

**ANTHELMINTIC EFFICACY OF *Flemingia vestita*: AN IN  
VITRO STUDY ON CARBOHYDRATE METABOLISM IN  
THE CESTODE, *Raillietina echinobothrida***

**ABSTRACT**



by

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**SUBMITTED**

in

**PARTIAL FULFILMENT OF THE REQUIREMENT OF THE DEGREE OF**

**DOCTOR OF PHILOSOPHY IN ZOOLOGY**

of

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## ABSTRACT

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The present work incorporates a study on the anthelmintic efficacy of *Flemingia vestita* Benth and Hooker (Family Fabaceae), a leguminous plant, the tuberous roots of which have a usage as vermifuge or vermicide in the indigenous traditional system of medicine in Meghalaya (Northeast India). In earlier studies, the root tuber peel extract of this plant and its major active component, genistein, were shown to cause flaccid paralysis and to be acting transtegumentally in trematode and cestode parasite. With a view to investing further the mode of action of these putatively anthelmintic phytochemicals, the crude-peel extract of *F. vestita*, genistein and the reference drug PZQ were tested in vitro against the cestode, *Raillietina echinobothrida*, in respect to the carbohydrate metabolism, the major energy yielding pathway in helminth parasites.

### Alterations in:

- glycogen level;
- activities of glycogen phosphorylase (GPase) and glycogen synthase (GSase), both active and total;
- physiological levels of some metabolites - glucose, lactate, pyruvate, malate and alanine;
- activities of some regulatory glycolytic enzymes - hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase (PK), phosphoenolpyruvate

carboxykinase (PEPCK), lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and malic enzyme (ME);

- \* activities of glucose 6-phosphate dehydrogenase (G6PDH), pyruvate carboxylase (PC) and fructose 1,6-bisphosphatase (FBPase) and
- \* physiological levels of some trace elements and changes in  $\text{Ca}^{2+}$  concentration, in particular

in treated parasites in comparison to controls form the parameters of this study. Standard techniques were used for biochemical assays and histochemical demonstrations of the mentioned parameters.

1. *R. echinobothrida* were collected in 0.9% PBS (pH 7.2) from freshly slaughtered domestic fowl and incubated at  $39 \pm 1$  °C with defined concentration of crude-peel extract (5 mg/ml), genistein (0.2 mg/ml) and PZQ (1 µg/ml) with simultaneous maintenance of controls till the onset of paralysis. Control parasites survived upto 72 h, whereas in the treated parasites paralysis set in about 5.9 h, 6.7 h and 2.9 h in case of crude root peel extract, genistein and PZQ, respectively. The treated parasites and controls were taken for various experiments.
2. After exposure to the plant crude-peel extract of *F. vestita* and genistein, the glycogen concentration was found to decrease by 15-44% in the parasites compared to their respective controls. The decrease in the physiological level of

glycogen was accompanied by an increase in the activity of GPase *a* by 29-39% and a decrease of activity of GSase *a* by 36-59% in treated parasites as compared to untreated controls, though without affecting the total activity of both the enzymes. PZQ also caused quantitative reduction in glycogen level and alterations in enzyme activities somewhat at par with the genistein treatment.

3. The glucose content in the treated worms decreased by 14-36%, whereas malate concentration increased by 49-134% as compared to controls. Both in controls and treated parasites, however, pyruvate content was not measurable, while alanine and lactate contents showed a decline by 7-31% in the parasites exposed to all test materials. Besides, the lactate content as effluxed into the incubation medium showed an increase of 9-44% in the case of treated parasites indicating an overall increase of lactate production.
4. While some enzymes of the glycolytic pathway showed an increase in their activities following exposure of the parasites to the various treatments, others showed a decline. The activities of HK, PFK, PEPCK and LDH increased by 33-39%, 41-125%, 44-49% and 55-67%, respectively and that of PK decreased by 14-26% in all treatments in comparison to the respective controls.
5. The cytosolic MDH got activated by 33-58% whereas mitochondrial MDH increased by 43-73% in the treated parasites. The MDH activity of in the tissue

homogenate was also found to be higher by 22-43% in treatments with crude-peel extract, genistein and PZQ. The ME activity in the tissue homogenate was found to be increased by 28-59% in the treated parasites; while the cytosolic ME activity showed an increase by 33-39%, there was no enhancement in mitochondrial ME activity.

6. The key regulatory enzyme of pentose phosphate pathway, G6PDH, was found to decrease by 23-31% in various treatments. FBPase, the enzyme of gluconeogenesis, did not show any changes during the treatment conditions while the activity of PC was significantly increased by 32-44% in treated parasites in comparison to the controls.
7. Using atomic absorption spectrophotometry, some trace elements, namely, lead (~ 25 µg/g dry tissue wt), iron (~ 1200 µg/g dry tissue wt), zinc (~ 400 µg/g dry tissue wt), magnesium (~1400 µg/g dry tissue wt), calcium (~ 400 µg/g dry tissue wt), chromium (~12 µg/g dry tissue wt) were detected in the cestode while manganese, cadmium and nickel were below level of detection.
8. The calcium concentration in the parasite tissue was found to decrease by 39-49% following with the test materials though the calcium concentration in the incubation media containing the test materials was found to increase by 94-118%, indicating thereby an efflux of  $\text{Ca}^{2+}$  from the parasite under the treatment itself.

9. The observations made in the present study are supported by six photomicrographs, eighteen tables and sixteen graphical column figures. Two hundred forty seven references are cited.
10. From the results obtained it can be hypothesized that due to the high energy demand of the parasites because of anthelmintic stress following exposure to the plant crude extract and genistein, the process of glycogenolysis and glycolysis got activated on the expense of other metabolic pathways of glucose utilization by regulating some key enzymes of these pathways. Changes in the homeostasis of  $Ca^{2+}$  could be one such regulating mechanism in the cestode parasites. The PEPCK/PK branch point provides an important clue for the switch over to the phosphoenolpyruvate-succinate pathway for the glucose metabolism and thus an avenue for anthelmintic attack. The phytochemicals of *F. vestita*, therefore, seem to influence the carbohydrate metabolism of the cestode, which may be a secondary target of action, the primary target being the tegumental interface of the parasite.

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## Declaration

*I, Bidyadhar Das, hereby declare that the subject matter of this thesis is the record of the work done by me, that the contents of this thesis did not form the basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.*

*This is being submitted to the North-Eastern Hill University for the degree of Doctor of Philosophy in Zoology.*

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## PREFACE

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*Medicinal plants provide an alternative to current practices involving chemotherapy. Some of the plant-derived components have been found to be comparable in their effect with commonly used drugs. The edible tuberous roots of *Flemingia vestita* (Fabaceae) are commonly used as anthelmintic against intestinal worms in traditional practice in Meghalaya (Northeast India). In a preliminary study the crude root-tuber peel extract of *F. vestita* was found to be effective against soft-bodied trematode and cestode parasites. Further studies indicated towards the mode of action of these phytochemicals, particularly targeting the enzymes associated with the tegument and also those involved in the nervous co-ordination, e.g., non-specific esterases, acetylcholine esterases and nitric oxide synthase.*

*As a sequel to the studies carried out so far in the quest for finding out the possible mode of action of the phytochemicals derived from the crude root-tuber peel of *F. vestita* the present study has been undertaken. Carbohydrate metabolism being the major energy-deriving pathway among the helminth parasites, it is aimed to study the carbohydrate metabolism under the influence, if any, of these phytochemicals. Alterations in the status/activity of the glycogen level, major enzymes involved in glycogen metabolism, metabolites and some regulatory enzymes of glycolysis, other selected regulatory enzymes related to carbohydrate metabolism and some trace elements, particularly  $Ca^{2+}$ , form the*

*parameters of the present study, for which *Roillietina echinobothrida*, the intestinal cestode from domestic fowl, has been used as the test parasite.*

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## INTRODUCTION

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Plants or their products provide an alternative to current practices involving chemotherapy (Didier et al., 1988; Robinson et al., 1990). Recent studies pertaining to the activity of plants, such as *Artocarpus lakoocha*, *Diospyros mollis*, *Mallotus philippinensis*, *Teloxys graveolens*, *Saussurea lappa*, *Albizia anthelmintica*, *A. lebbek*, *Alium sativum*, *Streblus asper*, *Buddleia asiatica*, *Uveria narum*, *Matteuccia orientalis*, to name a few have reported their anthelmintic efficacy in vitro against trematode, cestode and other helminth parasites (Charoenlarp et al., 1981, 1989; Maki et al., 1983; Gupta et al., 1984; Del Rayo Camacho et al., 1991; Akhtar and Riffat, 1991; Galal et al., 1991a,b; Soffar and Mokhtar, 1991; Akhtar and Ahmad, 1992; Chatterjee et al., 1992; Garg and Dengre, 1992; Hisham et al., 1992 and Shiramizu et al., 1993). In recent years, many other plants or their parts have been shown to have cidal activity against schistosomules of *Schistosoma mansoni*, metacestodes of *Hymenolepis diminuta* and larvae of filarid or other nematode parasites (Comely, 1990; Satrija et al., 1995; Ghosh et al., 1996; Khunkitti et al., 2000; Singh et al., 2000; Sparg et al., 2000; Molgaard et al., 2001; Al-Qarawi et al., 2001; Marley et al., 2003). Anthelmintic efficacy of some plant derived components has been found to be comparable or at par with commonly used broad spectrum drugs like albendazole, piperazine and diethylcarbamazine (Kalyani et al., 1989; Koko et al., 2000; Onyeyili et al., 2001; Enwerem et al., 2001; Temjenmongla and Yadav, 2003).

The mode of action is well known in respect to only a few commonly used anthelmintics. Some drugs, such as rafoxamide, praziquantel (PZQ), albendazole, fenbendazole, oxiclozanide, luxabendazole, oxfendazole, triclabendazole etc., have been reported to bring in ultrastructural alteration in the parasite's tegument (Mehlhorn et al., 1983; Schmahl and Mehlhorn, 1985; Jiang and Xia, 1992; Mackenstedt et al., 1993; Schmahl, 1993; Stoitsova and Gorchilova, 1994; Magambo, 1996; Apinhasmit and Sobhon, 1996; el Sayed and Allam, 1997; Mansoury, 1997; William et al., 2001; el-Sayad and Lotfy, 2002; Liang et al., 2002; Liu et al., 2003). Artemether, a derivative of the antimalarial artemisinin, injured the tegument by causing swelling, vesicle formation etc. in adults and juveniles of *Schistosoma* spp. (Xiao et al., 1996, 2000, 2001, 2002a,b). The primary target of action of ivermectin in *Echinococcus granulosus* is tegument-elicited alterations in rostellar disorganization, rigid paralysis, and eventually loss of viability (Perez-Serrano et al., 2001). The sulphoxide metabolite of triclabendazole and albendazole (Valbazen) caused blebbing, tegumental sloughing and spine loss in *Fasciola gigantica* and *F. hepatica* (Sukontason et al., 2000; Meaney, 2002; Buchanan, 2003). Certain drugs are known to act by binding to the structural proteins, such as collagen and tubulin, of the parasites (Eckert, 1986; Martin et al., 1997; Buchanan et al., 2003). Many of the broad spectrum anthelmintics affect the worms by blocking their central nervous system or the neuromuscular system by inhibiting the neuromodulators, such as bioamines, acetylcholine and neuropeptides, causing paralysis of the treated worms (Gustafsson, 1984; Raether, 1988; Vijayanathan and Raj 1991, 1992; Cox, 1994; Strote et al., 1997). Paraherquamide, 2-deoxoparaherquamide, and their structural analogs rapidly induced flaccid paralysis due to blockade of cholinergic neuromuscular

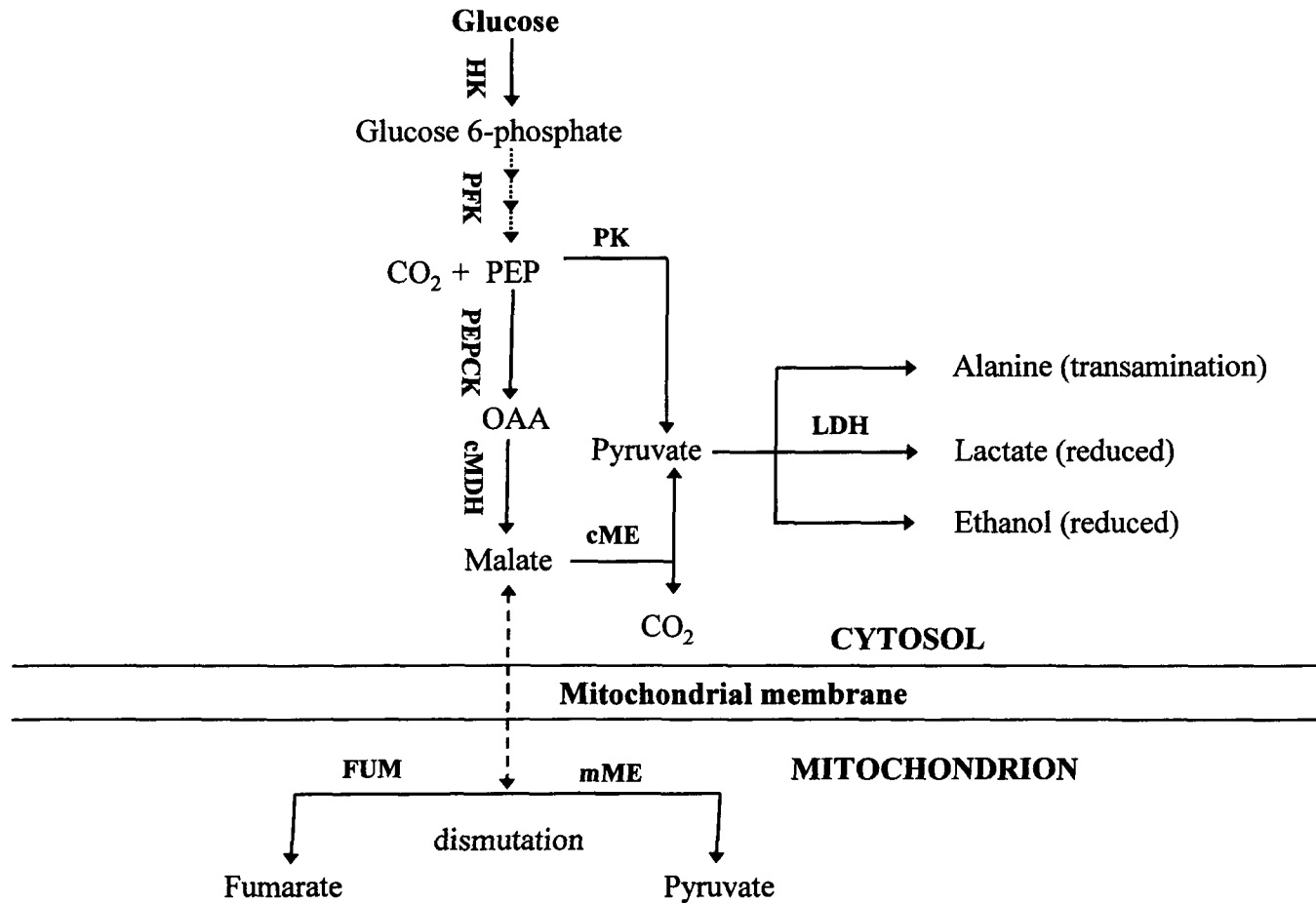
transmission in parasitic nematodes in vitro (Zinser et al., 2002). The anthelmintic emodepside stimulates release of an inhibitory neurotransmitter or neuromodulator to inhibit the muscle contraction elicited by acetylcholine or the neuropeptide in *Ascaris suum* (Willson et al., 2003). Some cholinergic anthelmintics (butamisole, morantel, metyridine and oxantel) and ivermectin were shown to influence the signaling pathways in nematode parasites. (Ros-Moreno et al., 1999; Raymond et al., 2000). Anthelmintics like PZQ mediate the schistosomicidal effect through nitric oxide (NO) (Ahmed et al., 1997; Ammar et al., 2002). Levamisole, morantel and pyrantel act as acetylcholine agonists at nicotinic acetylcholine receptors of nematodes, while avermectin interferes with signal transduction (Martin et al., 1991; Arena et al., 1992; Robertson et al., 1994; Martin et al., 1997; Richmond and Jorgensen, 1999). Drugs may also affect the amino acid metabolic pathways leading to more formation and accumulation of ammonia in the tissue to a toxic level, which in turn may cause neurological disorders (Cooper and Plum, 1987; Campbell, 1991) such as paralysis. Some anthelmintics are known to affect the worms by blocking their metabolic activities. Mebendazole significantly inhibits oxidative phosphorylation; however, PZQ exhibits high response to O<sub>2</sub> consumption in *Ancylostoma ceylanicum* (Srivastava et al., 1990). Rafoxamide was reported to affect the activity of major dehydrogenase enzymes of carbohydrate metabolism (Parveen et al., 1992). Diamphenethide and closulon inhibit glucose metabolism in *Fasciola*, whilst closantel uncouples oxidative phosphorylation (Martin et al., 1997). As cestodes and trematodes have a limited ability or are unable to metabolise lipids and amino acids, they mainly depend on glucose and other simple carbohydrates as the main energy source for various metabolic activities (Arme and Pappas, 1983; Bryant and Behm, 1989). So

carbohydrate metabolism, which is the main energy-yielding pathway, should expectedly be also prone to the action of drugs or phytochemicals. Fragmented and conflicting information is available regarding the anthelmintic efficacy of drugs on carbohydrate metabolism.

