Chapter 5
GENERAL DISCUSSION

A primary procedure often employed for evaluating anthelmintic-efficacy is direct observation on the viability of the helminthic worms upon exposure to the anthelmintic agent. Different classes of anthelmintic drugs are established to show profound effects on the physical activities, generally culminating into loss of mobility and mortality of helminth parasites in a dose-dependent manner (Togo et al., 1992; Urrea-Paris et al., 2000; Xiao et al., 2004; Hassan et al., 2003; Tippawangkosol et al., 2004). Following this basic technique, several plants have been effectively demonstrated to possess significant anthelmintic property against a variety of helminth parasites. The feasibility of anthelmintic usage of medicinal plants such as Allium sativum, Albizia lebbeck, Artemisia brevifolia, A. santonica, Carica papaya, Cardiospermum halicacabum, Melia azederach, Neurolaena lobata, Ocimum sanctum, Perilla frutescens, Polyalthia suaveolens and Zingiber officinale are already validated as potent antinematodals (Goto et al., 1990; Kasuya et al., 1990; Singh and Nagaich, 1999, 2000, 2002; Iqbal et al., 2001, 2004; El Garhy and Mahmoud, 2002; Szewczuk et al., 2003; Boonmars et al., 2005; Fujimaki et al., 2005; Nyasse et al., 2006). Flemingia vestita, Houttuynia cordata, Lasia spinosa, Lysimachia clethroides, Psidium guajava and Zanthoxylum limonella are authenticated as cestocidals (Soh et al., 1963; Kalyani et al., 1989; Tandon et al., 1997; Temjenmongla and Yadav, 2005); whereas Alpinia nigra, Buchholzia coriaceae, Cannabis sativa, F. vestita, Gynandropsis gynandra and Spilanthes oleracea are ascertained as trematocidals (Roy and Tandon, 1996, 1997, 1999; Tandon et al., 1997; Ajaiyeoba et al., 2001; Roy, 2001).
Results accrued from the present investigation indicate that *Acacia oxyphylla* and *Millettia pachycarpa*, two medicinal plants used by Mizo tribes, indeed have anthelmintic efficacy on the poultry gastrointestinal helminth parasites, the cestode *Raillietina echinobothrida* and the nematode *Ascaridia galli*. The cestode appeared to be the more susceptible helminth of the two when treated with ethanol, methanol and acetone extracts obtained from both *A. oxyphylla* stem bark and *M. pachycarpa* root bark, and a spontaneous loss of movement or paralysis ensued soon after incubation in the test media. It is also apparent that the plant extracts caused definitive fatality preceded by a lapse of paralytic state, which is consistent with other *in vitro* studies on cestocidal activities (Tandon *et al*., 1997; Temjenmongla and Yadav, 2005). There is a compelling indication of direct correlation between the concentrations of the plant extracts and the time taken to cause paralysis and death of the worms. However, none of the extracts of either of the plants caused significant mortality at the lowest concentration (0.5 mg/ml) tested. In general, plant extracts are known to contain a number of impurities beside the active components so that at a very low concentration, in the process anthelmintic effects may greatly diminish. The fact was comprehensively demonstrated by Diehle *et al*. (2004) for 19 different plant extracts in which the minimal effective dose was found to be $0.87 \pm 0.37$ (Mean $\pm$ SD) mg/ml. Though direct mortality did not occur at very low concentration, still a significant period of paralysis was noted, implying the possibility of efficacy at the lower doses through induction of reversible paralytic state during which the worms can be expelled from the host by peristalsis of the gut. Thus, the plant preparations are likely to function only as vermifugal at lower doses.

For both the plant materials, higher efficacies were noted for the ethanol and methanol extracts, while the acetone extracts showed least influence on motility and
survival of the worms. It has been heavily documented that ethanol and methanol extracts of anthelmintic plants generally give maximal effects (Akhtar and Riffat, 1991; Javed et al., 1994; Tangpu et al., 2004; Enwerem et al., 2001; Prashanth et al., 2001; Assis et al., 2003; Ademola et al., 2005a; Fujimaki et al., 2005; Iqbal et al., 2006a,b,e), while acetone extracts impart less or no activity from the same plants (McGaw et al., 2001). Comparative scrutiny of the efficacy of the two plant extracts also posited that *M. pachycarpa* is a more potent cestocide than *A. oxphylla* on the experimental cestode. The methanol and acetone extracts of *A. oxphylla*, in particular, did not show any cestocidal activity at the concentration lower than 5 mg/ml, though vermifugal potential could be inferred in terms of paralytic effect.

