). Therefore, I would like to suggest that if the mousee model system were able to be administered with at least a similar dose to that administered to *H. diminuta* infected rats, it might be possible to observe higher efficacy, perhaps similar between the 2 species.

Moving on to the next model system PLS was generally moderately efficacious against *H. diminuta in vivo* resulting in a significant, but relatively small, reduction in worm burdens and biomass. These effects were only partially dose dependant and specific inhibition by E-64 only confirmed the

role of cysteine proteinase as the active principles in papaya latex, affecting worm growth and hence size but not potent enough to cause worm expulsion.

Having observed these effects it was also important to determine whether these effects were permanent or temporary. In both models I attempted an experiment in which one group was killed immediately after completing the usual treatment regime whilst another group was killed and autopsied one week later. In *H. microstoma* there was no difference in worm count when worms were left to survive post treatment but in *H. diminuta* the worm elimination effects was magnified. The effects on worm growth, however, were not enhanced in either model. Perhaps the discrepancy between the 2 species was again due to the different doses applied but also it can be postulated that the surviving treated worms may not be able to withstand immunological attack to a degree comparable to that they show normally when they are fit and not threatened by anthelminics. It is possible that the longer term effects of treatment were mediated by a second phase of attack by the host system on already weakened worms, resulting in the worms succumbing eventually, given sufficient time.

One of the main limitations that I believe limited the efficacy of treatment with CPs on naturally growing adult worms in their normal hosts, was the concentration of anthelmintic, the exact dose that could be administered to the rodent hosts, without evident side effects and due consideration for animal welfare. While, because of the side effects observed at the higher dose levels, there was no possibility for increasing the dose further, there is another model that could be and was exploited to demonstrate the host-protective effects of CPs *in vivo*. *H. diminuta* is known to survive in the mouse host temporarily before being immunologically expelled (Hopkins *et al*., 1972a). The mouse is a non-permissive host because *H. diminuta* cannot grow to maturity in normal fully immunocompetent animals. If this window period, before expulsion, could be exploited, I would expect to see an enhanced CP effect because the parasite is in a non-permissive host in which there may be some physiological incompatibility and in which the worms are also subject to an effective immunological attack that may show synergism with the damaging consequences of exposure to CPs. Indeed, as predicted, PLS treatment of mice infected with *H. diminuta* resulted in marked significant reductions in all autopsy indices (Chapter 6). To extend these observations invoking an essential complementary role for the immune system, the primary hypothesis was modified invoking a compound effect partly mediated by the immune system. To test this idea, it was predicted that the effects would not be as marked when the immune system is suppressed. Indeed in the next experiment (Chapter 6) I demonstrated that when the immune system was suppressed by corticosteroids the effect of PLS at reducing worm burdens and biomass were less marked. The results from this chapter (Chapter 6) provide an interesting comparison between the effects seen in the permissive host model where efficacy against *H. diminuta* was moderate. It may also suggest that in terms of targeting tapeworm infections, CPs may be immunology dependant drugs, just as is thought to be the case in praziquantel and schistosomes (Hrckova and Velebny, 1997; Hrckova et al., 1998). This idea however needs to be further explored , exploiting mice with genetically knocked out immune systems, rather than those treated with a wide spectrum immusuppressive and anti-inflammary agent such as the corticosteroids employed here.

Having demonstrated systematically that CPs do indeed have potent effects on rodent cestodes it was worth extending the main research question concerning the degree to which CPs show a broad spectrum of activity by including another host-parasite system of the same class but of a different host and one with considerable economic importance. Horse tapeworm infections have recently been recognized as a significant cause of morbidity in horses (Proudman *et al*., 1998) and horses are animals of economic importance. Resistance to conventional anthelmintic is also a recognized problem in the horse industry (Matthews *et al*., 2004). If I could show that CPs also affect equine cestodes then there will be a real prospect for the development of CPs into novel classes of anthelmintic for horses. However, this approach had some limitations and was restricted to *in vitro* experiments as a license to conduct animal experiments involving horses was not available to me. To my surprise, not only was the horse cestode *A. perfoliata* susceptible to CPs, but the CPs were also observed to be many times more effiacacious *in vitro* compared to their effects against rodent cestodes (Chapter 8). Therefore, not only did this chapter (Chapter 8) extended the spectrum of activity to another cestode species, it also opened up a new chapter of opportunity for the development of CPs into a novel anthemintic for horses. Firstly, due to CPs being more efficacious at lower concentration in vitro and secondly because *A. perfoliata* resides in the ileo-caecal junction, it is probably exposed to prolonged exposure to CPs because experiments in rodents have shown clearly that CPs accumulate and reside for much longer in the hindgut segment compared with more anterior regions of the intestine(Chapter 3).

Lastly, with regards to CP side effects, although it has been observed in mice receiving PLS doses higher than the doses described in this thesis similar observations were not observed in rats receiving similar dose per kilo body weight. In fact oral dosing of papaya latex in sheep was sound and safe (Buttle *et al*., 2011). With regards to these side effects, although needs to be noted, it should never hinder the progress of developing CPs into useful novel anthelmintics.

Overall, although CPs appear to affect cestodes the efficacy is relatively less when compared to its efficacy against nematodes (Stepek et al., 2007c). It is even smaller when compared to the very high efficacy of conventional cestocidals like praziquantel (Thomas and Gonnert, 1977). Therefore it is not possible to immediately develop CPs into useful primary cestocidals at the moment. However with further refinement in formulation and delivery may perhaps improve CP efficacy as primary cestocidals. At the moment it does however fulfil the role of a broadspectrum anthelmintic primarily for nematodes with concurrent effects against cestodes.

9.2 Conclusion

Contrary to preliminary negative reports on CP efficacy against cestodes, in my thesis I have clearly demonstrated that CPs do indeed affect the rodent cestodes *H. microstoma* and *H. diminuta in vitro* and *in vivo*. Naturally occurring CPs were more efficacious than commercially available singular purified CPs. Efficacy was also enhanced by the synergistic effects of the immune system demonstrated in the non-permissive host. Moreover, efficacy was extended to include the equine cestode *A. perfoliata in vitro*. The results offer an interesting possibility that with further refinement, CPs may be developed

into broad spectrum anthelmintics that remove nematodes from their hosts and have significant effects also on any concurrently resident tapeworms. Novel anthelmintics are desperately needed at the current time to counter the onset of resistance to the synthetic anthelmintics that are used widely, and I hope sincerely that the experiments that I have reported here provide a baseline for the further exploration and development of CPs for the treatment of tapeworm infections of man and our domestic animals.

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