Glycogen is the major carbohydrate in both larval and adult cestodes and serves typically as the most important energy reserve in the parenchymatous tissue (Smyth and McManus, 1989). Several chemotherapeutic agents have been shown to influence glycogen metabolism in helminth parasites (Bueding, 1970; Schulman et al., 1982; Donahue, 1983). The two key regulatory enzymes in glycogen metabolism are glycogen phosphorylase (GPase) that releases the terminal glucose as glucose 1-phosphate and glycogen synthase (GSase) that extends the 'primed' glycogen by using UDP-glucose as glucosyl donor. The degradation and synthesis of glycogen are regulated depending upon the energy need under various physiological conditions mainly by regulating the activity of these two enzymes that are normally present in two forms - active (*a*) and less active (*b*), which are interconverted by phosphorylation by protein kinase, and dephosphorylation by protein phosphatase (Nelson and Cox, 2000). In addition, these enzymes can also be allosterically regulated by various modulators, such as  $\text{Ca}^{2+}$ , AMP and glucose 6-phosphate etc. (Bollen et al., 1998). The presence of GPase activity was demonstrated in *H. diminuta* (Read, 1951) and glycogenesis in cestodes was reported in vitro (Read and Simmons, 1963). GPase and GSase activities in cestodes are believed to be regulated qualitatively in a similar way to that of the host/mammalian system (Roberts, 1983). The *a* and *b* forms of GPase have been demonstrated in the

cysticercoids of *H. diminuta* and their interconversion is regulated by a 3', 5'-cyclic AMP-dependant protein kinase and a phosphorylase phosphatase (Moczon 1975, 1977). The enzymes necessary for galactose incorporation, namely galactokinase, galactose 1-phosphate uridyl transferase, UDP-galactose 4-epimerases, have been reported to be present in *H. diminuta* (Komuniecki and Roberts, 1977).

Glucose 1-phosphate, released by the glycogenolysis, is converted to glucose 6-phosphate by the enzyme phosphoglucomutase which is metabolized further. Depending upon the end products formed, the carbohydrate metabolism demonstrated among the parasitic helminths has been divided into three types by Bryant and Flockhart (1986). Of these, *type 1*, which is characterized by homolactic fermentation (Embden-Meyerhof pathway), is found in the ANU (Australian) strain of *H. diminuta*; *type 3* fermentation reported to be occurring in *Ascaris* and a number of other intestinal nematodes and in some trematodes, is characterized by yielding branched-chain fatty acids (e.g. 2-methylvalerate, 2-methylbutyrate) (McManus, 1987). Most of the cestodes are conventional to *type 2* category (Fig. 1), which is characterized by a CO<sub>2</sub>-fixation step (by phosphoenolpyruvate carboxykinase) and malate dismutation. Glucose and other simple carbohydrates, which are transported by the active transport as demonstrated in adult *H. diminuta* (Phifer, 1960; Pappas et al., 1974; Read et al., 1974; Uglem, 1976) and larvae (Arme et al., 1973), are metabolized following *type 2* pathway in most cestodes. Some anthelmintic drugs like niclosamide, PZQ and mebendazole were reported to cause inhibition of glucose uptake by the cestode (*Cotugnia digonopora*) and increase in the production of lactate (Pampori et al., 1984). In contrast, an enhancing



**Fig. 1.** Schematic diagram to show the respiratory pathways (*type 2*) in *Echinococcus* spp.. HK, hexokinase; PFK, phosphofructokinase; PEPCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; LDH, lactate dehydrogenase; cMDH, cytosolic malate dehydrogenase; cME, cytosolic malic enzyme; mME, mitochondrial malic enzyme; FUM, fumarase; PEP, phosphoenolpyruvate; OAA, oxaloacetate. (after McManus and Bryant, 1986)

effect of PZQ and fluoxetine at concentrations of 0.1-10  $\mu\text{M}$  and inhibitory effect above 10  $\mu\text{M}$  concentration was reported in *S. mansoni* by Harder et al. (1987a). Likewise, in *Cysticercus fasciolaris*, PZQ, mebendazole and some drug candidate compounds were reported to significantly lower the rate of glucose uptake and also to suppress the formation of lactate as a major end product of glycogen metabolism (Jain et al., 1992). In contrast, the drug-induced glycogen reduction in schistosomes was attributed to an inhibition of glycolysis rather than interference with glucose uptake (Xiao et al., 1997).

Glycolytic enzymes, such as hexokinase (HK), phosphofructokinase (PFK), aldolase, glyceraldehyde 3-phosphate dehydrogenase (G3PDH), pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), lactate dehydrogenase (LDH), and malic enzyme (ME) are reported to be present in cestodes (Arme and Pappas, 1983). Some of these enzymes are also regulated by several modulators (Nelson and Cox, 2000). Operation of the classical Embden-Meyerhof pathway has been confirmed by the demonstration of a highly active sequence of glycolytic enzymes in a variety of cestodes (Behm and Bryant, 1975; Beis and Barrett, 1979; McManus and Smyth, 1982; McManus and Sterry, 1982; Rahman and Mettrick, 1982). HK, which initiates the glucose catabolism via glycolysis and the pentose-phosphate pathway and activates the formation of glycogen and complex carbohydrates from glucose, was partially purified from *H. diminuta* and *Bothriocephalus scorpii* (Komuniecki and Roberts, 1977; Vykhrestyuk and Klochkova, 1984). PFK, the key regulatory enzyme of the pathway, which catalyses the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate,

has been investigated in adult *Moniezia expansa* and in plerocercoids of *Schistocephalus solidus* (Beis and Theophilidis, 1982). The properties of PK, another potential regulatory enzyme of glycolysis that converts phosphoenolpyruvate to pyruvate, have been investigated in a number of cestodes (Smyth and McManus, 1989). LDH, which catalyses the reversible terminal reaction in the glycolysis with the formation of lactate from pyruvate, is also reported to be in cestode spp. However, the properties of this enzyme have been investigated in details only in *Hymenolepis* spp. (Burke et al., 1972). The presence of malate dehydrogenase (MDH), which converts oxaloacetate to malate, has also been demonstrated in several cestodes (Smyth and McManus, 1989). ME, which oxidatively decarboxylates malate to pyruvate, has been investigated in *H. diminuta* (Fioravanti and Saz, 1980; Roberts, 1983) and also in a range of cestodes. The occurrence of PEPCK, which is involved in carboxylation of phosphoenolpyruvate to oxaloacetate with the production of ATP, has been reported from a number of cestodes, with more extensive studies in *M. expansa*, *H. diminuta* and *Spirometra erinacei* (Behm and Bryant, 1975; Moon et al., 1977; Fukumoto, 1985). It is predominantly cytosolic and regulated by a variety of modulators (Reynolds, 1980). The main role of PEPCK in cestodes appears to be opposite to that in vertebrates (Prescott and Campbell, 1965); in the former it is found to be involved in degradation of glucose molecule (Smyth and McManus, 1989) and has a greater affinity for phosphoenolpyruvate, metal ions and inhibitors (quinolinate and 3- mercaptopicolinate), whilst in the latter its main role is in gluconeogenesis. Because of the differing primary functions of PEPCK in cestodes and their hosts, this enzyme might be inhibited selectively and thus provide an avenue for anthelmintic attack (Reynolds, 1980).

Scanty information is available regarding the pentose phosphate pathway in cestodes. Its main role is to provide NADPH, needed for synthesis of fatty acid and pentoses (particularly D-ribose 5-phosphate) rather than production of ATP. This pathway also converts pentoses to hexoses, which are metabolized further following Embden-Meyerhof pathway of glycolysis (Smyth and McManus, 1989). A complete sequence of enzymes for pentose phosphate pathway has been demonstrated only in larval *E. granulosus* (Agosin and Aravena, 1960; Agosin and Repetto, 1961). However, the presence of first two enzymes of this pathway, namely glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PDH) have been reported in several cestodes, including both pseudophyllidean and cyclophyllidean (Körting and Barrett, 1977; McManus and Smyth, 1982; McMannus and Sterry, 1982; Roberts, 1983). Not much information is available regarding the presence of gluconeogenic pathway among cestodes. Pyruvate carboxylase (PC), which is involved in anaplerotic reaction in mitochondria and gluconeogenesis, has been reported in several cestodes (Unnikrishnan and Raj, 1995). Other regulatory enzymes of gluconeogenesis, namely, fructose 1,6-bisphosphatase (FBPase) that dephosphorylates the 6' phosphate from fructose 1,6-bisphosphate and glucose 6-phosphatase (G6Pase) that dephosphorylates the glucose 6-phosphate, are also present in some cestodes (Gupta et al., 1994). The functional significance of the pathway is under scrutiny in cestodes.

Trace elements like calcium, copper, manganese, magnesium, lead, iron, nickel, zinc and potassium are reported to be present in some helminth parasites, viz., amphistomid trematodes (Tandon and Roy, 1994), nematodes (Baruš et al., 1999;

Tenora et al., 1999, 2000) and cestodes (Baruš et al., 2000).  $\text{Ca}^{2+}$ , which is stored in calcareous corpuscles of many cestodes, especially the larvae, is intimately involved in both muscle contraction and many aspects of cell movement controlled by the cytoskeleton (Bryant and Behm, 1989). Some enzymes, like GPase, GSase and protein kinases, are allosterically regulated by various modulators, where  $\text{Ca}^{2+}$  plays a key role (Bollen et al., 1998). PZQ, calcium ionophore A-23187 and the benzodiazepine Ro 11-3128 disturbed the calcium homeostasis and caused paralysis in *S. mansoni* and also in cestodes by different mechanisms (Tayal et al., 1988; Bryant and Behm, 1989; Blair et al., 1994; Martin et al., 1997).

*Flemingia vestita* (Fabaceae), a leguminous plant, is mostly found in Meghalaya and other parts of Northeast India. The edible unpeeled root-tuber of *F. vestita* is conventionally used as anthelmintic in local traditional medicine against intestinal worms including cestodes. The active principles of the root-tuber peel extract were isolated and identified by Rao and Reddy (1991); these are found to be isoflavones and identified as genistein (0.25%), formononetin (0.035%), pseudobaptigenin (0.015%) and diadzein (0.01%). Genistein (4',5,7-trihydroxyisoflavone) is an inhibitor of tyrosine protein kinases and competitive inhibitor of several protein kinase reactions (Akiyama et al., 1987; O'Dell et al., 1991). By its inhibitory effect on NO production, it is reported to have beneficial effects on atherosclerosis and chronic inflammatory diseases (Sheu et al., 2001). Possible role of genistein in cell cycle, reflecting release from  $G_0$  into  $G_1$  cell cycle phase, is reported in *Candida albicans*, a fungus (Yazdanyar et al., 2001). It also

has anticancerous activity, particularly associated with a decreased breast cancer risk (Wiseman and Duffy, 2001).

In an earlier preliminary study, the alcoholic extract of the *F. vestita* root-tuber peel was reported to be effective against *A. suum* (Yadav et al., 1992). Further studies on the anthelmintic efficacy of the plant-derived materials provided evidences of the tuberous root of this plant being vermifugal/vermicidal against soft-bodied helminth parasites, i.e., cestodes and trematodes (Roy and Tandon, 1996). The plant-derived components were also shown to induce flaccid paralysis in the cestode parasite of domestic fowl, *Raillietina echinobothrida*, in vitro, accompanied by alterations in the tegumental architecture (Tandon et al., 1997; Pal and Tandon, 1998a) suggesting thereby a transtegumental action of these phytochemicals. In the treated parasites a pronounced decline was noticeable in the activity of the non-specific esterases and acetylcholine esterase (AChE). As the latter is associated with the nervous co-ordination in helminths, alteration in the AChE activity indicated towards acetylcholine, an inhibitory neurotransmitter in cestodes, as a potential target of action by the plant-derived components (Pal and Tandon, 1998b). Alterations in the activity of nitric oxide synthase, the enzyme that is associated with the synthesis of the nitrenergic neurotransmitter, NO, were also observed in *Fasciolopsis buski*, the giant intestinal trematode, following treatment with the same plant materials and genistein. (Tandon et al., 2001; Kar et al., 2002). Further, the activities of several enzymes that are associated with the tegument, namely acid- and alkaline phosphatases, adenosine triphosphatase and 5'-nucleotidase were also found to be altered in the treated cestodes (Pal and Tandon, 1998c). In vitro

treatment of the cestode, *R. echinobothrida*, with the crude-peel extract and genistein also caused alteration in the level of free amino acid pool and tissue ammonia (Tandon et al., 1998).

In the quest to find out clues for the mode of action of putative anthelmintic active principles of *F. vestita* on the metabolic pathways, it seems logical to investigate further and see whether or not the plant-derived components affect the carbohydrate metabolism of the parasite by altering or by regulating the key enzymes. For accomplishing this objective, the followings aspects, viz.:

- \* glycogen level;
- \* activities of GPase and GSase (both active and total);
- \* physiological levels of some metabolites - glucose, lactate, pyruvate, malate and alanine;
- \* activities of some regulatory glycolytic enzymes - HK, PFK, PEPCK, PK, LDH, MDH and ME;
- \* activities of G6PDH, PC and FBPase and
- \* physiological levels of some trace elements and changes in  $\text{Ca}^{2+}$  concentration, in particular

form the parameters for the present study on carbohydrate metabolism of the cestode, *R. echinobothrida* under the influence of the mentioned phytochemicals.

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## MATERIALS AND METHODS

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### ***Experimental Parasite***

Live cestodes, *Raillietina echinobothrida* (Megnin, 1880) (Class: Cestoda; Subclass: Eucestoda; Order: Cyclophyllidea; Family: Davaineidea) were collected from the intestine of freshly slaughtered domestic fowl (*Gallus domesticus*) at local abattoirs in 0.9% phosphate buffered saline (PBS, pH 7.2).

### ***Plant Materials***

*Flemingia vestita* Benth and Hooker (Family: Fabaceae), locally known as Soh-Phlang (in Khasi), is mostly found in West Khasi Hills of Meghalaya. It is a trailing herb (Plate 1) having a hairy stem of around 80-100 cm height and contains fleshy tuberous roots. The latter are edible and have been conventionally used against intestinal worms including cestodes in traditional medicine among local population. The alcoholic extract of root tuber peels of *F. vestita* has been reported to be vermifugal/vermicidal against soft-bodied parasites, i.e., cestodes and trematodes (Roy and Tandon, 1996).

### ***Alcoholic Extract from F. vestita***

Tuberous roots of *F. vestita* were collected from the neighboring villages (Weiloi, Pongkung, Tyrsad and others) of Shillong, Meghalaya in the months of October-November. The roots were peeled off and the peels, washed thoroughly with tap water

and dried at 50<sup>0</sup>C in the oven or in the sun. The dried peels of known weight were soaked in ethanol (100 g/litre) in a reflux flask. The suspension was refluxed for several hours and the cooled suspension was collected. This reflux process was repeated for several times. The solution was then passed first through a fine cloth and then filtered through Whatman filter paper. The clear solution was distilled and the semisolid residue was collected and kept in oven at 60<sup>0</sup>C for several days for complete evaporation of ethanol. Approximately 28 g residue was recovered from 100 g of dried root tuber peels of *F. vestita*.

### ***Isolation of Purified Active Principles***

The crude extract (~ 28%) obtained from the dried peels of *F. vestita* was thoroughly mixed with hexane and the supernatant was decanted. This process was repeated for several (~ 20) times in order to collect the hexane soluble components. The supernatant was distilled and the residue was passed through silica gel column using hexane, benzene and ethyl acetate as solvents. The column was prepared using hexane and benzene mixture (6:4 ratio) and silica gel 100-200 mesh (about 350 g). The sample was loaded on to the top of the column and elution was done with hexane:benzene mixture (about 300 ml) at the ratio of 6:4, 5:5, 4:6... and until pure benzene and then with benzene:ethyl acetate at the ratio of 9:1, 8:2, 6:4, 5:5...till pure ethyl acetate using approximately 300 ml of mixed solvent at each ratio.

Elute at 7:3::benzene:ethylacetate ratio was found to be genistein (Fig. 2). The genistein solution was concentrated through distillation in a water bath. Approximately 25 mg (0.25%) of genistein (4',5,7-trihydroxyisoflavone), which is the major active principle of the root peel, was purified from 10 g of the crude extract. The residue, i.e., crude extract minus genistein, was eluted further using methanol and contained a mixture of other isoflavones - formononetin, pseudobaptigenin and diadzein - in very low concentrations (Rao and Reddy, 1991).

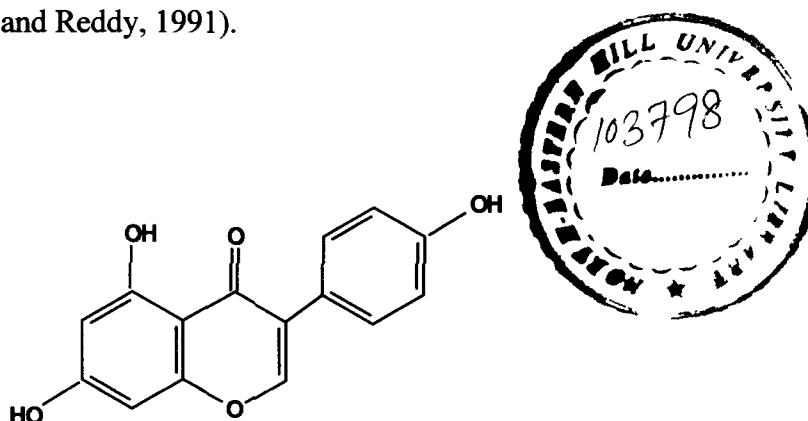


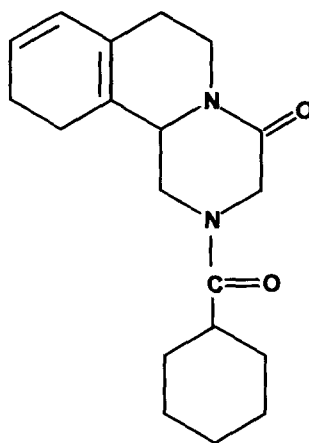
Fig. 2. Genistein

### ***Treatments in vitro***

The freshly collected live parasites, *R. echinobothrida* ( $\approx 0.2$  g fresh wt) were treated in vitro at  $39 \pm 1^{\circ}\text{C}$  in 10 ml of incubation media containing defined concentration of crude-peel extract of *F. vestita*, purified or synthetic genistein and PZQ dissolved in 1% dimethylsulfoxide (DMSO) with simultaneous maintenance of controls

for each treatment in PBS containing 1% DMSO, as reported previously by Tandon et al. (1997) till the onset of paralysis.