*A. galli* appeared to be relatively insensitive to the extracts of the two plants. Only higher doses of ethanol (2-20 mg/ml) and methanol (5-20 mg/ml) extracts of *A. oxphylla* showed significant efficacy characterized by a more or less simultaneous loss of movement and death. Similar observations were also reported by Tandon et al. (1997) using the root tuber extract of *F. vestita* on different nematodes such as *Ascaris suum*, *A. lumbricoides*, *A. galli* and *Heterakis gallinarum*. Therefore, it may be prudently speculated that *A. oxphylla* can influence considerable inhibition on the physical activity of the nematodes so that they can be effectively expelled from the intestine. In fact, this inhibitory mechanism is asserted to be the primary mode of action of standard drugs (Martin et al., 1997, 2002). On the other hand, treatment of the nematode with acetone extract of *A. oxphylla* and the extracts of *M. pachycarpa* failed to present any statistically significant efficacy as an antinematodal on the physical persistence of *A. galli*.
Within the last two decades, scanning electron microscopy has emerged into a powerful technique in helminthology, both in the taxonomy of helminths and the pharmacology of anthelmintics, providing morphological details at the ultrastructural level. Descriptions of the surface topographical changes on helminths are vital to understanding the nature and magnitude of anthelmintic effects, as tegument and cuticle form the direct interface with the host rendering them the primary target. Different anthelmintic drugs have been unequivocally shown to cause discernible topographical mutilation of varying degree on the teguments of cestodes (Becker et al., 1980a; Pool, 1985; Jain et al., 1989; Garcia-Dominguez et al., 1991; Stoitsova et al., 1992; Tandon et al., 1997; Pérez-Serrano et al., 1994, 2001; Elissondo et al., 2006; Naguleswaran et al., 2006) and trematodes (Becker et al., 1980b; Schmahl and Mehlhorn, 1985; Schmahl and Taraschewski, 1987; Anderson et al., 1988; Staudt et al., 1992; Xiao et al., 2001a,b,c, 2004; Meany et al., 2002, 2004; Keiser et al., 2006), and on the cuticle of nematodes (Strote et al., 1990; Boulos et al., 1992; El Gebaly et al., 1996; Tippawangkosol et al., 2004; Stepek et al., 2005, 2006, 2007).

Scanning electron micrographs from the present study revealed that the three extracts of both A. oxyphylla and M. pachycarpa are responsible for morphological alterations on the tegument of R. echinobothrida. While control cestode exhibited smooth surface due to finely organized filaments of the microtriches all over the tegument, the microtriches turned into dishevelled lumps in the plant extract-treated worms giving the tegument a coarse appearance. The intact scolex is provided with terminal rostellum that is lined with hooks, and four circular suckers bearing rows of straight pointed spines. The different extracts of the two plants caused considerable damage and deformity on the tegumental construction and on the suckers. The observations largely conform with that of Tandon et al. (1997), where the root tuber
extract of *F. vestita* caused deformity on the sucker, and clumping of the microtriches and tegumental vacuolisation on *R. echinobothrida*. It also turned out that the extent to which *M. pachycarpa* extracts damaged the tegumental integrity is comparatively greater than that caused by *A. oxyphylla* extracts and that the nature of their degenerative effects are quite different. Although surface damage due to *A. oxyphylla* extracts was prominent throughout the body of the cestodes, it was by and large typically confined to shrinkage and folding of the tegument. By contrast, the characteristic effect of *M. pachycarpa* extracts was exceptionally devastating with inexorable truncation and erosion of the tegument, in addition to complete destruction of the suckers and microtriches surrounding the scolex.

Different pharmaceutical drugs are also shown to cause conspicuous topographical distortions on a number of cestodes. Praziquantel (PZQ) caused deformity of microtriches, tegumental corrugations and vacuolization on *Hymenolepis nana* (Becker et al., 1980a). *H. diminuta* recovered form amoscanate-treated rats revealed bleb formation and erosion of microtriches from the tegument, as well as disappearance of microvilli from nephridial canals (Nelson and Saz, 1983). PZQ also caused formation of bubbles and vacuolization on the tegument of adult *Bothriocephalus acheilognathi*, with bursting of the mature proglottids affecting mass expulsion of eggs at higher dose of the drug (Pool, 1985). Extensive reduction in the number of microtriches and complete degeneration of the tegument were seen upon exposure to mebendazole and its metabolite in *Cysticercus fasciolaria* (Jain et al., 1989), and in *Taenia solium* cysticerci upon treatment with oxfendazole (Liu et al., 2003) and PZQ (Garcia-Dominguez et al., 1991). The *in vivo* effects of the anthelmintics taenifugin, VUFB 14170 and VUFB 15269 on *Hymenolepis fraterna* was pronounced on the apical surface of the tegument, where there was blebbing as
well as accumulation of membrane fragments over the microtrich tips (Stoitsova et al., 1992). Formation of numerous blebs on the tegument which became detached, leaving debris only, rostellar disorganization, loss of the microtriches, increased lipid deposition and depleted glycogen reserves were observed for pure ABZ and ABZ sulphoxide combination therapy on the human cestode *Echinococcus granulosus* (Pérez-Serrano et al., 1994). The detrimental effect on the tegumental integrity in *H. microsoma* is accompanied by replenished secretion of glycoconjugates that are known to protect the tegument (Schmidt, 1998). Erosion of the surface microvilli layer, body flattening and elongation, development of irregular protuberances and depressions were described for *Mesocestoides vogae* after incubation with PZQ, and the extent of alterations corresponded to incubation time and concentrations (Hrckova et al., 1998). PZQ and ABZ combination treatment of *E. granulosus* also resulted in the loss of sucker concavity, separation and disintegration of the germinal layers, loss of microtriches and destruction of the tegument (Urrea-Paris et al., 2000). Damaging effects described for ABZ and nitazoxanide are quite similar and typified by reductions in number and length of the microtriches, and vesiculation on the germinal layer of the metacestodes of *E. granulosus* and *E. multilocularis* (Stettler et al., 2003; Walker et al., 2004). The effects of flubendazole on *E. granulosus* include contraction and shrinkage of the soma region, formation of blebs on the tegument, rostellar disorganization, loss of hooks and destruction of microtriches (Elissondo et al., 2006).