At the concentration of 50 mg/ml crude extract, 0.5 mg/ml genistein and 0.01 mg/ml praziquantel (PZQ) the time taken for paralysis and death of the treated worms post incubation was about 0.3 h and 6.5 h, 2.1 h and 13.35 h, and 0.47 h and 6.1 h, respectively. However, with a view to allowing sufficient time for a significant effect and in order to prolong the time for onset of paralysis, lower concentrations of the test materials were used; these were 5 mg/ml crude-peel extract, 0.2 mg/ml genistein and 0.001 mg/ml PZQ. On exposure to these dosages, paralysis in the treated worms occurred at 5.9 h, 6.7 h and 2.9 h, respectively. Synthetic genistein (Sigma, Code no. 6649) was also used. PZQ (Fig. 3) was used as the reference drug.



**Fig. 3.** Praziquantel

For all analyses, therefore, parasites exposed to these lower concentrations of the test materials were used, since it allowed incubation time range between 3 and 6 h. In controls, the parasites survived up to ~ 72 h in PBS. For each set of treatment, the parasites were taken from a single host.

## ***Estimations***

### ***1. Glycogen***

Alkali-soluble glycogen was estimated using anthrone reagent following the method of Seifter et al. (1950). Approximately 500 mg of tissue was taken and digested in 3 ml of 30% KOH at 70<sup>0</sup>C in a water bath. After cooling the solution, 0.2 ml of saturated Na<sub>2</sub>SO<sub>4</sub> was added and mixed thoroughly to assist precipitation of the glycogen. The alkali soluble-glycogen was precipitated out by adding 5 ml of 95% ethanol and centrifuged for 10 min at 10,000 x g. This process was repeated for three times in order to isolate the total glycogen present in the tissue. Glycogen was then estimated using 0.2% anthrone reagent in concentrated H<sub>2</sub>SO<sub>4</sub>. D-glucose released from glycogen under acid condition gets dehydrated in the presence of conc. H<sub>2</sub>SO<sub>4</sub> to yield 5-hydroxymethylfurfural, which gets reduced with anthrone reagent to give a brown coloured complex. The optical density (OD) was measured at 620 nm using a visible spectrophotometer (Systronics). The concentration of glycogen was calculated against a standard graph prepared with different known concentrations of glycogen.

## ***2. Metabolites***

### ***a. Tissue Processing***

A 10% homogenate (w/v) of the treated parasites and controls was prepared in 50 mM Tris-HCl buffer (pH 7.4) using a motor driven Potter-Elvehjem type glass homogenizer fitted with a Teflon pestle. The homogenate was treated with 2 M perchloric acid (PCA) in the ratio of 1:0.5 (homogenate:PCA), followed by centrifugation at 10,000 x g for 10 min to precipitate out proteins and other macromolecules from the tissue. The supernatant was decanted out and neutralised with 2 M NaOH. The resultant supernatant was taken for the estimation of different metabolites of carbohydrate metabolism.

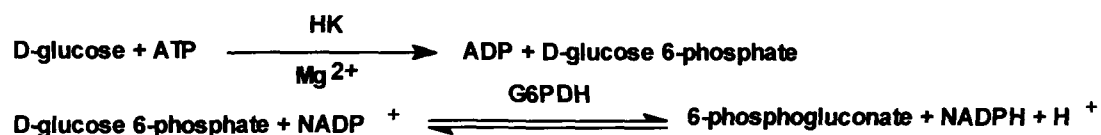
### ***b. Estimations***

Concentrations of the different metabolites of carbohydrate metabolism, viz., glucose, pyruvate, alanine, malate and lactate were estimated enzymatically following the method of Bergmeyer (1974). For all the metabolites, the OD was measured at 340 nm in a UV-Visible spectrophotometer (Beckman, DU 640) using a quartz cuvette of 1.5 ml having 1 cm light pathway.

#### ***Glucose***

For the estimation of glucose, the total glucose was converted to 6-phosphogluconate following two-step enzymatic reactions. In the auxiliary reaction, glucose is phosphorylated by ATP to glucose 6-phosphate in the presence of excess of HK. In the

NADP<sup>+</sup>-dependent indicator reaction, glucose 6-phosphate is oxidized by G6PDH to give 6-phosphogluconate with quantitative reduction of NADP<sup>+</sup> by the following reaction:



The reaction mixture in a final volume of 1 ml contained:

|                     |   |            |
|---------------------|---|------------|
| TRA buffer (pH 7.6) | - | 100 μmoles |
| MgCl <sub>2</sub>   | - | 10 μmoles  |
| NADP <sup>+</sup>   | - | 0.5 μmole  |
| ATP                 | - | 0.9 μmole  |
| G6PDH               | - | 5 units    |
| HK                  | - | 5 units    |

The reaction mixture was incubated at 37°C for 30 min (sufficient enough to convert all the glucose to glucose 6-phosphate). A control was also run simultaneously, which contained the reaction mixture as mentioned above and distilled water in place of the processed sample. The concentration of D-glucose present in different samples was calculated taking the difference in the OD values between the control and the sample and by taking  $6.22 \times 10^6$  as molar extinction coefficient for NADPH.

***Lactate***

Lactate is converted to pyruvate in the presence of LDH along with equimolar reduction of  $\text{NAD}^+$  to NADH by the following reaction:



The reaction mixture in a final volume of 1 ml contained the following:

|                   |   |                      |
|-------------------|---|----------------------|
| Glycine           | - | 50 $\mu\text{moles}$ |
| Hydrazine hydrate | - | 42 $\mu\text{moles}$ |
| $\text{NAD}^+$    | - | 0.9 $\mu\text{mole}$ |
| LDH               | - | 5 units              |

The reaction mixture was incubated at  $37^\circ\text{C}$  for 90 min to convert all the lactate to pyruvate. The control contained distilled water in place of the processed sample. The lactate concentration was calculated from the OD difference between the control and the sample and by taking  $6.22 \times 10^6$  as molar extinction coefficient for NADH.

***Pyruvate***

Pyruvate is converted to lactate in the presence of LDH along with the equimolar oxidation of NADH by the following reaction:



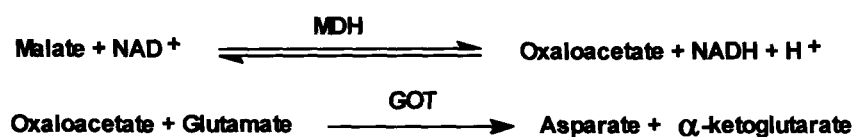
The reaction mixture of 1 ml contained the following:

|                          |   |                 |
|--------------------------|---|-----------------|
| Tris-HCl buffer (pH 7.4) | - | 50 $\mu$ moles  |
| NADH                     | - | 0.2 $\mu$ moles |
| LDH                      | - | 10 units        |

The reaction mixture was incubated at 37°C for 30 min to convert the total pyruvate in the sample to lactate with the maintenance of control, which contained distilled water in place of the sample. The difference in the OD values between the control and the sample was taken for the calculation of pyruvate concentration and by using  $6.22 \times 10^6$  as molar extinction coefficient for NADH.

### ***Malate***

Malate is converted to oxaloacetate in the first reaction with equimolar reduction of  $\text{NAD}^+$  to NADH by the enzyme MDH. In the indicator reaction, the oxaloacetate is converted to  $\alpha$ -ketoglutarate by glutamate oxaloacetate transaminase (GOT) in the presence of glutamate.



The reaction mixture of 1 ml contained the following:

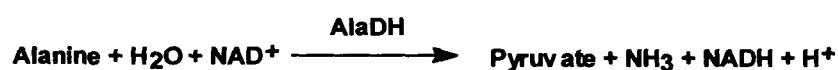
|                          |   |                 |
|--------------------------|---|-----------------|
| Tris-HCl buffer (pH 7.4) | - | 100 $\mu$ moles |
|--------------------------|---|-----------------|

|                  |   |                |
|------------------|---|----------------|
| Glutamate        | - | 1 $\mu$ mole   |
| NAD <sup>+</sup> | - | 0.9 $\mu$ mole |
| MDH              | - | 10 units       |
| GOT              | - | 10 units       |

The reaction mixture was incubated at 37<sup>0</sup>C for 30 min to convert the total malate to oxaloacetate present in the sample with the maintenance of control, which contained distilled water in place of the sample. The malate concentration was calculated taking the difference in OD values between the control and the sample and by taking  $6.22 \times 10^6$  as molar extinction coefficient for NADH.

### *Alanine*

Alanine is converted to pyruvate in the presence of alanine dehydrogenase (AlaDH) along with the equimolar conversion of NAD<sup>+</sup> to NADH by the following reaction:



The reaction mixture for alanine in a final volume of 1 ml contained:

|                                     |   |                 |
|-------------------------------------|---|-----------------|
| 3-amino-1-propanol buffer (pH 10.0) | - | 100 $\mu$ moles |
| NAD <sup>+</sup>                    | - | 0.9 $\mu$ mole  |
| AlaDH                               | - | 10 units        |

The reaction mixture was incubated at 37<sup>0</sup>C for 90 min to convert all the alanine to pyruvate in the sample with the maintenance of control, which contained distilled water in place of sample. The difference in OD values between the control and the sample was taken for the calculation of the alanine concentration and by using  $6.22 \times 10^6$  as molar extinction coefficient for NADH.

## ***Enzyme Assays***

### ***a. Tissue Processing***

For assaying the activities of GPase and GSase, a 10% homogenate (w/v) of the treated worms and controls was prepared in a homogenizing buffer containing 20 mM imidazole-HCl buffer (pH 7.2), 100 mM NaF, 10 mM EDTA, 10 mM EGTA, 15 mM 2-mercaptoethanol and 0.1 mM PMSF (Russel and Storey, 1995). The homogenate was centrifuged at 10,000 x g at 4<sup>0</sup>C for 10 min and the supernatant was used for the enzyme assays.

For assaying the activity of some selected regulatory glycolytic and other enzymes of carbohydrate metabolism, a portion of frozen tissue was thawed in ice and a 10% homogenate (w/v) of each sample was prepared in a homogenizing buffer containing 50 mM Tris-HCl (pH 7.4), 0.3 M sucrose, 1 mM EDTA, 2 mM MgCl<sub>2</sub> and 3 mM 2-mercaptoethanol. The homogenate was treated with 0.5% Triton X-100 in 1:1 ratio for 30 min, followed by sonication for 30 sec using a sonicator (Soniprep 150) for proper breakage of mitochondria. The homogenate was then centrifuged at 10,000 × g for 15

min (Beckman J2-HS) and the resultant supernatant was used for all the enzyme assays. All these steps were carried out at 4<sup>0</sup>C.

### ***b. Sub-cellular Fraction***

Sub-cellular fractionation was carried out following the method of Dkhar et al. (1991). A 20% homogenate of fresh frozen parasites was prepared in a fractionating buffer containing 50 mM Tris-HCl (pH 7.4) buffer, 0.3 M sucrose, 1 mM EDTA, 2 mM MgCl<sub>2</sub> and 3 mM 2-mercaptoethanol. The sub-cellular fractions such as nuclear, mitochondrial and cytosolic (soluble) were separated out by differential centrifugation of the homogenate. The homogenate was centrifuged at 600 × g for 10 min to pellet out the cell debris and nuclei. The supernatant was decanted and kept aside. The loose pellet was again resuspended in equal volume of the fractionation buffer, homogenized for the second time as described previously to aid breaking of unbroken cells and again centrifuged at 600 × g for 10 min. The supernatant was poured out, pooled with the first supernatant and the pellet was taken as nuclear fraction. The pooled supernatant was centrifuged at 10,000 × g for 30 min to give a well defined and firm pellet (mitochondrial fraction). The supernatant so acquired from this centrifugation step was taken as the cytosolic fraction. The nuclear and mitochondrial fractions were resuspended in the same volume of the fractionation buffer. All the fractions as well as a part of the crude homogenate (kept separately before centrifugation) were treated with 0.5% Triton X-100 in 1:1 ratio for 30 min. The crude homogenate and the mitochondrial fractions were sonicated for 30 sec for proper breakage of mitochondria. LDH was used as the cytosolic marker and glutamate dehydrogenase (GDH) enzyme was used as the

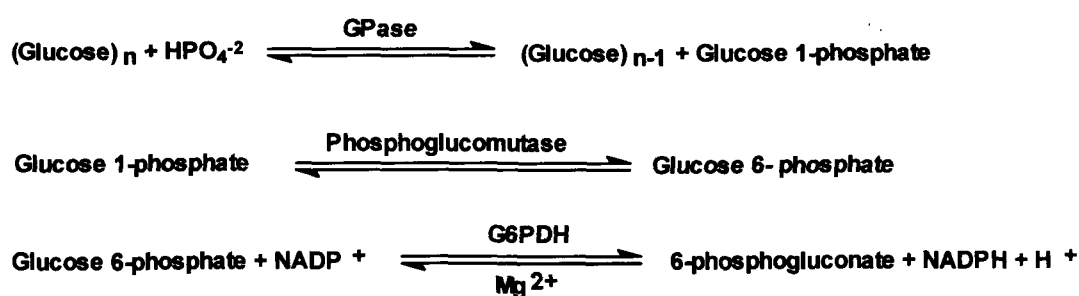
mitochondrial marker to access the complete separation process of different sub-cellular fractions. All the steps were carried out at 4<sup>0</sup>C.

### *c. Assays*

For all the enzyme assays, the OD readings were taken in a quartz cuvette of 1.5 ml volume having 1 cm light pathway, using a UV-Visible spectrophotometer (Beckman, DU 640) fitted with a peltier temperature controlled system.

#### ***Glycogen Phosphorylase (GPase; EC 2.4.1.1)***

The GPase (1,4- $\alpha$ -D-Glucan: orthophosphate  $\alpha$ -D-glucosyltransferase) activity was assayed following the method of Moon et al. (1989). Glucose 1-phosphate released by the action of GPase from glycogen is converted to glucose 6-phosphate by the enzyme phosphoglucomutase. In the indicator reaction, glucose 6-phosphate is oxidised to 6-phosphogluconate by G6PDH with simultaneous reduction of NADP<sup>+</sup> by the following equation:



The assay mixture in a final volume of 1 ml contained:

|                                     |   |                 |
|-------------------------------------|---|-----------------|
| Potassium phosphate buffer (pH 7.2) | - | 60 $\mu$ moles  |
| NADP <sup>+</sup>                   | - | 0.5 $\mu$ mole  |
| Glucose 1,6-bisphosphate            | - | 5 $\mu$ moles   |
| AMP                                 | - | 2.5 $\mu$ moles |
| Phosphoglucomutase                  | - | 5 units         |
| G6PDH                               | - | 5 units         |
| Glycogen                            | - | 10 mg           |
| Tissue extract                      | - | 100 $\mu$ l     |

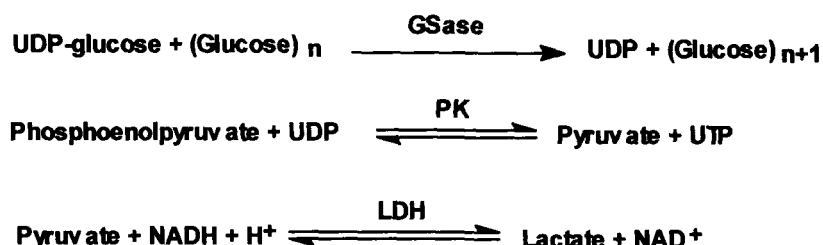
The reaction mixture also contained 10  $\mu$ moles caffeine (1,3,7-trimethylxanthine, cAMP phosphodiesterase inhibitor) for measurement of GPase *a*, and no caffeine for measurement of total GPase (*a* + *b*) activity.

The assay mixture without the tissue extract was preincubated at 38<sup>0</sup>C for 5 min. The tissue extract was added to the preincubated reaction mixture in order to start the reaction. The increase in OD was recorded at 340 nm at 30 sec interval for 10 min and the linear increase in OD values was used for calculating the GPase activity. The amount of NADP<sup>+</sup> reduced was calculated taking  $6.22 \times 10^6$  as molar extinction coefficient value for NADPH for expressing the GPase activity. One unit of GPase activity was expressed as that amount of enzyme which catalyzed the reduction of 1  $\mu$ mole of NADP<sup>+</sup> to NADPH per min at 38<sup>0</sup>C.

Percent GPase *a* represents the ratio of GPase *a* to total GPase x 100.

***Glycogen Synthase (GSase; EC 2.4.1.11)***

GSase (UDP glucose: glycogen 4- $\alpha$ -glucosyltransferase) was assayed following the method of Passoneau and Rottenberg (1973). The UDP released after incorporation of UDP-glucose to the primed glycogen is converted to UTP by PK with conversion of phosphoenolpyruvate to pyruvate. In the indicator reaction, pyruvate is converted to lactate with equimolar oxidation of NADH by LDH as depicted in the following reaction:



The assay mixture in a final volume of 1 ml contained:

|                               |   |                 |
|-------------------------------|---|-----------------|
| Imidazole-HCl buffer (pH 7.5) | - | 60 $\mu$ moles  |
| Phosphoenolpyruvate           | - | 5 $\mu$ moles   |
| UDP-glucose                   | - | 6 $\mu$ moles   |
| NADH                          | - | 0.15 $\mu$ mole |
| KCl                           | - | 150 $\mu$ moles |
| MgCl <sub>2</sub>             | - | 15 $\mu$ moles  |
| Glycogen                      | - | 2 mg            |
| PK                            | - | 10 units        |

|                |   |             |
|----------------|---|-------------|
| LDH            | - | 10 units    |
| Tissue extract | - | 100 $\mu$ l |

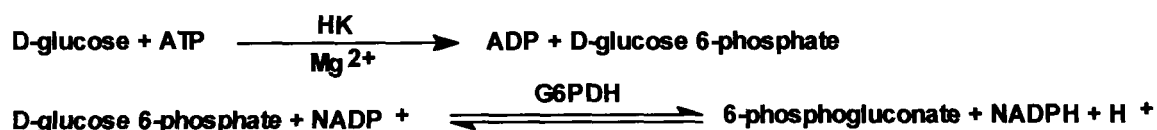
The reaction mixture also contained 5  $\mu$ moles glucose 6-phosphate while measuring the total GSase ( $a + b$ ) activity.

The tissue extract was added to the preincubated assay mixture at 38<sup>0</sup>C for 5 min. The reaction was started by the addition of the tissue extract and the decrease in OD was recorded at 340 nm at 30 sec interval for 10 min and the linear decrease in OD values was taken for calculating the GSase activity. The amount of NADH oxidized was calculated taking  $6.22 \times 10^6$  as molar extinction coefficient value for NADH for expressing the GSase activity. One unit of GSase activity was expressed as that amount of enzyme which oxidized 1  $\mu$ mole of NADH to NAD<sup>+</sup> per min at 38<sup>0</sup>C.

Percent GSase  $a$  represents the ratio of GSase  $a$  to total GSase x 100.

#### ***Hexokinase (HK; EC 2.7.1.1)***

The HK (ATP: D-hexose 6-phosphotransferase) activity was measured following the method of Bergmeyer (1974) which is a two-step enzymatic reaction. In the auxiliary reaction, D-glucose is phosphorylated to D-glucose 6-phosphate in the presence of HK and in the indicator reaction glucose 6-phosphate is oxidized by G6PDH to give 6-phosphogluconate with simultaneous equimolar reduction of NADP<sup>+</sup> to NADPH by the following reaction:



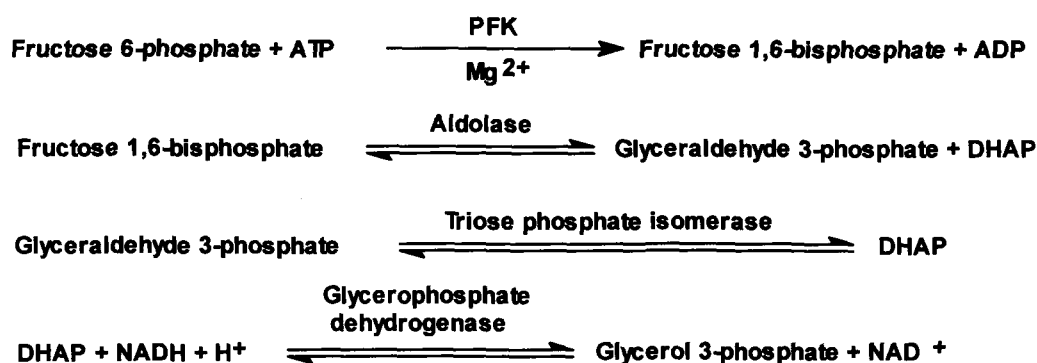
The reaction mixture in a final volume of 1 ml contained:

|                         |   |                |
|-------------------------|---|----------------|
| Tri-HCl buffer (pH 7.4) | - | 50 $\mu$ moles |
| D-glucose               | - | 5 $\mu$ moles  |
| NADP <sup>+</sup>       | - | 0.2 $\mu$ mole |
| ATP                     | - | 0.9 $\mu$ mole |
| MgCl <sub>2</sub>       | - | 5 $\mu$ moles  |
| G6PDH                   | - | 10 units.      |
| Tissue extract          | - | 50 $\mu$ l     |

The assay mixture was preincubated without the tissue extract at 38<sup>0</sup>C for 5 min. The tissue extract was added to the assay mixture in order to start the reaction. The increase in OD was recorded at 340 nm at 30 sec interval for 10 min and the linear increase in OD values was taken for calculating the HK activity. The amount of NADP<sup>+</sup> reduced was calculated taking 6.22  $\times 10^6$  as molar extinction coefficient value for NADPH for expressing the HK activity. One unit of HK activity was expressed as that amount of enzyme which catalyzed the reduction of one  $\mu$ mole of NADP<sup>+</sup> per min at 38<sup>0</sup>C.

**Phosphofructokinase (PFK; EC 2.7.1.11)**

PFK (ATP: D-fructose 6-phosphate 1-phosphotransferase) was assayed by the modified method of Buckwitz et al. (1988). The four-step coupled reaction involves in the first place the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate by PFK, in the second auxiliary reaction the latter is cleaved to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DHAP) in presence of aldolase. In the next step glyceraldehyde 3-phosphate is reversibly converted to DHAP by the enzyme triose phosphate isomerase. In the indicator reaction DHAP is converted to glycerol 3-phosphate by glycerophosphate dehydrogenase with quantitative oxidation of NADH as depicted in the following reaction:



The reaction mixture in a final volume of 1 ml contained:

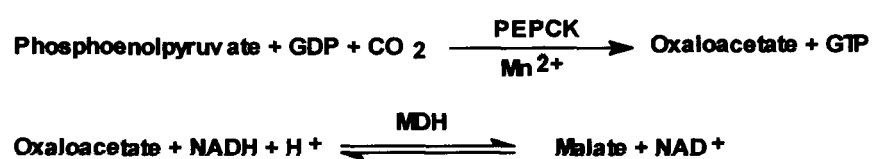
|                      |   |                |
|----------------------|---|----------------|
| Tris-HCl (pH 7.2)    | - | 80 $\mu$ moles |
| Fructose 6-phosphate | - | 5 $\mu$ moles  |
| NADH                 | - | 0.2 $\mu$ mole |
| ATP                  | - | 0.8 $\mu$ mole |
| MgCl <sub>2</sub>    | - | 0.9 $\mu$ mole |

|                                 |   |                |
|---------------------------------|---|----------------|
| KCl                             | - | 90 $\mu$ moles |
| K <sub>2</sub> HPO <sub>4</sub> | - | 1 $\mu$ mole   |
| Aldolase                        | - | 8.0 $\mu$ g    |
| Triose phosphate isomerase      | - | 3.3 $\mu$ g    |
| Glycerophosphate dehydrogenase  | - | 3.3 $\mu$ g    |
| Tissue extract                  | - | 100 $\mu$ l    |

The assay mixture without the tissue extract was preincubated at 38<sup>0</sup>C for 5 min. The reaction was started by the addition of the tissue extract to the preincubated reaction mixture. The decrease in OD was recorded at 340 nm at 30 sec interval for 10 min. and the linear decrease in OD values was taken for calculating the PFK activity, using  $6.22 \times 10^6$  as molar extinction coefficient value for NADH. One unit of PFK activity was expressed as that amount of enzyme which catalyzed the oxidation of 1 $\mu$ mole of NADH per min at 38<sup>0</sup>C.

### ***Phosphoenolpyruvate Carboxykinase (PEPCK; EC 4.1.1.32)***

PEPCK (Orthophosphate: oxaloacetate carboxykinase) was assayed by the method described by Mommsen et al. (1985). Oxaloacetate so formed by the enzyme PEPCK is further converted to malate in the presence of excess of MDH where equimolar amount of NADH is oxidized to NAD<sup>+</sup> in the following reaction:



The reaction mixture in a final volume of 1 ml contained:

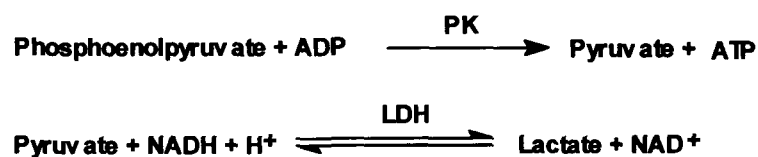
|                          |   |                 |
|--------------------------|---|-----------------|
| Tris-HCl buffer (pH 7.4) | - | 50 $\mu$ moles  |
| Phosphoenolpyruvate      | - | 4.5 $\mu$ moles |
| NADH                     | - | 0.15 $\mu$ mole |
| GDP                      | - | 0.6 $\mu$ mole  |
| NaHCO <sub>3</sub>       | - | 20 $\mu$ moles  |
| MnCl <sub>2</sub>        | - | 1 $\mu$ mole    |
| MDH                      | - | 5 units         |
| Tissue extract           | - | 20 $\mu$ l      |

The assay mixture without the tissue extract was preincubated for 5 min at 38<sup>0</sup>C. The reaction was started by the addition of the tissue extract to the preincubated reaction mixture and the decrease in OD was noted at 340 nm at 30 sec interval for 10 min. The linear decrease in OD values was considered for calculating the PEPCK activity taking  $6.22 \times 10^6$  as molar extinction coefficient value for NADH. One unit of PEPCK activity was expressed as the amount of enzyme that catalyzed the oxidation of 1  $\mu$ mole of NADH per min at 38<sup>0</sup>C.

***Pyruvate Kinase (PK; EC 2.7.1.40)***

The PK (ATP: pyruvate 2-0-phosphotransferase) activity was assayed following the method of Bücher and Pfeleiderer (1955). This is a two-step reaction where phosphoenolpyruvate is converted to pyruvate by PK in the auxiliary reaction and then

pyruvate gets converted to lactate in the presence of LDH, with equimolar oxidation of NADH by the following reaction:



The reaction mixture in a final volume of 1 ml contained:

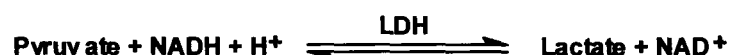
|                           |   |                 |
|---------------------------|---|-----------------|
| Imidazole buffer (pH 7.6) | - | 50 $\mu$ moles  |
| Phosphoenolpyruvate       | - | 5 $\mu$ moles   |
| NADH                      | - | 0.2 $\mu$ mole  |
| ADP                       | - | 1.5 $\mu$ moles |
| KCl                       | - | 120 $\mu$ moles |
| MgSO <sub>4</sub>         | - | 30 $\mu$ moles  |
| LDH                       | - | 10 units        |
| Tissue extract            | - | 50 $\mu$ l      |

The assay mixture without the tissue extract was preincubated at 38<sup>0</sup>C for 5 min. The reaction was started by the addition of the tissue extract to the preincubated reaction mixture and the decrease in OD was recorded at 340 nm at 30 sec interval for 10 min. The linear decrease in OD values was considered for calculating the PK activity taking  $6.22 \times 10^6$  as molar extinction coefficient for NADH. One unit of PK activity was

expressed as that amount of enzyme that catalyzed the oxidation of 1  $\mu$ mole of NADH per min at 38<sup>0</sup>C.

***Lactate Dehydrogenase (LDH; EC 1.1.1.27)***

The LDH (L-Lactate: NAD<sup>+</sup> oxidoreductase) activity was determined following the method of Vorhaben and Campbell (1972). This is a bimolecular reaction where pyruvate is converted to lactate by LDH with quantitative oxidation of NADH by the following reaction:



The reaction mixture in a final volume of 1 ml contained:

|                                  |   |                |
|----------------------------------|---|----------------|
| Sodium phosphate buffer (pH 7.4) | - | 30 $\mu$ moles |
| Pyruvate                         | - | 5 $\mu$ moles  |
| NADH                             | - | 0.2 $\mu$ mole |
| Tissue extract                   | - | 50 $\mu$ l     |

The assay mixture without the tissue extract was preincubated for 5 min at 38<sup>0</sup>C min. The tissue extract was added to the preincubated reaction mixture in order to start the reaction. The decrease in OD was recorded at 340 nm at 30 sec interval for 10 min and the linear decrease in OD values was taken for calculating the LDH activity taking  $6.22 \times 10^6$  as a molar extinction coefficient value for NADH. One unit of LDH activity

was expressed as that amount of enzyme which catalyzed the oxidation of 1  $\mu$ mole of NADH to NAD<sup>+</sup> per min at 38<sup>0</sup>C.

***Malate Dehydrogenase (MDH; EC 1.1.1.37)***

The MDH (L-malate: NAD<sup>+</sup> oxidoreductase) activity was assayed following the method of Kun and Volfin (1966). This is a second order reaction where oxaloacetate is converted to malate by the enzyme with equimolar oxidation of NADH by the following reaction:



The reaction mixture in a final volume of 1 ml contained:

|                                  |   |                 |
|----------------------------------|---|-----------------|
| Sodium phosphate buffer (pH 7.4) | - | 100 $\mu$ moles |
| Oxaloacetate                     | - | 6 $\mu$ moles   |
| NADH                             | - | 0.2 $\mu$ mole  |
| Tissue extract                   | - | 50 $\mu$ l      |

The assay mixture without the tissue extract of the respective (total, cytosolic and mitochondrial) fraction was preincubated at 38<sup>0</sup>C for 5 min. The reaction was started by addition of the tissue extract of different fractions to the preincubated reaction mixture. The decrease in OD was noted at 340 nm at 30 sec interval for 10 min and the linear decrease in OD values was used for calculating the MDH activity taking  $6.22 \times 10^6$  as

molar extinction coefficient value for NADH. One unit of MDH activity was expressed as that amount of enzyme which catalyzed the oxidation of 1  $\mu$ mole of NADH per min at 38<sup>0</sup>C.

**Malic Enzyme (ME; EC 1.1.1.40)**

The ME (L-malate: NADP<sup>+</sup> oxidoreductase) activity was assayed following the method of Bergmeyer (1974). This is a single-step reaction where pyruvate is converted to malate by ME with quantitative oxidation of NADPH by the following reaction:



The reaction mixture in a final volume of 1 ml contained:

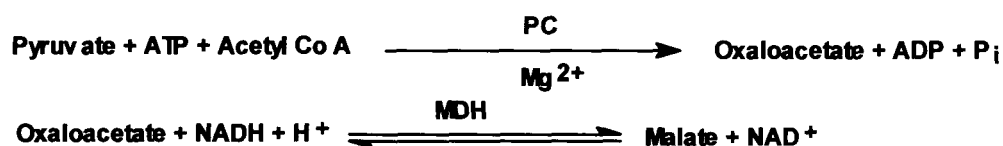
|                          |   |                |
|--------------------------|---|----------------|
| Tris-HCl buffer (pH 7.4) | - | 50 $\mu$ moles |
| Pyruvate                 | - | 5 $\mu$ moles  |
| NADPH                    | - | 0.2 $\mu$ mole |
| NaHCO <sub>3</sub>       | - | 5 $\mu$ moles  |
| Tissue extract           | - | 50 $\mu$ l     |

The assay mixture without the tissue extract (respective fractions) was preincubated at 38<sup>0</sup>C for 5 min. The reaction was started by the addition of the respective fraction of tissue extract to the preincubated reaction mixture. The decrease in OD was recorded at 340 nm at 30 sec interval for 10 min and the linear decrease in OD values was used for calculating the ME activity taking 6.22  $\times 10^6$  as molar extinction

coefficient value for NADPH. One unit of ME activity was expressed as that amount of enzyme which catalyzed the oxidation of 1  $\mu$ mole of NADPH per min at 38<sup>0</sup>C.

***Pyruvate Carboxylase (PC; EC 6.4.1.1)***

PC (Pyruvate: CO<sub>2</sub> ligase) was assayed following the method of Moon and Mommsen (1987) with a two-step enzymatic reaction. Oxaloacetate so formed by PC is further converted to malate in the presence of excess MDH, where equimolar amount of NADH is oxidized by the following reaction:



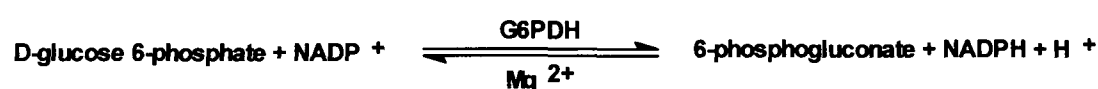
The reaction mixture in a final volume of 1 ml contained:

|                          |   |                 |
|--------------------------|---|-----------------|
| Tris-HCl buffer (pH 7.8) | - | 100 $\mu$ moles |
| Acetyl CoA               | - | 0.1 $\mu$ mole  |
| NaHCO <sub>3</sub>       | - | 15 $\mu$ moles  |
| Pyruvate                 | - | 10 $\mu$ moles  |
| NADH                     | - | 0.15 $\mu$ mole |
| MgCl <sub>2</sub>        | - | 5 $\mu$ moles   |
| MDH                      | - | 10 units        |
| Tissue extract           | - | 20 $\mu$ l      |

The tissue extract was added to the assay mixture that was preincubated at 38<sup>0</sup>C for 5 min. The decrease in OD was noted at 340 nm at 30 sec interval for 10 min and the linear decrease in OD values was taken for expressing the PC activity taking  $6.22 \times 10^6$  as molar extinction coefficient value for NADH. One unit of PC activity was expressed as that amount of enzyme which catalyzed the oxidation of 1  $\mu$ mole of NADH per min at 38<sup>0</sup>C.