The present study reveals that *A. galli* seemed to be relatively insensitive to the plant extracts with respect to morphological alterations conforming to the mortality tests. In fact, the three extracts of *M. pachycarpa* and acetone extract of *A. oxyphylla* failed to produce discernible destructive impact on the nematode at all concentrations tested. Only the ethanol and methanol extracts of *A. oxyphylla* stem bark affected
distinctive changes on the cuticular topography. Collapse of the lips, shrinkage of the cuticle at the anterior cephalic region and formation of blisters are the distinctive effects. Nematodes being hard-bodied worms are not easily killed or greatly affected by anthelmintics of any kind. In fact it has been conclusively established that the antinematodal drugs act not by direct lethal effect but through paralytic immobilization of the worms by which they are then swept out of the intestinal tract of the host by peristaltic movements (Martin et al., 1997, 2002). A number of in vitro experiments have also supported the fact that after complete paralysis, the nematodes could be gradually revived in control buffer solutions (Iqbal et al., 2004). Even trematodes devoid of complex cuticle can be affected in similar pattern. Extensive structural damage in human schistosomes could be restored to normal after in vivo treatment in mice with artemisin derived from the leaf of Artemisia annua (Xiao et al., 2000b).

Recent investigations have lucidly evoked the vermicidal application of exogenous cysteine proteinases on veterinary enteroparasites. Specific cysteine proteinases are isolated from different fruits such as Actinidia chinensis, Ananas comosus, Asclepias sinaica, Carica papaya, Ficus carica and F. benjamina, and are tested to have practical application as antinematodal agents. SEM observations coherently resolved that the primary mechanism of action anthelmintic cysteine proteinases involves targeting the cuticular architecture of different nematodes characterized by obvious progressive damage with initial appearance of transverse wrinkles and folds, which appeared to give rise to sloughing, and then followed by blistering of the outer cuticle (Stépek et al., 2005, 2006, 2007; Hoste et al., 2006). Albendazole (ABZ) reportedly caused structural deterioration on the cuticular surface of Trichinella spiralis upon in vivo treatment (Hrčkova et al., 1993). Surface distortion and loss of regular cuticular
annulations were conspicuous on the infective stage of *Brugia malayi* (Tippawangkosol *et al.*, 2004). *Wuchereria bancrofti* adult worms recovered from patients with lymphatic filariasis subjected to ABZ and diethylcarbamazine combination therapy exhibited morphological alterations including a swollen band whose mid-region presented a row of puffed-up and deflated structures, formation of long digitiform, spherical and spike-like projections at the anterior region, and leaf-like expansion on the general cuticle (Oliveira-Menezes *et al.*, 2007). *Angiostrongylus contonensis* developed severe shrinkage and formation of rounded leaf-like expansions on the cuticle throughout the body after treatment *in vivo* with imidacloprid and moxidectin combination (Schmahl *et al.*, 2007). Thus, the developments of wrinkles and blisters on *A. galli* cuticle after treatment with ethanol and methanol extracts of *A. oxyphylla* in the present study lend support to the fact that the plant extracts possess antinematodal property.

The modes of action of anthelmintics are diverse, reflecting the natural differences in the physiology of the parasite and their potential hosts. Anthelmintic drugs are established to reach target parasites by either oral ingestion or by diffusion through the external surface (‘cuticle’ in nematodes or ‘tegument’ in cestodes and trematodes), or some combination of both routes (Thompson and Geary, 1995). The cuticle or tegument is metabolically active and morphologically specialised to perform selective absorption of nutrients, secretion of glycoproteins for immunoprotection, osmoregulation and (insofar as it supports sense organs) sensory reception. Consequently, trans-cuticular and trans-tegumental passive diffusion is the principal mechanism of anthelmintic entry into the helminths (Freeman *et al.*, 2003; Mottier *et al.*, 2003, 2006; Alvarez *et al.*, 2007). Apparently, it has been firmly documented that one of the hallmark effects of any anthelmintic is destruction of the worm’s surface.
(Xiao et al., 2000; William et al., 2001; McKinstry et al., 2003; Rivera et al., 2004); obviously due to the fact that these tegumental and cuticular structures are the primary interfaces vital for absorption of nutrients and perception of the surrounding environment.