***Glucose 6-phosphate Dehydrogenase (G6PDH; EC 1.1.1.49)***

The G6PDH activity was measured following the method of DeMoss (1955). D-glucose 6-phosphate is oxidised to 6-phosphogluconate by G6PDH with simultaneous equimolar reduction of NADP<sup>+</sup> to NADPH by the following reaction:



The reaction mixture in a final 1 ml contained:

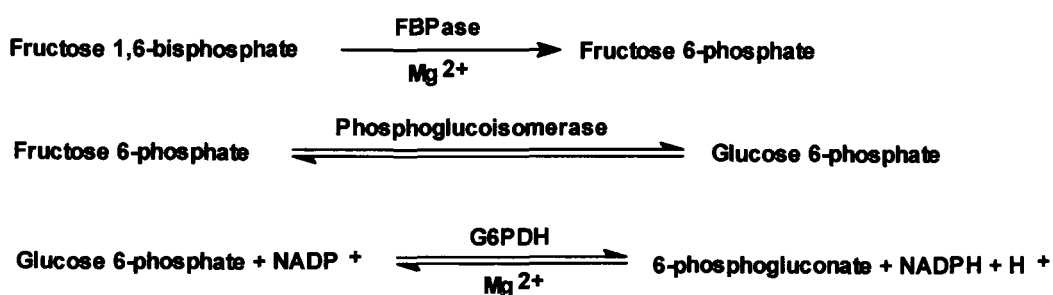
|                          |   |                |
|--------------------------|---|----------------|
| Tris-HCl buffer (pH 7.4) | - | 50 $\mu$ moles |
| MgCl <sub>2</sub>        | - | 10 $\mu$ moles |
| NADP <sup>+</sup>        | - | 0.2 $\mu$ mole |
| D-glucose 6-phosphate    | - | 5 $\mu$ moles  |
| Tissue extract           | - | 50 $\mu$ l     |

The assay mixture was preincubated without the tissue extract at 38<sup>0</sup>C for 5 min. The tissue extract was added to the assay mixture in order to start the reaction. The

increase in OD was recorded at 340 nm at 30 sec interval for 10 min and the linear increase in OD values was taken for calculating the G6PDH activity. The amount of NADP<sup>+</sup> reduced was calculated taking  $6.22 \times 10^6$  as molar extinction coefficient value for NADPH for expressing the G6PDH activity. One unit of G6PDH activity was expressed as that amount of enzyme which catalyzed the reduction of 1  $\mu$ mole of NADP<sup>+</sup> per min at 38<sup>0</sup>C.

***Fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11)***

FBPase (D-fructose 1,6-bisphosphate 1-phosphohydrolase) was assayed by the method described by Mommsen et al. (1985) with a three-step enzymatic reaction. Fructose 6-phosphate so formed by FBPase is further converted to glucose 6-phosphate in the presence of excess of phosphoglucosomerase. In the NADP<sup>+</sup>-dependent indicator reaction glucose 6-phosphate is oxidised to 6-phosphogluconate in the presence of G6PDH with quantitative reduction of NADP<sup>+</sup> to NADPH by the following reaction:



The reaction mixture in a final volume of 1 ml contained:

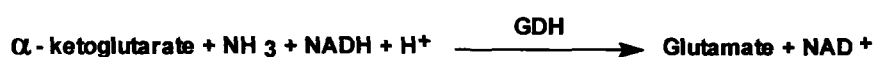
|                          |   |                 |
|--------------------------|---|-----------------|
| Tris-HCl buffer (pH 7.4) | - | 50 $\mu$ moles  |
| F 1,6-bisphosphate       | - | 0.15 $\mu$ mole |

|                          |   |               |
|--------------------------|---|---------------|
| NADP <sup>+</sup>        | - | 0.2 μmole     |
| MgCl <sub>2</sub>        | - | 15 μmoles     |
| G6PDH                    | - | 10 units      |
| Phosphoglucose isomerase | - | 400-600 units |
| Tissue extract           | - | 50 μl.        |

The tissue extract was added to the previously preincubated reaction mixture at 38<sup>0</sup>C for 5 min. The increase in OD was recorded at 340 nm at 30 sec interval for 10 min and the linear increase in OD values was used for calculating the FBPase activity taking  $6.22 \times 10^6$  as molar extinction coefficient value for NADPH. One unit of FBPase activity was expressed as that amount of enzyme which catalyzed the reduction of 1 μmole of NADP<sup>+</sup> to NADPH per min at 38<sup>0</sup>C.

### ***Glutamate Dehydrogenase (GDH; EC 1.4.1.3)***

GDH (L-Glutamate: NAD(P)<sup>+</sup> oxidoreductase) was assayed following the method of Olson and Anfinsen (1952) with certain modifications by Das et al. (1991). α-ketoglutarate is converted to glutamate by GDH with quantitative oxidation of NADH by the following reaction:



The reaction mixture in a final volume of 1 ml contained:

|                                     |   |                 |
|-------------------------------------|---|-----------------|
| Potassium phosphate buffer (pH 8.5) | - | 100 $\mu$ moles |
| Ammonium chloride                   | - | 50 $\mu$ moles  |
| $\alpha$ -ketoglutarate             | - | 25 $\mu$ moles  |
| NADH                                | - | 0.2 $\mu$ mole  |
| EDTA                                | - | 0.2 $\mu$ mole  |
| Tissue extract                      | - | 50 $\mu$ l      |

The tissue extract was added to the assay mixture preincubated at 38<sup>0</sup>C for 5 min. The decrease in O.D was recorded at 340 nm at 30 sec interval for 10 min and the linear decrease in O.D values was taken for expressing the GDH activity taking  $6.22 \times 10^6$  as molar extinction coefficient value for NADH. One unit of GDH activity was expressed as that amount of enzyme which catalyzed the oxidation of 1  $\mu$ mole of NADH per min at 38<sup>0</sup>C.

#### ***d. Protein Estimation***

Protein was estimated following the method of Lowry et al. (1951) using bovine serum albumin as the standard.

#### ***e. Specific Activity***

Specific activity of the enzymes was expressed as the units of enzyme activity per mg protein:

### ***Measurement of Trace Elements***

The parasite fresh tissue (~ 2 g) was dried using a lyophilizer (Heto Lyolab 3000). The dried tissue was ground using a mortar and pestle and approximately 500 mg of the powdered sample was digested in 10 ml of concentrated HNO<sub>3</sub> for 12 h at 50<sup>0</sup>C and then kept for 1-2 h on a hot plate for complete evaporation of the acid, followed by addition of 10 ml deionised double distilled water. The solution was filtered and the volume was finally made to 100 ml by adding deionised double distilled water. The solution was used for the analysis of Ca<sup>2+</sup> and other trace elements using Perkin Elemer atomic absorption spectrophotometer (Model 3110).

The incubation media were also collected after taking out the treated parasites and parasites in controls. The solutions were centrifuged at 600 x g for 20 min and the supernatant of the respective incubation medium was taken for the analysis of effluxed Ca<sup>2+</sup>.

### ***Histochemistry***

As a visual confirmation of the biochemical analysis, histochemical methods were also used for demonstration of some parameters.

#### ***a. Glycogen***

Glycogen was histochemically demonstrated following the Best's method (1906) as described by Pearse (1968). Paraffin sections (6-7 μm thick) of the bouin-fixed

material were brought to absolute alcohol and placed for 2 min in 1% celloidin. The dried sections were passed through alcohol gradation to water and stained in Ehrlich's haemalum for 5 min. These sections were rinsed and differentiated rapidly in 1% acid alcohol and then rinsed in water. After staining in Best's carmine [15 ml of stock solution (2 g carmine, 1 g  $\text{KCO}_3$ , 5 g KCl and 20 ml ammonia to 60 ml distilled water) with 12.5 ml of ammonia and 12.5 ml of methyl alcohol] for 30 min, the sections were differentiated in Best's differentiator (8 ml absolute alcohol, 4 ml methyl alcohol and 10 ml distilled water) without previous rinsing. The sections were washed in 80% alcohol, dehydrated in absolute alcohol, cleared in xylene and mounted in DPX.

***b. Hexokinase (HK)***

HK was demonstrated histochemically following the method of Meijer (1967) as described in Pearse (1972). Fresh frozen cryostat sections (15  $\mu\text{m}$ ) were fixed by drying and incubated at 38<sup>0</sup>C for 2 h. The incubation medium contained 30 mg D-glucose, 2.5 mg  $\text{NADP}^+$ , 5.5 mg ATP, 20 mg  $\text{MgCl}_2$ , 2.5 mg NBT, 2 ml 40 mM imidazole buffer (pH 7.5), 3.8 ml 6% gelatin and 5  $\mu\text{l}$  G6PDH per 10 ml of incubation mixture. The sections were washed briefly and fixed for 30 min in 4% neutral buffered formaldehyde and mounted in glycerine jelly. Dark blue formazan granules, formed by the reduction of NBT by NADPH formed in the reaction, indicate the sites of activity of the enzyme.

***c. Lactate Dehydrogenase (LDH)***

LDH was demonstrated following the method of Jacobsen (1969) as described by Pearse (1972). Fresh frozen cryostat sections (15  $\mu\text{m}$ ) were fixed for 10 min in cold

acetone (0-4 °C), followed by treatment with CHCl<sub>3</sub> at -15°C for 10 min. The sections were washed in cold acetone and dried in air followed by incubation in final incubation medium for 2 h. The final incubation medium contained equal quantities of solution A and solution B and 1.5 mM NAD<sup>+</sup> (added just before incubation). [Solution A contained 200 mM L-lactate, 9.8 mM NBT, 10 mM NaCN, 0.66 mM PMS, 33 g PVA in 100 ml of 0.1 M Tris buffer (pH 7.2), whereas solution B contained 40 g PVA in 100 ml of 50 mM Tris buffer (pH 7.2)]. The sections were washed in warm water (45°C) and fixed in neutral formol-calcium for 15 min. Monoformazans were removed by brief treatment with acetone and the sections, mounted in glycerine jelly. Dark blue formazan granules, formed by the reduction of NBT by NADH formed in the reaction, indicate sites of the enzyme activity.

#### ***d. Malate Dehydrogenase***

MDH was demonstrated by following the procedure described by Pearse (1972). 15 µm thick cryostat sections were immersed in incubation medium and incubated aerobically for 1 h. Each 10 ml of incubation medium contained 2 mg NAD<sup>+</sup>, 0.1 ml of stock substrate solution and 0.9 ml of stock incubating medium. [10 ml of stock substrate solution contained 1 M L-malate, which was neutralized by 0.9 ml of 40% NaOH. The stock incubating medium (9 ml) contained 2.5 ml of NBT (4 mg/ml), 2.5 ml 0.2 M Tris-HCl buffer (pH 7.4), 1 ml of 5 mM MgCl<sub>2</sub> and 3 ml distilled water]. The sections were then immersed in 15% formol saline for 15 min. After washing in running water for 2 min, the sections were dehydrated in graded alcohol, cleared in xylene and

mounted in DPX. Dark blue formazan granules, formed by the reduction of NBT by NADH formed in the reaction, indicate sites of the MDH activity.

***e. Glucose 6-phosphate Dehydrogenase (G6PDH)***

The activity of G6PDH was demonstrated following the procedure described by Pearse (1972). The cryostat sections (15  $\mu\text{m}$  thick) were covered with sufficient amount of incubation medium and incubated aerobically for 1 h. 10 ml of incubation medium contained 2 mg  $\text{NADP}^+$ , 0.1 ml of stock substrate solution, 0.1 ml of 0.1 M NaCN and 0.9 ml of stock incubating medium. [10 ml of stock substrate solution contained 1 M glucose 6-phosphate which is neutralized by 0.6 ml of 1N HCl. The stock incubating medium (9 ml) contained 2.5 ml of NBT (4 mg/ml), 2.5 ml of 0.2 M Tris-HCl buffer (pH 7.4), 1 ml of 5 mM  $\text{MgCl}_2$  and 3 ml distilled water]. The sections were immersed in 15% formol saline for 15 min. After washing in running water for 2 min, the sections were dehydrated in graded alcohol, cleared in xylene and mounted in DPX. Dark blue formazan granules, formed by the reduction of NBT by NADPH formed in the reaction, indicate sites of activity of the enzyme.

***Chemicals***

Synthetic genistein was obtained from Sigma Chemicals (St. Louis, USA). Praziquantel (PZQ (2-[cyclohexylcarbonyl]-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1a]isoquinolin-4-one), Droncit), a broad-spectrum cestocide, was from Bayer. All enzymes and co-enzymes were obtained either from Sigma Chemicals, USA or from Roche,

Germany. Other chemicals used were of analytical grade and obtained from local sources such as Sisco Research Laboratory, E-Merck etc. Deionized double glass-distilled water was used for all preparations.

### ***Statistical analysis***

Data collected from three to five replicates were statistically analyzed and are presented as mean  $\pm$  SEM. Comparisons of the paired mean values between the experimental and respective controls were made using Student's *t*-test (Croxtton et al., 1982) and  $P < 0.05$  was regarded as statistically significant.

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## RESULTS AND OBSERVATIONS

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The cestode, *Raillietina echinobothrida*, could survive very well at  $39 \pm 1^{\circ}\text{C}$  in 0.9% PBS (pH 7.2) containing 1% DMSO for  $72 \pm 0.05$  h. Following treatment with crude-peel extract of *Flemingia vestita* at a concentration of 5 mg/ml, paralysis in the cestode occurred at  $5.9 \pm 0.05$  h. When the cestode was treated with genistein at a concentration of 0.2 mg/ml, paralysis set in at  $6.7 \pm 0.04$  h, whereas in the medium containing PZQ (1  $\mu\text{g}/\text{ml}$ ), the reference drug paralysis ensued after  $2.9 \pm 0.05$  h (Table 1).

### ***Glycogen and its Metabolism***

Table 2 and Fig. 4 show the glycogen content in the control and treated cestode (*R. echinobothrida*). The physiological level of glycogen in the control parasite was found to be quite high and averaging to be about 66 mg/g wet wt, which decreased significantly from 12 h onwards during post incubation period as control. The glycogen level in the parasite decreased significantly compared to the respective controls at different hours of treatment with crude-peel extract, genistein and PZQ. The maximum percentage decrease of glycogen was recorded to be 44, 35 and 28% after treating with crude-peel extract, genistein and PZQ, respectively, either after 12 h or 6 h of incubation. This decrease of glycogen content in treated cestode was supported by histochemical studies

also (Plate 2; Figs. a-d); the intensity of colour, indicating the glycogen concentration, decreased significantly in all the treatments compared to respective controls.

Table 3 and Figs. 5 and 6 show the effects of crude-peel extract, genistein and PZQ on the activities of GPase (both active and total) and GSase (both active and total), the two key enzymes related to glycogen metabolism. All the three treatments caused significant changes in the activities of only the active forms of GPase and GSase, with no significant effect on the total activity of either enzyme. GPase *a*, which constituted about 72-74% of the total GPase activity in the control parasite, increased significantly by 29, 39 and 30% in the parasite treated with crude-peel extract, genistein for 6 h and PZQ for 3 h, respectively. However, GSase *a* activity, which constituted about 56-68% of the total GSase activity, decreased significantly by 59, 36 and 38%, respectively, in similar treatments.

Table 4 and Figs. 5 and 6 show the changes of specific activities of GPase and GSase in the parasite under different treatments. The pattern of changes of specific activity was almost similar to that of the tissue activity. All the treatments did not affect the total specific activity ( $a + b$ ) of both GPase and GSase enzymes except for the active form of both. The specific activity of GPase *a*, which constituted about 71-76% of the total specific activity, increased significantly by 28, 39 and 30%, respectively, during treatments with crude-peel extract, genistein and PZQ. Whereas in the case of GSase *a*, which constituted about 53-68% of the total specific activity, decreased significantly by

The changes in the efflux of lactate by the treated parasites are depicted in Table 7 and Fig. 9. There was a significant increase of lactate efflux of about 44 and 23%, respectively, by the parasites treated with the crude-peel extract and PZQ, whereas there was only 9% increase (not significant) of lactate efflux by the genistein-treated parasite. The net lactate production by the parasite both under treated and untreated condition can be calculated by subtracting the net decrease of tissue lactate level from the total lactate excreted by the parasite in the incubation medium at paralysis time. There was a significant increase in the net lactate production of about 20 and 16% by the parasite treated with the crude-peel extract and PZQ, respectively. No significant change in the pH of the incubation medium was observed in any of the treated conditions (Table 7).

Table 8 and Figs. 10-12 show the effects of the crude-peel extract of *F. vestita*, genistein and PZQ on tissue activities of some selected enzymes of glycolysis, namely HK, PFK, PEPCK, PK and LDH in *R. echinobothrida*. As such the physiological level of activity of PEPCK in the parasite was found to be highest, averaging to be about 18.85 units/g wet wt, followed by LDH (4.53 units/g wet wt), HK (1.68 units/g wet wt) and PFK (1.52 units/g wet wt). The activity of PEPCK in the parasite increased significantly by 49, 45 and 44%, respectively, in treatments with crude-peel extract, genistein and PZQ. Whereas in the case of PK, a significant decrease in the activity (of about 26%) was observed when the parasite was treated with crude-peel extract, only a little decrease of activity (of about 15%, non-significant) was observed while treating with genistein and PZQ. The tissue activity of HK in the parasite increased significantly

under all the three treated conditions. The percentage increase of HK activity was 33, 39 and 36 respectively, compared to controls during treatment with crude-peel extract, genistein and PZQ. The change of HK activity was further supported by histochemical demonstration (Plate 3; Figs. a-f). The intensity of colour of formazan particles, which indicated the activity of the enzyme, increased significantly in all the treatments in respect to the control. The activity of PFK which is the pace maker of the glycolytic pathway, increased by 125% in PZQ treatment, and by 49 and 41% in genistein and crude-peel extract treatments, respectively, in comparison to the respective controls. The activity of LDH increased by 67% in genistein treatment, and by 55 and 58% in treatments with crude-peel extract and PZQ, respectively. Histochemically also, alteration in the LDH activity under the influence of the various treatments was demonstrable; the activity of the enzyme increased significantly in all the treatments in respect to the control (Plate 4; Figs. a-f).

Table 9 and Figs. 10-12 show the changes of specific activity of HK, PFK, PK, PEPCK and LDH in the parasite under different treatments. The pattern of these changes was almost similar to that of the tissue activity. The specific activity of HK increased maximally by 41% while treating with genistein, and by 34% both in the crude-peel extract and PZQ treatments. In the case of PFK, the specific activity increased maximally by 127% while treating with PZQ, and 43 and 49%, respectively, during treatment with crude-peel extract and genistein. In the case of PEPCK, the specific activity increased maximally by 52% while treating with crude-peel extract, and 46 and

42%, respectively, during treatment with genistein and PZQ. Like the tissue activity, the specific activity of PK decreased by 23, 14 and 16%, in crude-peel extract, genistein and PZQ treatments, respectively; whereas the specific activity of LDH increased maximally by 68% in genistein-treated and 60 and 58% in the case of crude-peel extract and PZQ treated parasites, respectively.