Tegumental destruction, especially around the oral and ventral suckers, associated with pit formation and severe vacuolisation in various trematodes have been heavily documented on treatment with a number of drugs (Mehlhorn et al., 1983; Schmahl and Mehlhorn, 1985; Schmahl and Taraschewski, 1987; Fairweather et al., 1987; Anderson et al., 1988; Schmahl, 1993; Stitt and Fairweather, 1993; Anderson et al., 1998; Veerakumari and Munuswamy, 1999; Xiao et al., 2000; William et al., 2001; Liang et al., 2002; Rivera et al., 2004). A stepwise formation of vesicles, followed by vacuolisation and disruption of the dorsal tubercles, and eventual erosion of the surface layer were clearly demonstrated (Becker et al., 1980a,b; Bricker et al., 1983; Shaw and Erasmus, 1983; Shaw, 1990). Abnormal secretion of vesicles was also conspicuous in the suckers of *R. echinobothrida* exposed to the ethanol extracts of both *A. oxyphylla* and *M. pachycarpa* in the present study. Vacuolisation and pit formation were also observed in different trematodes upon treatment with the extracts of *F. vestita* (Roy and Tandon, 1996). Mirazid, derived from *Commiphora molmol*, was also shown to cause disruption of the tegument and collapse of tubercles in *S. mansoni* after 10 minutes exposure and extensive swelling and complete loss of spines covering the tubercles after 30 minutes (Hassan et al., 2003).

The absence of a digestive system and hermaphroditism in cestodes offers a simplification in the interpretation of the physiological functions of the tegument and the underlying internal organs. Absorption of chemical materials into the body of the
worm is functionally analogous to those normally associated with intestinal tissue. Consequently, all interactions between the worm and its environment necessarily take place across the tegument (Thompson and Geary, 1995). The benzimidazole group of anthelmintics, with ABZ as the most widely used, also exploit this tegumental property. Benzimidazoles (BZs) are by far the best understood anthelmintic agents of all in terms of mechanism of action, prevalence of resistance and the molecular basis behind such resistance (Lacey, 1990; Sangster, 1999; Geerts and Gryseels, 2000, 2001; Köhler, 2001; McKeller and Jackson, 2004). BZs are construed to enter the cestode body by passive diffusion through the tegument (Alvarez et al., 1999, 2007; Mottier et al., 2006). Moreover, soon after the drugs exert localized tegumental disruption, increased permeability of the tegumental membrane to the drugs occur, resulting in increased intensity of destruction (Mottier et al., 2003). These BZs are resolutely determined to bind selectively and with high affinity to the β-subunit of helminth microtubule proteins, tubulins, causing disruption of the microtubule dynamic equilibrium, and with that, cell lysis (Schmidt, 1998; Köhler, 2001). By binding specifically to free β-tubulin, BZs inhibit the polymerization of α- and β-tubulin molecules and the microtubule-dependent uptake of glucose, ensuing starvation then the worms are paralysed and killed (Lacey, 1990).

Piperazine (PPZ, diethylenediamine) is an anthelmintic commonly used against animal and a drug of choice in bird ascariasis. It has been rationally established that transcuticcular diffusion is the predominant pathway by which anthelmintic drugs enter the nematode body (Alvarez et al., 2001, 2007). PPZ once inside the body acts to block neuromuscular transmission in the nematode by hyperpolarizing the neuronal membrane an agonist at GABA-gated chloride channels, which leads to flaccid paralysis (Martin et al., 1997). The parasites, paralysed and depleted of energy are
expelled by peristalsis. In *Litomosoides carinii*, the mitochondria of muscle cells considerably swollen before disintegration, followed by disintegration of the myofilaments and vacuolization of the cytoplasm after treatment with PPZ (Franz et al., 1990a). PPZ also inhibited the activity of a moulting-specific enzyme, inorganic pyrophosphatase of *A. suum*, thus, interfering with growth and ecdysis of the cuticle and causing damage to gut tissues of the larvae (Islam et al., 2006). Most of the degenerative effects confined to the anterior cephalic region on *A. galli* as observed also follow the known facts that the anterior portion of nematodes are specifically sensitive to and targeted by anthelmintics (Strote et al., 1997).

Histological studies have revealed that anthelmintic agents induce considerable changes in the internal structural features of helminthes, resulting in loss of normal cellular conformity in vital tissues. In the present observations, different extracts of both *A. oxyphylla* and *M. pachycarpa* affected the disintegration of tegumental layer, muscle fibres, parenchyma network, and pygnosis of egg cells in *R. echinobothrida*. The magnitude of tissue damage clearly corresponds to the concentration of the extracts, which implies that the cestocidal activities of these plant extracts are concentration-dependent. Moreover, the lowest concentration (0.5 mg/ml) tested for all the extracts of both plants failed to bring about any apparent structural alteration in the cestode. It turned out from histological observations that the common destructive effects of the two plant materials are degeneration of the muscle tissue and the parenchyma cells.

Comparable to the present findings, accompanying tegumental destruction, accumulation of granules in the tegumental cells and disorganization of the tegumental layer have been described for the effects of different BZs in *C. fasciolaria* (Jain et al.,
1989) and *Taenia solium* cysticerci (Liu *et al.*, 2003). ABZ caused increased vesiculation within the tegumentary cytons together, an increase in lipid deposits and depletion of glycogen reserves, with some intact nuclei being discernible in the protoscolex parenchyma of *E. granulosus* (Pérez-Serrano *et al.*, 1994). In addition, separation of the laminated and germinal layers, degeneration of the parenchyma cells, vacuolization and formation of abnormal lipid droplets and autophagosomes were observed in case of the combined treatment of ABZ and PZQ (Urrea-Paris *et al.*, 2000). Similar degenerative effects were seen in flubendazole-treated cestodes (Elissondo *et al.*, 2005). Ivermectin caused contraction of the distal cytoplasm in *E. granulosus* accompanied by depolarization of the tegumental membrane, followed by rostellar disorganization, rigid paralysis and eventually loss of viability (Pérez-Serrano *et al.*, 2001). PZQ and ABZ combination treatment of *E. granulosus* also resulted vesiculation of the cytoplasm and formation of autophagosomes (Urrea-Paris *et al.*, 2000). ABZ and nitazoxanide caused comparable degenerations such as increased cytoplasmic vacuoles, separation of the germinal layer from the tegument and production of lipid droplets in the metacestodes of *E. granulosus* and *E. multilocularis* (Stettler *et al.*, 2003; Walker *et al.*, 2004). Progressive degeneration, characterized by overall tegument disintegration, muscle fibre fragmentation and necrosis, diffused parenchyma organization accompanied by recurrent destrobilization were observed in *Mesocestoides corti* after ABZ treatment (Markoski *et al.*, 2006).

The susceptibility of the nematode to the tested plant extracts in terms of tissue damage is comparatively very low. Only higher concentrations (5, 10, and 20 mg/ml) of ethanol and methanol extracts of *A. oxyphylla* caused obvious structural alterations in *A. galli*, in which the muscle layer, uterine wall and intestinal epithelial linings appeared degraded to a great extent. The gut wall of helminth parasites, being the
absorptive surface for nutrients, is particularly receptive to the destructive effects of anthelmintic drugs (Gorchilova et al., 1988; Kaur and Sood, 1993). The necrotic effects were most profound on the conformation of the ovaries. The fine layers of the egg cells in the ovaries were hampered with their membranes defaced; the ovarian wall also dissolved dispersing the eggs into the pseudocoel cavity. Thus, female nematodes in general are more easily or conspicuously affected, certainly due to their elaborate reproductive structures, reciprocal to their morphological features, which is consistent with several studies on the influence of drugs on different nematodes (Franz et al., 1987, 1990a,b).

On the other hand, histological alterations are better defined in nematodes, which can be attributed to a more distinctive organization and complex structures of their anatomy. Kaur and Sood (1983) reported disorganisation of the muscle layer and vacuolisation of the intestinal epithelium in *Haemonchus contortus* exposed to tetramisole. *Onchocerca volvulus* microfilariae exposed to amocarzine and milbemycin resulted in increased numbers of vacuoles in the cytoplasm and clefts between cuticle and hypodermis, disintegration of the cytoplasm, myofilaments and mitochondria of the muscle cells, associated with progressive separation of the cuticle from the hypodermis (Strote, 1989; Strote et al., 1990). Tribendimidine also caused severe disruption of the cuticle and intestinal epithelium in *Necator americanus* (Xiao et al., 1989). Comprehensive disintegration of muscle cells was observed in *L. carinii* after treatment with two benzothiazole derivatives (Franz et al., 1987); additionally, blebs were formed on the luminal side of the intestinal membrane and microtubuli disappeared from the intestinal cells of *L. carinii* and *B. malayi* after treatment with amoscanate (Franz et al., 1990a) and flubendazole (Franz et al., 1990b). Morphological effects of cyclosporin A on *Trichinella spiralis* were in the form of
disorganization of the cuticle especially between the hypodermal pores which appeared somewhat thickened and irregular, wrinkling of the outer layer that later to be peeled off in adults, while smoothing of the cuticle with obliteration of the grooves between the cuticular ridges were observed as the earliest surface larval changes (Boulos et al., 1992). Mebendazole and ivermectin treatment resulting in topographic changes were in the form of disorganized cuticle and absence of surface uniformity on Capillaria hepatica (El Gebaly et al., 1996). Similar observation was made on A. suum when treated with the root tuber extracts of F. vestita (Yadav et al., 1992). PPZ in combination a depsipeptide BAY 44-4400 caused vacuolization in the hypodermal layer, degeneration of the muscle fibres, distension of the nerve fibre axons, and rupture of the epithelial cells of Heterakis spumosa (Nicolay et al., 2000). The principal mode of action of flubendazole was based on the complete reduction of microtubule polymerisation inside the cells of adult Toxocara canis and A. suum, which apparently led to the complete destruction of the hypodermis, muscle layer and intestine (Hanser et al., 2003). The epithelial cells of the nematode intestine are particularly susceptible to different classes of broad spectrum anthelmintics. Extensive comparative studies of Aukštikalnienė et al. (2000, 2005) had demonstrated that the intestinal epithelial cells of T. canis filled with vacuoles and granules, associated with degeneration of the microvilli and swelling upon treatment with ABZ, levamisole, pyrantel pamoate and nitroscanate. The anthelmintic action took considerably long time in the nematode than in cestode and trematode. The complexity of the structure of the nematode cuticle compared with the external tegument of the trematode is responsible for such a difference in drug diffusion and the subsequent influence on vital organs between the species (Alvarez et al., 2001).
A number of bio-active chemicals in *M. pachycarpa* had been reported including several prenylated isoflavonoids, dihydroflananol and chalcones from the seed (Singhal *et al.*, 1980, 1981, 1983), and rotenoids such as rotenone, cis-12a-hydroxyretene, rot-2'-enonic acid and cis-12a-hydroxyrot-2'-enonic acid from the root (Singhal *et al.*, 1981). Recent chemical analyses have yielded a number of novel prenylated isoflavones including erysenegalensein E, isoerysenegalensein E, 6,8-diprenylorobol, furowanin A, furowanin-A (2) and B (3), millewanins-F (1), -G (1) and -H (2), and auriculasin from the leaves, which were all demonstrated to have significant antiestrogenic activity (Ito *et al.*, 2004; Okamoto *et al.*, 2006; Ito *et al.*, 2006a,b), supporting the evidence of its use in Chinese medicine as cancer therapy. Plant-derived isoflavonoids are well-known bio-active compounds having huge potential implications in medicine as anti-inflammatory, anticancer, antibacterial, antiplasmodial, antioxidant, antiosteoporosis and hepatoprotective agents (Laupattarakasem *et al.*, 2004; Bedani and Rossi, 2005; Shon *et al.*, 2007).