The MDH activity in *R. echinobothrida* was found to be localized both mitochondria and cytosol with 40% of the activity in the former and 50% in the later (Table 10; Fig. 13). Both the mitochondrial and cytosolic MDH were affected in the parasite under different treated conditions. The mitochondrial MDH activity was stimulated significantly under all treated conditions with an increase of activity by 44, 43 and 73%, respectively; during treatment with crude-peel extract, genistein and PZQ. The cytosolic MDH activity was also stimulated significantly under similar conditions with a percentage increase of 36, 33 and 58, respectively. The mitochondrial activity appeared to be stimulated more than the cytosolic one. Histochemically the MDH activity in the parasite tissue was demonstrated (Plate 5; Figs. a-f). The intensity of stain of formazan particles, which is indicative of the enzyme activity, increased significantly in all the treatments in comparison to the control.

As shown in Table 11 and Fig. 13, the specific activity of MDH, both in the mitochondria and cytosol, was also increased significantly in the parasite under all treated conditions. The percentage increase of the mitochondrial MDH activity was 46,

44 and 73, respectively, with crude-peel extract, genistein and PZQ, while that of the cytosolic one increased by 35, 33 and 58, respectively, with crude-peel extract, genistein and PZQ.

Like MDH, ME was also found to be localized both in the mitochondria and cytosol of this parasite, distributing about 35 and 60% of the activity in the mitochondria and cytosol, respectively (Table 12; Fig. 14). Only the cytosolic ME was affected under different treated conditions in the parasite. The percentage increase in the activity of the cytosolic ME was 36, 39 and 33, respectively, during treatment with crude-peel extract, genistein and PZQ.

Table 13 and Fig. 14 show the changes of specific activity of both mitochondrial and cytosolic ME under different treated conditions. Like that of the tissue activity, the specific activity of only the cytosolic ME was affected with different treatments with a percentage increase of 33, 40 and 25 respectively, with crude-peel extract, genistein and PZQ.

### ***Enzymes of other Metabolic Pathways***

The effect of different test materials on the activity of G6PDH, PC and FBPase was also studied in the parasite (Table 14; Figs. 15 and 16). The tissue activity of G6PDH was significantly decreased by 31, 28 and 23%, respectively, in treatments with crude-peel extract, genistein and PZQ. In histochemical demonstration also, the intensity

of formazan particles, which indicates the activity of the enzyme, decreased significantly in all the treatments in comparison to the control (Plate 6; Figs. a-f). Whereas the tissue activity of PC was significantly increased by 44, 32 and 33%, respectively, while treating with crude-peel extract, genistein and PZQ; the tissue activity of FBPase, however, was not affected significantly by any of the treatments.

The pattern of changes of specific activity of these enzymes under different treatments was almost similar to that of the tissue activity (Table 15 Figs. 15 and 16). The percentage decrease of the G6PDH specific activity was 32, 27 and 23, respectively, in treatments with crude-peel extract, genistein and PZQ. The PC specific activity increased by 43, 27 and 39%, respectively, under similar treatments. Like the tissue activity, the specific activity of FBPase was not affected significantly in any of the treated conditions.

### ***Trace Elements***

Using atomic absorption spectroscopy, the presence of some trace elements could be detected in *R. echinobothrida*; their concentration in quantitative terms in the parasite tissue is depicted in Table 16 and Fig. 17. Out of the elements detected, the concentration of magnesium was found to be highest (~1400 µg) and that of chromium, the lowest (~12 µg), while manganese, cadmium and nickel were below the level of detection. A significant amount of Ca<sup>2+</sup> (350-400 µg/g dry tissue wt) was found to be present in control parasites. However, a significant decline in the concentration of tissue

$\text{Ca}^{2+}$  was recorded in the parasite exposed to all the test materials (Table 17; Fig. 18). The  $\text{Ca}^{2+}$  concentration decreased significantly by 49, 39 and 45%, respectively, while treating with crude-peel extract, genistein and PZQ. In corroboration to this decrease in tissue  $\text{Ca}^{2+}$  concentration as influenced by these treatments, the  $\text{Ca}^{2+}$  concentration in the incubation media containing the crude-peel extract, genistein and PZQ was found to be increased by 100, 118 and 94%, respectively, indicating thereby an increased efflux of  $\text{Ca}^{2+}$  into the medium (Table 18; Fig. 19).

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## **TABLES**

**Table 1.** Efficacy of root-tuber peel extract of *F. vestita*, genistein and PZQ on *R. echinobothrida* in vitro\*. Values are expressed as mean  $\pm$  SEM (n = 3).

| Treatment<br>(mg/ml)              | Time (h) taken for paralysis (P) and death (D) |                                     |
|-----------------------------------|--|-------------------------------------|
|                                   | P  | D                                   |
| Control<br>(in 0.9% PBS)          | -  | 72 $\pm$ 0.05                       |
| Crude peel extract<br>5.0<br>50.0 | 5.9 $\pm$ 0.05<br>0.03 $\pm$ 0.01              | 46.1 $\pm$ 0.04<br>6.5 $\pm$ 0.4    |
| Genistein<br>0.2<br>0.5           | 6.7 $\pm$ 0.04<br>4.4 $\pm$ 0.07               | 49.2 $\pm$ 0.08<br>19.08 $\pm$ 0.02 |
| PZQ<br>0.001<br>0.01              | 2.9 $\pm$ 0.05<br>0.47 $\pm$ 0.07              | 9.8 $\pm$ 0.17<br>6.1 $\pm$ 0.34    |

\* Data as per ref. Tandon et al. (1997) and reconfirmed (n = 5) by repeated incubations.

**Table 2.** Effects of different test materials on glycogen content (mg/g wet wt) in *R. echinobothrida* in vitro. Values are expressed as mean  $\pm$  SEM (n = 3).

| Treatment (mg/ml)                    | Concentration of glycogen** post incubation |   |   |  |  |
|--------------------------------------|---|---|---|--|--|
|                                      | 0 h*  | 3 h                                     | 6 h                                     | 12 h   | 24 h   |
| <b>1.a. Control</b><br>(in 0.9% PBS) | 66.53 $\pm$ 4.17                            | -                                       | 56.97 $\pm$ 4.41                        | 36.04 $\pm$ 2.64 <sup>1</sup>                  | 20.9 $\pm$ 2.71 <sup>2</sup>                   |
| <b>1.b. Crude peel extract</b> (5.0) | -   | -                                       | 47.93 $\pm$ 2.32 <sup>a</sup><br>(- 16) | 20.24 $\pm$ 2.73 <sup>b</sup><br>(- 44) [- 46] | 14.54 $\pm$ 2.05 <sup>a</sup><br>(- 30) [- 69] |
| <b>2.a. Control</b>                  | 65.74 $\pm$ 4.44                            | -                                       | 57.28 $\pm$ 4.12                        | 40.14 $\pm$ 1.62 <sup>1</sup>                  | 25.89 $\pm$ 3.36 <sup>2</sup>                  |
| <b>2.b. Genistein</b> (0.2)          | -   | -                                       | 48.83 $\pm$ 1.94 <sup>a</sup><br>(- 15) | 26.19 $\pm$ 2.18 <sup>b</sup><br>(- 35) [- 39] | 18.74 $\pm$ 1.36 <sup>a</sup><br>(- 28) [- 61] |
| <b>3.a. Control</b>                  | 67.56 $\pm$ 5.00                            | 63.78 $\pm$ 3.59                        | 45.42 $\pm$ 2.90 <sup>1</sup>           | -  | -  |
| <b>3.b. PZQ</b> (0.001)              | -   | 51.16 $\pm$ 3.91 <sup>a</sup><br>(- 20) | 32.53 $\pm$ 0.44 <sup>a</sup><br>(- 28) | -  | -  |

\* Glycogen content in freshly recovered parasite.

\*\* Percentage decrease (-) in glycogen content in treated worms from respective controls is given in parentheses and that to 0 h, in square brackets.

<sup>1,2</sup>: P value significant at < 0.05 and < 0.01, respectively in starved controls compared with the 0 h control.

<sup>a, b</sup>: P value significant at < 0.05 and < 0.01, respectively, in treatments compared with their respective controls.

**Table 3.** Effects different test materials on tissue activity (units/g wet wt) of GPase and GSase of *R. echinobothrida* in vitro. Values are expressed as mean  $\pm$  SEM (n = 3).

| Treatment<br>(mg/ml)                    | Enzyme activity (units/g wet wt) of |   |                           |   |
|---|-------------------------------------|---|---------------------------|---|
|   | GPase                               |   | GSase                     |   |
|   | Total (a+b)                         | Active (a)                                  | Total (a+b)               | Active (a)                                  |
| <b>1.a. Control</b><br>(in 0.9% PBS)    | 1.92 $\pm$ 0.12                     | 1.43 $\pm$ 0.02 (74)                        | 0.73 $\pm$ 0.04           | 0.41 $\pm$ 0.06 (56)                        |
| <b>1.b. Crude peel extract</b><br>(5.0) | 2.09 $\pm$ 0.25<br>[+ 9]            | 1.84 $\pm$ 0.25 (88)<br>[+ 29] <sup>a</sup> | 0.65 $\pm$ 0.03<br>[- 10] | 0.17 $\pm$ 0.04 (27)<br>[- 59] <sup>b</sup> |
| <b>2.a. Control</b>                     | 2.28 $\pm$ 0.43                     | 1.66 $\pm$ 0.31 (73)                        | 1.68 $\pm$ 0.17           | 1.15 $\pm$ 0.08 (68)                        |
| <b>2.b. Genistein</b><br>(0.2)          | 2.49 $\pm$ 0.27<br>[+ 9]            | 2.30 $\pm$ 0.17 (92)<br>[+ 39] <sup>a</sup> | 1.54 $\pm$ 0.13<br>[- 8]  | 0.74 $\pm$ 0.14 (48)<br>[- 36] <sup>a</sup> |
| <b>3.a. Control</b>                     | 2.42 $\pm$ 0.53                     | 1.74 $\pm$ 0.49 (72)                        | 1.29 $\pm$ 0.02           | 0.86 $\pm$ 0.08 (67)                        |
| <b>3.b. PZQ</b><br>(0.001)              | 2.45 $\pm$ 0.28<br>[+ 1]            | 2.26 $\pm$ 0.33 (92)<br>[+ 30] <sup>a</sup> | 1.16 $\pm$ 0.09<br>[- 10] | 0.53 $\pm$ 0.07 (46)<br>[- 38] <sup>a</sup> |

Percentage of GPase *a* and GSase *a* tissue activities out of the total (*a* + *b*) is given in parentheses.

Percentage increase (+) or decrease (-) of GPase *a* and GSase *a* tissue activities in the treated worms compared to their respective controls is given in square brackets.

One unit of enzyme activity is defined as that amount of enzyme which catalyzed 1 $\mu$ mol of NADP<sup>+</sup> reduction (in case of GPase) or NADH oxidation (in case of GSase) per min at 38<sup>o</sup>C.

<sup>a</sup>:  $P < 0.05$ ; <sup>b</sup>:  $P < 0.01$

GPase - glycogen phosphorylase; GSase - glycogen synthase

**Table 5.** Physiological levels of different metabolites related to carbohydrate metabolism in *R. echinobothrida*. Values are expressed as mean  $\pm$  SEM (n = 3).

| <b>Metabolites</b> | <b>Concentration (<math>\mu</math>moles/g wet wt)</b> |
|--------------------|---|
| Glucose            | 26.58 $\pm$ 1.12                                      |
| Lactate            | 9.35 $\pm$ 0.56                                       |
| Malate             | 2.01 $\pm$ 0.25                                       |
| Pyruvate           | BLD   |
| Alanine            | 7.45 $\pm$ 0.91                                       |

BLD - Below the level of detection

**Table 6.** Effects of different test materials on some intermediary metabolites of glycolysis in *R. echinobothrida* in vitro. Values are expressed as mean  $\pm$  SEM (n = 3).

| Treatment<br>(mg/ml)                              | Concentration of metabolites at paralysis time ( $\mu$ moles/g wet wt) |          |                                    |                                    |                                      |
|---|--|----------|------------------------------------|------------------------------------|--------------------------------------|
|   | Glucose  | Pyruvate | Lactate                            | Alanine                            | Malate                               |
| <b>1. a. Control</b><br>(in 0.9% PBS)             | 23.69 $\pm$ 0.62   | BLD      | 8.33 $\pm$ 0.64                    | 5.35 $\pm$ 0.13                    | 1.64 $\pm$ 0.28                      |
| <b>1. b. Crude peel extract (5.0)</b><br><i>P</i> | 16.16 $\pm$ 1.36<br>(-36)<br>< 0.05                                    | „        | 5.75 $\pm$ 0.55<br>(-31)<br>< 0.05 | 4.97 $\pm$ 0.18<br>(-7)<br>N.S.    | 3.35 $\pm$ 0.12<br>(+104)<br>< 0.001 |
| <b>2. a. Control</b>                              | 19.4 $\pm$ 1.89  | BLD      | 9.5 $\pm$ 1.24                     | 4.54 $\pm$ 0.37                    | 1.76 $\pm$ 0.42                      |
| <b>2. b. Genistein (0.2)</b><br><i>P</i>          | 15.74 $\pm$ 0.2<br>(-19)<br>< 0.05                                     | „        | 7.9 $\pm$ 0.34<br>(-24)<br>< 0.05  | 3.42 $\pm$ 0.29<br>(-25)<br>< 0.05 | 4.11 $\pm$ 0.34<br>(+134)<br>< 0.001 |
| <b>3. a. Control</b>                              | 18.71 $\pm$ 1.12   | BLD      | 11.7 $\pm$ 0.76                    | 6.31 $\pm$ 0.34                    | 1.44 $\pm$ 0.16                      |
| <b>3. b. PZQ (0.001)</b><br><i>P</i>              | 16.18 $\pm$ 0.39<br>(-14)<br>N.S.                                      | „        | 9.3 $\pm$ 1.04<br>(-21)<br>< 0.05  | 5.69 $\pm$ 0.25<br>(-10)<br>N.S.   | 2.14 $\pm$ 0.1<br>(+49)<br>< 0.01    |

Percentage increase (+) and decrease (-) of metabolites compared to respective controls are given in parentheses.

BLD - Below the level of detection

N.S. - Not significant

**Table 7.** Effects of different test materials on lactate efflux by *R. echinobothrida* into the culture medium and pH changes of the culture medium (PBS, pH 7.2). Values are expressed as mean  $\pm$  SEM (n = 3).

| Treatment (mg/ml)                                | Lactate efflux ( $\mu$ moles/g body wt/h) | pH after 1 h                    | pH at paralysis time            |
|--|---|---------------------------------|---------------------------------|
| <b>1.a. Control</b><br>(in 0.9% PBS)             | 1.78 $\pm$ 0.04                           | 7.44 $\pm$ 0.004                | 6.78 $\pm$ 0.06                 |
| <b>1.b. Crude peel extract</b> (5.0)<br><i>P</i> | 2.56 $\pm$ 0.22<br>(+44)<br>< 0.05        | 7.02 $\pm$ 0.01<br>(-6)<br>N.S. | 6.32 $\pm$ 0.02<br>(-7)<br>N.S. |
| <b>2.a. Control</b>                              | 1.35 $\pm$ 0.15                           | 7.45 $\pm$ 0.007                | 7.1 $\pm$ 0.1                   |
| <b>2.b. Genistein</b> (0.2)<br><i>P</i>          | 1.67 $\pm$ 0.19<br>(+9)<br>N.S.           | 7.47 $\pm$ 0.6<br>(0)<br>N.S.   | 6.5 $\pm$ 0.004<br>(-8)<br>N.S. |
| <b>3.a. Control</b>                              | 1.45 $\pm$ 0.29                           | 7.47 $\pm$ 0.02                 | 7.27 $\pm$ 0.004                |
| <b>3.b. PZQ</b> (0.001)<br><i>P</i>              | 1.89 $\pm$ 0.62<br>(+23)<br>< 0.05        | 7.48 $\pm$ 0.004<br>(0)<br>N.S. | 7.27 $\pm$ 0.05<br>(0)<br>N.S.  |

Percentage increase (+) and decrease (-) of metabolites or pH changes compared to respective controls are given in parentheses.

**Table 8.** Effects of different test materials on tissue activity (units/g wet wt) of HK, PFK, PEPCK, PK and LDH of *R. echinobothrida* in vitro. Values are expressed as mean  $\pm$  SEM (n = 4).

| Treatment (mg/ml)                                | Enzyme activity (units/g wet wt) of |                                      |                                      |                                     |                                     |
|--|-------------------------------------|--------------------------------------|--------------------------------------|-------------------------------------|-------------------------------------|
|  | HK                                  | PFK                                  | PEPCK                                | PK                                  | LDH                                 |
| <b>1.a. Control</b><br>(in 0.9% PBS)             | 1.96 $\pm$ 0.02                     | 1.45 $\pm$ 0.17                      | 15.87 $\pm$ 0.11                     | 1.07 $\pm$ 0.16                     | 4.51 $\pm$ 0.06                     |
| <b>1.b. Crude peel extract (5.0)</b><br><i>p</i> | 2.61 $\pm$ 0.12<br>(+ 33)<br>< 0.05 | 2.04 $\pm$ 0.1<br>(+ 41)<br>< 0.05   | 23.68 $\pm$ 1.18<br>(+ 49)<br>< 0.01 | 0.79 $\pm$ 0.13<br>(- 26)<br>< 0.05 | 7 $\pm$ 1.05<br>(+ 55)<br>< 0.01    |
| <b>2.a. Control</b>                              | 1.79 $\pm$ 0.13                     | 1.77 $\pm$ 0.06                      | 16.74 $\pm$ 0.37                     | 1.07 $\pm$ 0.16                     | 4.51 $\pm$ 0.06                     |
| <b>2.b. Genistein (0.2)</b><br><i>p</i>          | 2.48 $\pm$ 0.23<br>(+ 39)<br>< 0.05 | 2.64 $\pm$ 0.15<br>(+ 49)<br>< 0.01  | 24.33 $\pm$ 0.64<br>(+ 45)<br>< 0.05 | 0.92 $\pm$ 0.15<br>(- 14)<br>N. S.  | 7.55 $\pm$ 0.66<br>(+ 67)<br>< 0.01 |
| <b>3.a. Control</b>                              | 1.28 $\pm$ 0.12                     | 1.34 $\pm$ 0.04                      | 23.93 $\pm$ 0.97                     | 1.09 $\pm$ 0.12                     | 4.57 $\pm$ 0.04                     |
| <b>3.b. PZQ (0.001)</b><br><i>p</i>              | 1.74 $\pm$ 0.03<br>(+ 36)<br>< 0.05 | 3.01 $\pm$ 0.3<br>(+ 125)<br>< 0.001 | 34.31 $\pm$ 0.37<br>(+ 44)<br>< 0.05 | 0.93 $\pm$ 0.12<br>(- 15)<br>N. S.  | 7.21 $\pm$ 0.25<br>(+ 58)<br>< 0.01 |

Percentage increases (+) or decreases (-) of enzyme activity compared to respective controls is given in parentheses.

One unit of enzyme activity is defined as that amount of enzyme which catalyzed 1  $\mu$ mole of NADP<sup>+</sup> reduction (in case of HK) or NADH oxidation (in case of PFK, PEPCK, PK and LDH) per min at 38<sup>o</sup>C.

HK - Hexokinase; PFK - Phosphofructokinase; PEPCK - Phosphoenolpyruvate carboxykinase; PK - Pyruvate kinase; LDH - Lactate dehydrogenase

**Table 9.** Effects of different test materials on the specific activity (units/mg protein) of HK, PFK and PEPCK, PK and LDH of *R. echinobothrida* in vitro. Values are expressed as mean  $\pm$  SEM (n = 4).

| Treatment (mg/ml)                                | Specific activity (units/mg protein) of |   |                                     |                                       |                                       |
|--|---|---|-------------------------------------|---------------------------------------|---------------------------------------|
|  | HK                                      | PFK                                     | PEPCK                               | PK                                    | LDH                                   |
| <b>1.a. Control</b><br>(in 0.9% PBS)             | 0.041 $\pm$ 0.002                       | 0.03 $\pm$ 0.001                        | 0.33 $\pm$ 0.02                     | 0.022 $\pm$ 0.001                     | 0.094 $\pm$ 0.001                     |
| <b>1.b. Crude peel extract (5.0)</b><br><i>p</i> | 0.055 $\pm$ 0.001<br>(+ 34)<br>< 0.05   | 0.043 $\pm$ 0.001<br>(+ 43)<br>< 0.05   | 0.5 $\pm$ 0.03<br>(+ 52)<br>< 0.01  | 0.017 $\pm$ 0.001<br>(- 23)<br>< 0.05 | 0.15 $\pm$ 0.002<br>(+ 60)<br>< 0.01  |
| <b>2.a. Control</b>                              | 0.037 $\pm$ 0.003                       | 0.037 $\pm$ 0.004                       | 0.35 $\pm$ 0.03                     | 0.022 $\pm$ 0.002                     | 0.094 $\pm$ 0.002                     |
| <b>2.b. Genistein (0.2)</b><br><i>p</i>          | 0.052 $\pm$ 0.002<br>(+ 41)<br>< 0.05   | 0.055 $\pm$ 0.001<br>(+ 49)<br>< 0.01   | 0.51 $\pm$ 0.04<br>(+ 46)<br>< 0.05 | 0.019 $\pm$ 0.001<br>(- 14)<br>N. S.  | 0.158 $\pm$ 0.003<br>(+ 68)<br>< 0.01 |
| <b>3.a. Control</b>                              | 0.029 $\pm$ 0.001                       | 0.03 $\pm$ 0.004                        | 0.38 $\pm$ 0.02                     | 0.025 $\pm$ 0.001                     | 0.103 $\pm$ 0.004                     |
| <b>3.b. PZQ (0.001)</b><br><i>p</i>              | 0.039 $\pm$ 0.003<br>(+ 34)<br>< 0.05   | 0.068 $\pm$ 0.003<br>(+ 127)<br>< 0.001 | 0.52 $\pm$ 0.03<br>(+ 42)<br>< 0.05 | 0.021 $\pm$ 0.002<br>(- 16)<br>N. S.  | 0.163 $\pm$ 0.002<br>(+ 58)<br>< 0.01 |

Percentage increases (+) or decreases (-) of specific activity compared to respective controls is given in parentheses.

Specific activity of the enzymes was expressed as the units of enzyme activity per mg protein.

Abbreviations are same as Table 8.

**Table 10.** Effects of different test materials on tissue activity of malate dehydrogenase (MDH) (units/g wet wt) in *R. echinobothrida* in vitro. Values are expressed as mean  $\pm$  SEM (n = 4).

| Treatment<br>(mg/ml)                     | MDH activity (units/g wet wt) in |                                  |                                  |
|--|----------------------------------|----------------------------------|----------------------------------|
|  | Homogenate                       | Mitochondrial<br>fraction        | Cytosolic<br>fraction            |
| <b>1.a. Control</b><br>(in 0.9% PBS)     | 73.23 $\pm$ 4.32                 | 26.89 $\pm$ 0.75<br>(37)         | 37.77 $\pm$ 1.25<br>(52)         |
| <b>1.b. Crude peel<br/>extract (5.0)</b> | 89.61 $\pm$ 2.68<br>[22]         | 39.4 $\pm$ 1.52<br>(44)<br>[47]  | 51.32 $\pm$ 0.98<br>(57)<br>[36] |
| <i>p</i>                                 | < 0.05                           | < 0.05                           | < 0.05                           |
| <b>2.a. Control</b>                      | 65.28 $\pm$ 2.41                 | 22.01 $\pm$ 1.09<br>(34)         | 38.8 $\pm$ 1.02<br>(59)          |
| <b>2.b. Genistein<br/>(0.2)</b>          | 81.69 $\pm$ 0.99<br>[25]         | 31.51 $\pm$ 0.98<br>(39)<br>[43] | 51.7 $\pm$ 0.56<br>(63)<br>[33]  |
| <i>p</i>                                 | < 0.05                           | < 0.05                           | < 0.05                           |
| <b>3.a. Control</b>                      | 46.39 $\pm$ 4.12                 | 18.95 $\pm$ 0.65<br>(41)         | 24.93 $\pm$ 0.79<br>(54)         |
| <b>3.b. PZQ<br/>(0.001)</b>              | 66.56 $\pm$ 2.55<br>[43]         | 32.71 $\pm$ 2.05<br>(49)<br>[73] | 39.44 $\pm$ 2.03<br>(59)<br>[58] |
| <i>p</i>                                 | < 0.05                           | < 0.01                           | < 0.01                           |

Percentage of tissue activity in the mitochondrial and cytosolic fraction compared to the activity in the homogenate is given within parentheses. Percentage increase of activity compared to respective controls is given within square brackets.

One unit of enzyme activity is defined as that amount of enzyme which catalyzed 1  $\mu$ mole of NADH oxidation per min at 38<sup>o</sup>C.

**Table 11.** Effects of different test materials on the specific activity (units/mg protein) malate dehydrogenase (MDH) in *R. echinobothrida* in vitro. Values are expressed as mean  $\pm$  SEM (n = 4).

| Treatment<br>(mg/ml)                     | Specific activity of MDH (units/mg protein) in |                           |                          |
|--|--|---------------------------|--------------------------|
|  | Homogenate                                     | Mitochondrial<br>fraction | Cytosolic<br>fraction    |
| <b>1.a. Control</b><br>(in 0.9% PBS)     | 1.6 $\pm$ 0.03                                 | 0.59 $\pm$ 0.05<br>(37)   | 0.83 $\pm$ 0.025<br>(52) |
| <b>1.b. Crude peel<br/>extract (5.0)</b> | 1.96 $\pm$ 0.02                                | 0.86 $\pm$ 0.02<br>(44)   | 1.12 $\pm$ 0.09<br>(57)  |
| <i>p</i>                                 | [23]<br>< 0.05                                 | [46]<br>< 0.05            | [35]<br>< 0.05           |
| <b>2.a. Control</b>                      | 1.43 $\pm$ 0.04                                | 0.48 $\pm$ 0.009<br>(34)  | 0.85 $\pm$ 0.02<br>(59)  |
| <b>2.b. Genistein<br/>(0.2)</b>          | 1.79 $\pm$ 0.03                                | 0.69 $\pm$ 0.008<br>(39)  | 1.13 $\pm$ 0.05<br>(63)  |
| <i>p</i>                                 | [25]<br>< 0.05                                 | [44]<br>< 0.05            | [33]<br>< 0.05           |
| <b>3.a. Control</b>                      | 1.18 $\pm$ 0.02                                | 0.48 $\pm$ 0.06<br>(41)   | 0.64 $\pm$ 0.009<br>(54) |
| <b>3.b. PZQ<br/>(0.001)</b>              | 1.69 $\pm$ 0.05                                | 0.83 $\pm$ 0.05<br>(49)   | 1.01 $\pm$ 0.03<br>(59)  |
| <i>p</i>                                 | [43]<br>< 0.05                                 | [73]<br>< 0.01            | [58]<br>< 0.01           |

Percentage of specific activity in the mitochondrial and cytosolic fractions compared to the activity in the homogenate is given within parentheses. Percentage increase of specific activity compared to respective controls is given within square brackets.

Specific activity of the enzymes was expressed as the units of enzyme activity per mg protein.

**Table 12.** Effects of different test materials on tissue activity (units/g wet wt) of malic enzyme (ME) in *R. echinobothrida* in vitro. Values are expressed as mean  $\pm$  SEM (n = 5).

| Treatment<br>(mg/ml)                     | ME activity (units/g wet wt) in |                           |                          |
|--|---------------------------------|---------------------------|--------------------------|
|  | Homogenate                      | Mitochondrial<br>fraction | Cytosolic<br>fraction    |
| <b>1.a. Control</b><br>(in 0.9% PBS)     | 0.18 $\pm$ 0.005                | 0.08 $\pm$ 0.002<br>(44)  | 0.11 $\pm$ 0.002<br>(61) |
| <b>1.b. Crude peel<br/>extract (5.0)</b> | 0.23 $\pm$ 0.009                | 0.09 $\pm$ 0.001<br>(39)  | 0.15 $\pm$ 0.005<br>(65) |
| <i>p</i>                                 | [+ 28]<br>< 0.05                | [+ 13]<br>N. S.           | [+ 36]<br>< 0.05         |
| <b>2.a. Control</b>                      | 0.29 $\pm$ 0.007                | 0.07 $\pm$ 0.003<br>(24)  | 0.18 $\pm$ 0.003<br>(62) |
| <b>2.b. Genistein</b><br>(0.2)           | 0.37 $\pm$ 0.006                | 0.08 $\pm$ 0.004<br>(22)  | 0.25 $\pm$ 0.001<br>(68) |
| <i>p</i>                                 | [+ 28]<br>< 0.05                | [+ 14]<br>N. S.           | [+ 39]<br>< 0.05         |
| <b>3.a. Control</b>                      | 0.22 $\pm$ 0.005                | 0.1 $\pm$ 0.008<br>(36)   | 0.15 $\pm$ 0.002<br>(54) |
| <b>3.b. PZQ</b><br>(0.001)               | 0.35 $\pm$ 0.008                | 0.11 $\pm$ 0.006<br>(31)  | 0.2 $\pm$ 0.003<br>(57)  |
| <i>p</i>                                 | [+ 59]<br>< 0.05                | [+ 10]<br>N. S.           | [+ 33]<br>< 0.05         |

Percentage of tissue activity in the mitochondrial and cytosolic fraction fractions compared to the activity in the homogenate is given within parentheses. Percentage increase of activity compared to respective controls is given within square brackets.

One unit of enzyme activity is defined as that amount of enzyme which catalyzed 1  $\mu$ mole of NADPH oxidation per min at 38<sup>o</sup>C.

**Table 13.** Effects of different test materials on specific activity (units/mg protein) of malic enzyme (ME) in *R. echinobothrida* in vitro. Values are expressed as mean  $\pm$  SEM (n = 5).

| Treatment<br>(mg/ml)                     | Specific activity of ME (units/mg protein) in |                           |                       |
|--|---|---------------------------|-----------------------|
|  | Homogenate                                    | Mitochondrial<br>fraction | Cytosolic<br>fraction |
| <b>1.a. Control</b><br>(in 0.9% PBS)     | 0.005   | 0.002<br>(40)             | 0.003<br>(60)         |
| <b>1.b. Crude peel<br/>extract (5.0)</b> | 0.006   | 0.0025<br>(42)            | 0.004<br>(67)         |
| <i>p</i>                                 | [+ 20]<br>< 0.05                              | [+ 25]<br>N. S.           | [+ 33]<br>< 0.05      |
| <b>2.a. Control</b>                      | 0.008   | 0.002<br>(25)             | 0.005<br>(63)         |
| <b>2.b. Genistein<br/>(0.2)</b>          | 0.01  | 0.002<br>(20)             | 0.007<br>(70)         |
| <i>p</i>                                 | [+ 28]<br>< 0.05                              | [-]<br>N. S.              | [+ 40]<br>< 0.05      |
| <b>3.a. Control</b>                      | 0.006   | 0.003<br>(50)             | 0.004<br>(67)         |
| <b>3.b. PZQ<br/>(0.001)</b>              | 0.01  | 0.003<br>(30)             | 0.005<br>(60)         |
| <i>p</i>                                 | [+ 67]<br>< 0.05                              | [-]<br>N. S.              | [+ 25]<br>< 0.05      |

Percentage of specific activity in the mitochondrial and cytosolic fraction compared to the activity in the homogenate is given within parentheses. Percentage increase of specific activity compared to respective controls is given within square brackets.

Specific activity of the enzymes was expressed as the units of enzyme activity per mg protein.

**Table 14.** Effects of different test materials on tissue activity (units/g wet wt/min) of PC, G6PDH and FBPase in *R. echinobothrida* in vitro. Values are expressed as mean  $\pm$  SEM (n = 4).

| Treatment (mg/ml)                                | Enzyme activity (units/g wet wt/min) of |                                      |                                   |
|--|---|--------------------------------------|-----------------------------------|
|  | G6PDH                                   | PC                                   | FBPase                            |
| <b>1.a. Control</b><br>(in 0.9% PBS)             | 1.27 $\pm$ 0.02                         | 6.39 $\pm$ 0.38                      | 1.1 $\pm$ 0.02                    |
| <b>1.b. Crude peel extract (5.0)</b><br><i>p</i> | 0.88 $\pm$ 0.006<br>(- 31)<br>< 0.05    | 9.21 $\pm$ 0.2<br>(+ 44)<br>< 0.05   | 0.98 $\pm$ 0.15<br>(-11)<br>N. S. |
| <b>2.a. Control</b>                              | 1.55 $\pm$ 0.1                          | 6.68 $\pm$ 0.29                      | 1.1 $\pm$ 0.02                    |
| <b>2.b. Genistein (0.2)</b><br><i>p</i>          | 1.12 $\pm$ 0.15<br>(- 28)<br>< 0.05     | 8.82 $\pm$ 0.38<br>(+ 32)<br>< 0.05  | 1.05 $\pm$ 0.08<br>(- 5)<br>N. S. |
| <b>3.a. Control</b>                              | 3.2 $\pm$ 0.29                          | 8.46 $\pm$ 1.32                      | 1.68 $\pm$ 0.09                   |
| <b>3.b. PZQ (0.001)</b><br><i>p</i>              | 2.48 $\pm$ 0.45<br>(- 23)<br>< 0.05     | 11.23 $\pm$ 1.03<br>(+ 33)<br>< 0.05 | 1.45 $\pm$ 0.06<br>(-14)<br>N. S. |

Percentage increase (+) or decrease (-) in enzyme activity in treated worms from respective control is given in parentheses.

One unit of enzyme activity is defined as that amount of enzyme which catalyzed 1  $\mu$ mole of NADP<sup>+</sup> reduction (in case of G6PDH and FBPase) or NADH oxidation (in case of PC) per min at 38°C.

PC - Pyruvate carboxylase; G6PDH - Glucose 6-phosphate dehydrogenase; FBPase - Fructose 1,6-bisphosphatase

**Table 15.** Effects of different test materials on specific activity (units/mg protein) of PC, G6PDH and FBPase of *R. echinobothrida* in vitro. Values are expressed as mean  $\pm$  SEM (n = 4).

| Treatment<br>(mg/ml)                             | Specific activity (units/mg protein) of |                                     |                                      |
|--|---|-------------------------------------|--------------------------------------|
|  | G6PDH                                   | PC                                  | FBPase                               |
| <b>1.a. Control</b><br>(in 0.9% PBS)             | 0.028 $\pm$ 0.002                       | 0.14 $\pm$ 0.03                     | 0.024 $\pm$ 0.002                    |
| <b>1.b. Crude peel extract (5.0)</b><br><i>p</i> | 0.019 $\pm$ 0.006<br>(- 32)<br>< 0.05   | 0.2 $\pm$ 0.02<br>(+ 43)<br>< 0.05  | 0.022 $\pm$ 0.001<br>(-8)<br>N. S.   |
| <b>2.a. Control</b>                              | 0.034 $\pm$ 0.001                       | 0.15 $\pm$ 0.02                     | 0.024 $\pm$ 0.002                    |
| <b>2.b. Genistein (0.2)</b><br><i>p</i>          | 0.024 $\pm$ 0.001<br>(- 27)<br>< 0.05   | 0.19 $\pm$ 0.03<br>(+ 27)<br>< 0.05 | 0.023 $\pm$ 0.001<br>(- 4)<br>N. S.  |
| <b>3.a. Control</b>                              | 0.04 $\pm$ 0.003                        | 0.18 $\pm$ 0.01                     | 0.037 $\pm$ 0.001                    |
| <b>3.b. PZQ (0.001)</b><br><i>p</i>              | 0.031 $\pm$ 0.004<br>(- 23)<br>< 0.05   | 0.25 $\pm$ 0.03<br>(+ 39)<br>< 0.05 | 0.032 $\pm$ 0.003<br>(- 14)<br>N. S. |

Percentage increase (+) or decrease (-) in enzyme specific activity in treated worms from respective controls is given in parentheses.