A number of *Millettia* species have been reported to contain several classes of isoflavonoids as their principal chemical constituents indicating a variety of positive therapeutic activities (Perrett *et al.*, 1994; Sritularak *et al.*, 2002; Palazzino *et al.*, 2003; Yankep *et al.*, 2003; Ngamga *et al.*, 2005; Ketcha Wanda *et al.*, 2006). Members of the genus *Millettia* as rich sources of isoflavonoids is particularly appealing in the light of several reports on their anthelmintic efficacy. Dichloromethane extract of *Millettia thonningii* contains isoflavonoids such as robustic acid, alpinumisoflavone and dimethylalpinumisoflavone, and was shown to have potent trematocidal activity against cercariae, miracidia and adults of *Schistosoma mansoni* (Squire and Whitfield, 1989; Perrett *et al.*, 1994; Perrett and Whitfield, 1995a), specifically by disrupting energy metabolism and osmoregulation through inhibition of mitochondrial electron...
transport enzyme (Lyddiard and Whitfield, 2001; Lyddiard et al., 2002). Isoflavones such as diadzein, genistein, formonectin, pseudobaptigenin were identified in the fleshy tuberous root of *F. vestita* (Rao and Reddy, 1991), which is consumed directly by Khasi tribes for deworming gastrointestinal parasites. Pseudobaptigenin, also reportedly present in *Millettia griffoniana* (Ngamga et al., 2005), was experimentally shown to have significant cestocidal activity on *R. echinobothrida* (Tandon et al., 1997). A series of experimental evaluations has convincingly demonstrated that genistein is the most active component from *F. vestita* exhibiting vermicidal activity on the trematodes *Fascioloopsis buski* and *Artyfechinostomum sufrartyfex* (Roy and Tandon, 1996; Kar et al., 2002, 2004; Kar and Tandon, 2004), and on the cestode *R. echinobothrida* (Tandon et al., 1997, 1998; Pal and Tandon 1998a,b; Das et al., 2004a,b,c). Fascinatingly, mortality effects and structural deformities observed in the cestode upon treatment with *M. pachycarpa* extracts and rotenone from the present study strikingly correspond to those that were demonstrated for genistein.

The crude extract and genistein of *F. vestita* produced prominent structural alterations on *R. echinobothrida* extending from destruction of tegument, microtriches and suckers to vacuolization and muscle distortion in the internal structures (Tandon et al., 1997; Pal and Tandon, 1998c). In acting through the tegument, the phytochemicals of *F. vestita* caused drastic suppression in the activity of vital enzymes (Pal and Tandon, 1998a,b), which can be attributed to extensive damage on the cestode topography. In addition, energy metabolism in the cestode is greatly impaired leading to stress (Tandon et al., 2003; Das et al., 2004a,b), which explains the tegumental damage and paralytic effects. Bricker et al. (1983) had clearly demonstrated the correlation between contraction and vacuolisation of the parasite tegument and the concentration of Ca\(^{2+}\). Disruption of tegumental organization leading
to vacoulsation and pit formation on the tegument is the result of disturbances in ion
($\text{Ca}^{2+}$) flux across the parasite membrane (Mehlhorn et al., 1983; Schmahl and
Mehlhorn, 1985). The paralyzing activity is further supported by the findings that $\text{Ca}^{2+}$
homeostasis is interrupted in the cestode tissue by decreasing $\text{Ca}^{2+}$ accumulation and
increasing its efflux (Das et al., 2006), as $\text{Ca}^{2+}$ is the fundamental ion for muscle
contraction. Concomitantly, the primary mechanism of action of drugs such as PZQ
appears to be disturbance on calcium homeostasis, causing sustained $\text{Ca}^{2+}$-dependent
muscle contraction, resulting in flaccid paralysis of the worms (Valle et al., 2003;
Kohn et al., 2001, 2003). Naguleswaran et al. (2006) further observed that synthetic
derivatives of genistein, Rm6423 and Rm6426, induced considerable damage in
human cestodes, $E$. granulosus and $E$. multilocularis, rendering them nonviable by
inducing truncation of microtriches, nuclear pyknosis and vesiculations; in addition,
Rm6423 specifically induced dramatic breakdown of the structural integrity of the
metacestode germinal layer. Therefore, it is highly probable to envisage that similar
cestocidal activity demonstrated for $M$. pachycarpa extracts may be quite similar to
the effects of $F$. vestita isoflavonoids, genistein in particular, on the viability and
structural integrity of $R$. echinobothrida; and that isoflavonoids, if not exclusive, are
the active compounds responsible for the anthelmintic activity.

Of the two plant materials investigated, only the ethanol and methanol extracts of
$A$. oxyphylla appeared to have significantly feasible anthelmintic effect on the
nematode in terms of viability and structural damage. It is well-documented that
members of the genus of $Acacia$ are generally rich in saponins and tannins (Rama
Devi and Prasad, 1991; Seigler, 2003). Certain saponins are experimentally validated
to possess potent antimicrobial (Mandal et al., 2005), and antinematodal activities
(Ghosh et al., 1993; Sarkar et al., 1998; Deepak et al., 2002). Two triterpenoid
saponins, named acaciaside A and acaciaside B, have been isolated from *A. auriculiformis* that specifically caused absolute reduction in the burden of nematodes including *Setaria cervi* (Ghosh et al., 1993) and *Dirofilaria immitis* (Chakraborty et al., 1995; Sarkar et al., 1998), and of the cestode *Hymenolepis dimunita* (Ghosh et al., 1996). Other types of saponins such as asperocide and strebloside were also shown to have potent nematicidal activity on *Acanthocheilonema viteae*, *L. carinii* and *B. malayi* (Chatterjee et al., 1992). The cellular actions of these saponins are destruction of cell membranes by inducing peroxidation, increasing energy metabolism and inhibiting glucose uptake of the parasites (Singh et al., 1994, 1998; Sinha Babu et al., 1997, 2005; Nandi et al., 2004). Inference from the present investigation overwhelmingly corroborates to the nature of these anthelmintic effects, which makes it tempting to assert that the broad-spectrum activity of *A. oxyphylla* stem bark in causing deleterious effect on the cestodes and nematodes may be attributed to saponins.

A good number of tannin-rich plants such as *Acacia nilotica*, *A. karoo*, *A. mearnsii*, *Castanea sativa*, *Cichorium intybus*, *Corylus avellana*, *Hedysarum coronarium*, *Lespedeza culeata*, *Lotus corniculatus*, *L. pedunculatus*, *Quercus robur*, *Rubus fructicosus* and *Sarothamnus scoparius* have been experimentally shown to possess effective deworming properties on important nematodes of livestock animals (Hoskin et al., 2000; Kahiya et al., 2003; Marley et al., 2003; Paolini et al., 2003, 2004; Tzamaloukas et al., 2005, 2006; Bahuaud et al., 2006; Cenci et al., 2007), with their tannins component confirmed as the principal anthelmintic compounds (Molan et al., 2000, 2004; Barrau et al., 2005; Brunet and Hoste, 2006; Hoste et al., 2006). It has been evoked that the mechanism of antinematodal action of these tannins are due to their high-affinity protein binding property. The nematode cuticle is known to be a
proline- and hydroxyproline-rich structure that covers not only the nematode body, but also gastrointestinal and reproductive tracts (Thompson and Geary, 1995). Probably by binding to these structural proteins, tannins caused detrimental effects in the nematodes. This ability plausibly explains the cuticular destruction on *Trichostrongylus culubriformis* observed by SEM; in addition, massive lesions have been described in the digestive and reproductive tracts of different nematodes, with the external cuticle, the intestine or the uterus being principal targets (Hoste *et al.*, 2006). There are also clinical evidences that the antinematodal activities are enhanced by elevated immune response in the host due the application of these tannins (Tzamaloukas *et al.*, 2005, 2006). In spite of such evidential speculation, identification and specific activity of the precise chemical constituents of *A. oxyphylla* stem bark responsible for the broad-spectrum anthelmintic activity is yet undefined.

The occurrence of trace metals such as cadmium, calcium, cobalt, copper, iron, lead, nickel, magnesium, manganese, nickel, potassium, selenium and zinc has been documented for different helminth parasites, ranging from the amphistomid trematodes (Tandon and Roy, 1994), nematodes (Barus *et al.*, 1999), to cestodes (Tenora *et al.*, 2000; Vijayalakshmi *et al.*, 2003), including *R. echinobothrida* (Das *et al.*, 2006). These trace elements play a significant role in the physiology, growth and development, the sequestration of free radicals and in the cellular antioxidant defense system, metabolism and immuno-tolerance of parasites. The manifold role of calcium is recognized ranging from regulation of the electrolyte level, maintenance of intercellular ionic bridges, neuro-motor functions and palatalizing metabolic reactions within the cell. Moreover, different trace metals are attributed to play significant roles in host-parasite interactions (Koski and Scott, 2003).
The present study also indicates the presence of calcium, magnesium, sodium and potassium in *R. echinobothrida*, at the concentrations of 296.2, 953, 435.7 and 132.8 \( \mu \text{g/g dry tissue weight} \), respectively. The cestodes treated with the different extracts of *A. oxyphylla* and *M. pachycarpa*, rotenone and albendazole evidently affected significant disturbances in the physiological homeostasis of these four trace metals. Therefore, the anthelmintic activities are highly correlated to reduction in the levels of the trace metals, which presumably leads to decreased vital metabolic activities within the cells, eventually resulting in paralysis, and subsequent death.

The occurrence of vital enzymes, viz acid phosphatase (AcPase) and alkaline phosphatase (AlkPase) have been clearly demonstrated both histochemically and biochemically in a number of helminth parasites (Pappas, 1991; Pappas and Leiby, 1986; Stettler *et al.*., 2001), including *R. echinobothrida* (Pal and Tandon, 1998b). These enzymes have been unequivocally revealed to be intimately associated with the tegument and subtegumental regions of cestodes and trematodes, as well as the cuticle of nematodes (Kwak and Kim, 1996; Buchmann, 1998; Fetterer and Rhoads, 2000). It is also firmly established that between the two tegumental enzymes, AlkPase is the major active enzyme in cestodes (Pappas, 1991; Pal and Tandon, 1998b; Stettler *et al.*, 2001), where they are posited to be involved in the digestive and/or absorptive functions, and modulation of host-parasite interaction (Poljakova-Krustena *et al.*, 1983; Lawton *et al.*, 1997; Stettler *et al.*, 2001).

Commercial synthetic drugs such as isatin, hexacholorophene, luxabendazole, nitazoxanide, praziquantel and thiabendazole reportedly induce detectable alterations in the activities of the tegumental phosphatases in different helminths (McCracken and Taylor, 1983; Fallon *et al.*, 1994; Stettler *et al.*, 2003). Especially in *E. multilocularis,*
the cestocidal effects of ABZ characterized by progressive degeneration and
destruction of the tissue evidently correlate with AlkPase activity, suggesting that the
phosphatase activity is an ideal parameter for performing first-round in vitro tests on
the efficacy of a large number of antiparasitic compounds (Stettler et al., 2001).

Extracts from certain medicinal plants, including Butea monosperma, Embelia
ribes, and Roltlesia tinctoria reportedly influenced drastic decrease in the activities of
both AcPase and AlkPase in the trematode, Paramphistomum cervi (Chopra et al.,
1991). The root tuber peel extract and genistein from Flemingia vestita similarly
causd significant reduction of the enzymes in R. echinobothrida (Pal and Tandon,
1998b), and in the fluke, Fasciolopsis buski (Kar and Tandon, 2004), comparable to
those of the standard pharmaceuticals, praziquantel and oxyclozanide, respectively.

In the present study, the cestodes exposed to 20 mg/ml of the extracts of both A.
oxiphylla and M. pachycarpa, rotenone and ABZ showed significant inhibition in
their AcPase and AlkPase activities. Comparable results were reported in a human
tapeworm E. multilocularis metacestode in which acute inhibition of AlkPase activity
by 23% and a complete inhibition of glucose uptake following treatment with isatin, a
known inhibitor of phosphatase, were observed (Delabre-Defayolle et al., 1989).
Similar results were also obtained for the both the AlkPase and AcPase activity in H.
dimunita (Pappas, 1988, 1991). These results suggest that there is direct correlation
between the activities of tegumental phosphatases and glucose uptake in cestodes.
Thus, the observed reduction in the two tegumental enzymes also might be associated
with inhibition or reduced uptake of glucose in R. echinobothrida leading to gradual
loss of motor activity due to deprivation of energy source and, thus, culminated in
paralysis. It also implies that transtegumental diffusion is obviously the primary route of action of the extracts of the two plants.

It, therefore, become acceptable to appreciate that there are pragmatic reasons for pursuing traditional-based phytomedicines to ameliorate the current conundrum in the control of pervasive helminth infections that put the well-being of both humans and our livestock in jeopardy. Experimental results from the present study evidently advocate that there is a sound scientific basis for the use of *A. oxyphylla* stem bark and *M. pachycarpa* root bark as gastrointestinal helminthic remedies. However, it is important to understand that plant products, such as cysteine proteinases, or secondary metabolites, such as alkaloids, glycosides and tannins, though are shown to possess anti-parasitic properties, may also have anti-nutritional effects, such as reduced food intake and performance, and serious physiological side effects in the host (Githiori *et al.*, 2006). Moreover, there is another practical caution to take into account that the tested anthelmintic plants appear to be helminth-specific, as *A. oxyphylla* is evidently effective on both the cestode and nematode, while *M. pachycarpa* is indicating cestocidal activity only. Further, identification of the prospective anthelmintic compounds from these two plants is beyond conclusive comprehension from this investigation in terms of their chemical nature, effective dose, safety and mode of action underpinning the cellular and structural alterations. Therefore, further laboratory and field experimentations are crucially required prior to their legitimate adoption as a genuine practice for the control of helminth infections both in humans and animals.