Specific activity of the enzymes was expressed as the units of enzyme activity per mg protein.

Abbreviations are same as Table 14.

**Table 16.** Levels of trace elements ( $\mu\text{g/g}$  dry tissue wt) present in *R. echinobothrida*. Values are expressed as mean  $\pm$  SEM (n = 5).

| <b>Trace elements</b> | <b>Concentration (<math>\mu\text{g/g}</math> dry tissue wt)</b> |
|-----------------------|---|
| Calcium               | 393.8 $\pm$ 13.54   |
| Chromium              | 11.7 $\pm$ 0.87   |
| Manganese             | BLD   |
| Cadmium               | BLD   |
| Lead                  | 23.5 $\pm$ 2.13   |
| Nickel                | BLD   |
| Iron                  | 1184.8 $\pm$ 42.57  |
| Zinc                  | 387.1 $\pm$ 7.42  |
| Magnesium             | 1395.9 $\pm$ 32.86  |

BLD – Below the level of detection

**Table 17.** Changes in the concentration of  $\text{Ca}^{2+}$  ( $\mu\text{g/g}$  dry tissue wt) at the time of paralysis caused due to treatment with different test materials in *R. echinobothrida* in vitro. Values are expressed as mean  $\pm$  SEM ( $n = 4$ ).

| Treatment (mg/ml)  | $\text{Ca}^{2+}$ ( $\mu\text{g/g}$ dry tissue wt)         |
|--|---|
| <b>1.a. Control</b><br>(in 0.9% PBS)<br><b>1.b. Crude peel extract (5.0)</b><br><p style="text-align: right;"><i>p</i></p> | 266.67 $\pm$ 31.29<br>136.59 $\pm$ 6.54<br>[49]<br>< 0.05 |
| <b>2.a. Control</b><br><b>2.b. Genistein (0.2)</b><br><p style="text-align: right;"><i>p</i></p>                           | 266.67 $\pm$ 31.29<br>162.5 $\pm$ 4.12<br>[39]<br>< 0.05  |
| <b>3.a. Control</b><br><b>3.b. PZQ (0.001)</b><br><p style="text-align: right;"><i>p</i></p>                               | 256.88 $\pm$ 25.36<br>142.12 $\pm$ 5.86<br>[45]<br>< 0.05 |

Percentage decrease of  $\text{Ca}^{2+}$  concentration compared to respective controls is given in parentheses.

**Table 18.** Effects of root-tuber peel extract of *F. vestita*, genistein and PZQ on  $\text{Ca}^{2+}$  efflux ( $\mu\text{g/g}$  dry body wt/h) by *R. echinobothrida* into the culture medium. Values are expressed as mean  $\pm$  SEM (n = 4).

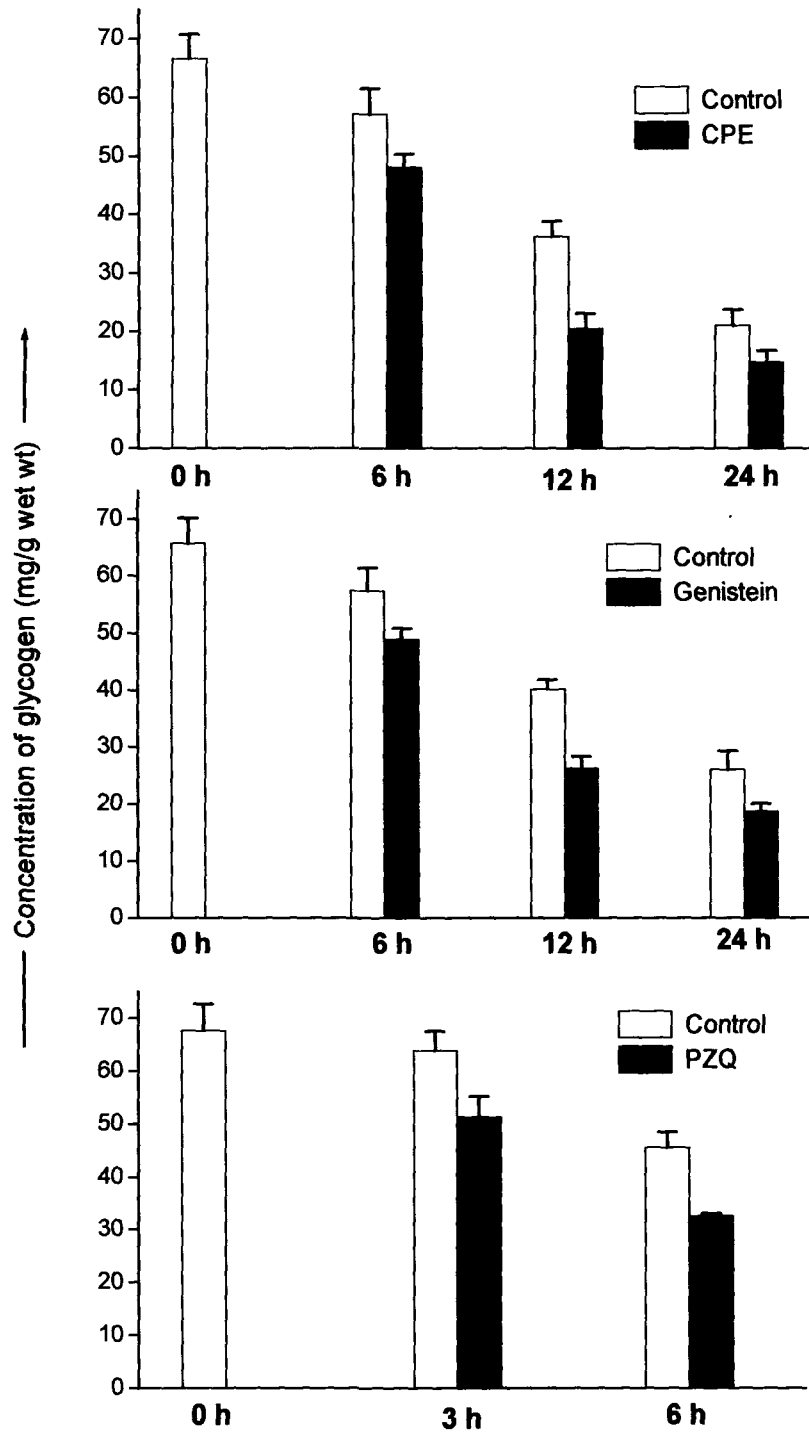
| Treatment (mg/ml)                | $\text{Ca}^{2+}$ efflux ( $\mu\text{g/g}$ dry body wt/h) |
|----------------------------------|--|
| 1.a. Control<br>(in 0.9% PBS)    | 12.2 $\pm$ 0.56  |
| 1.b. Crude peel<br>extract (5.0) | 24.39 $\pm$ 1.02<br>(100)                                |
| <i>p</i>                         | < 0.001  |
| 2.a. Control                     | 12.2 $\pm$ 0.85  |
| 2.b. Genistein (0.2)             | 26.56 $\pm$ 0.98<br>(118)                                |
| <i>p</i>                         | < 0.001  |
| 3.a. Control                     | 15.59 $\pm$ 0.45   |
| 3.b. PZQ (0.001)                 | 30.3 $\pm$ 0.79<br>(94)                                  |
| <i>p</i>                         | < 0.01   |

Percentage increase of  $\text{Ca}^{2+}$  efflux compared to the control is given in parentheses.

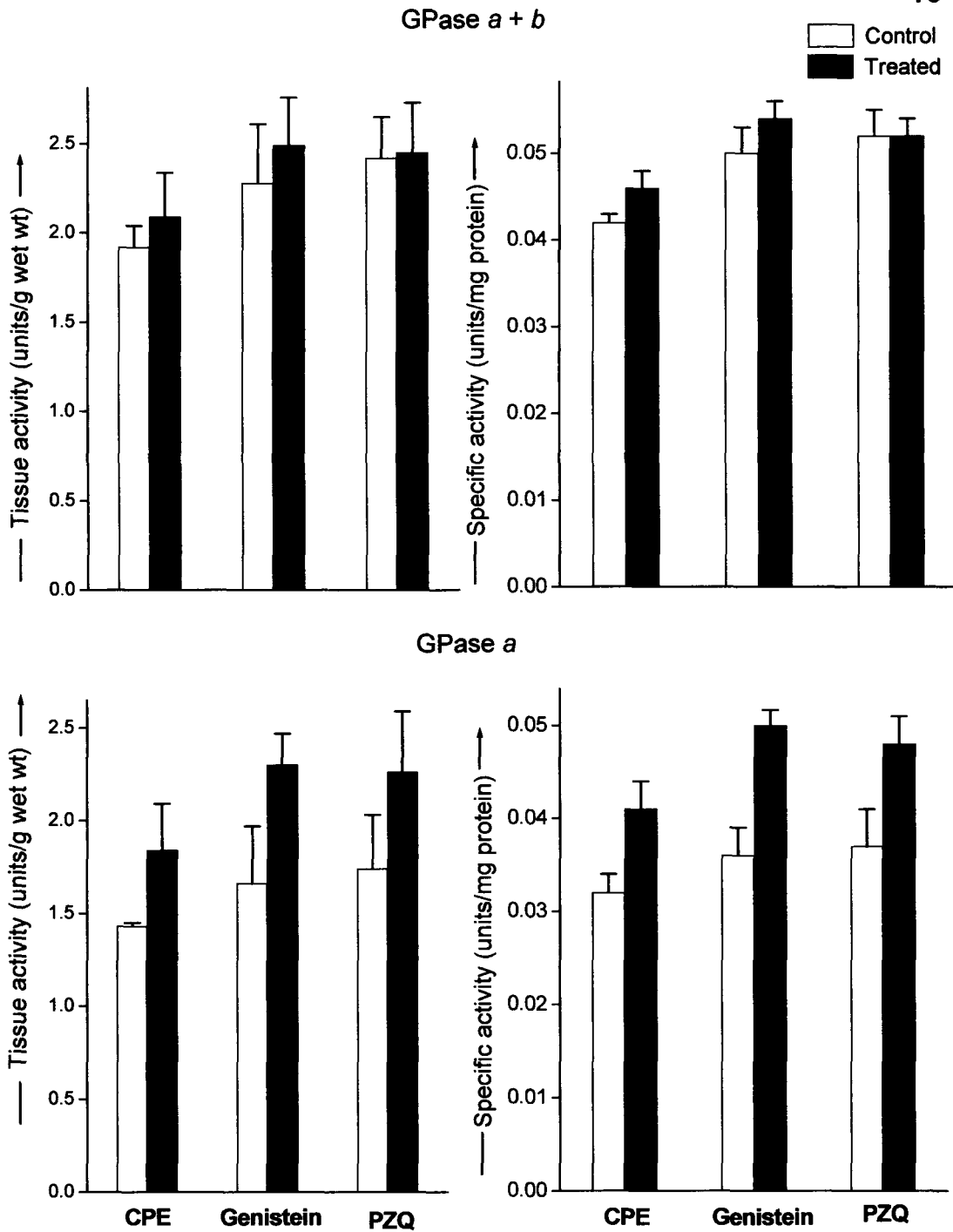
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## FIGURES

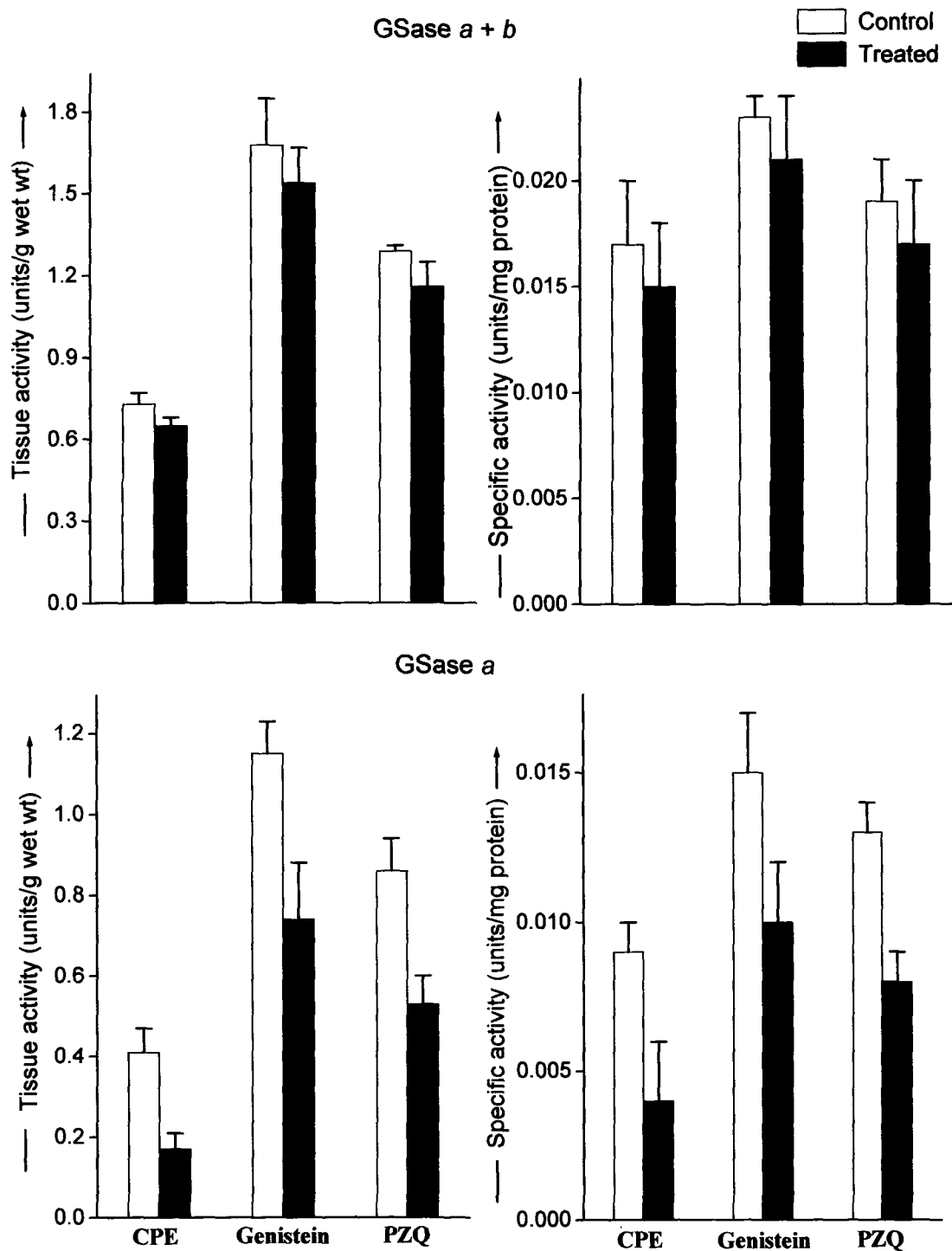


**Fig. 4.** Effects of different test materials on glycogen level (mg/g wet wt) in *R. echinobothrida*. Values are plotted as mean  $\pm$  SEM (n = 3). CPE - Crude-peel extract of *F. vestita*; PZQ - Praziquantel

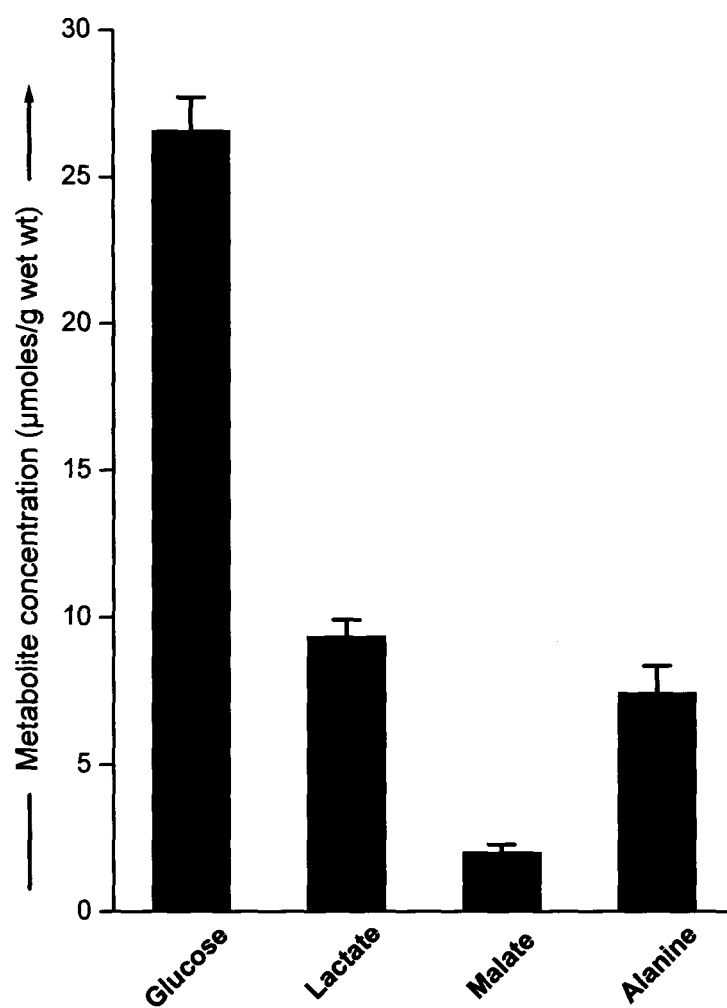


**Fig. 5.** Effects different test materials on tissue activity (units/g wet wt) and specific activity (units/mg protein) of GPase a + b (total) and GPase a (active) in *R. echinobothrida*. Values are plotted as mean  $\pm$  SEM (n = 3).

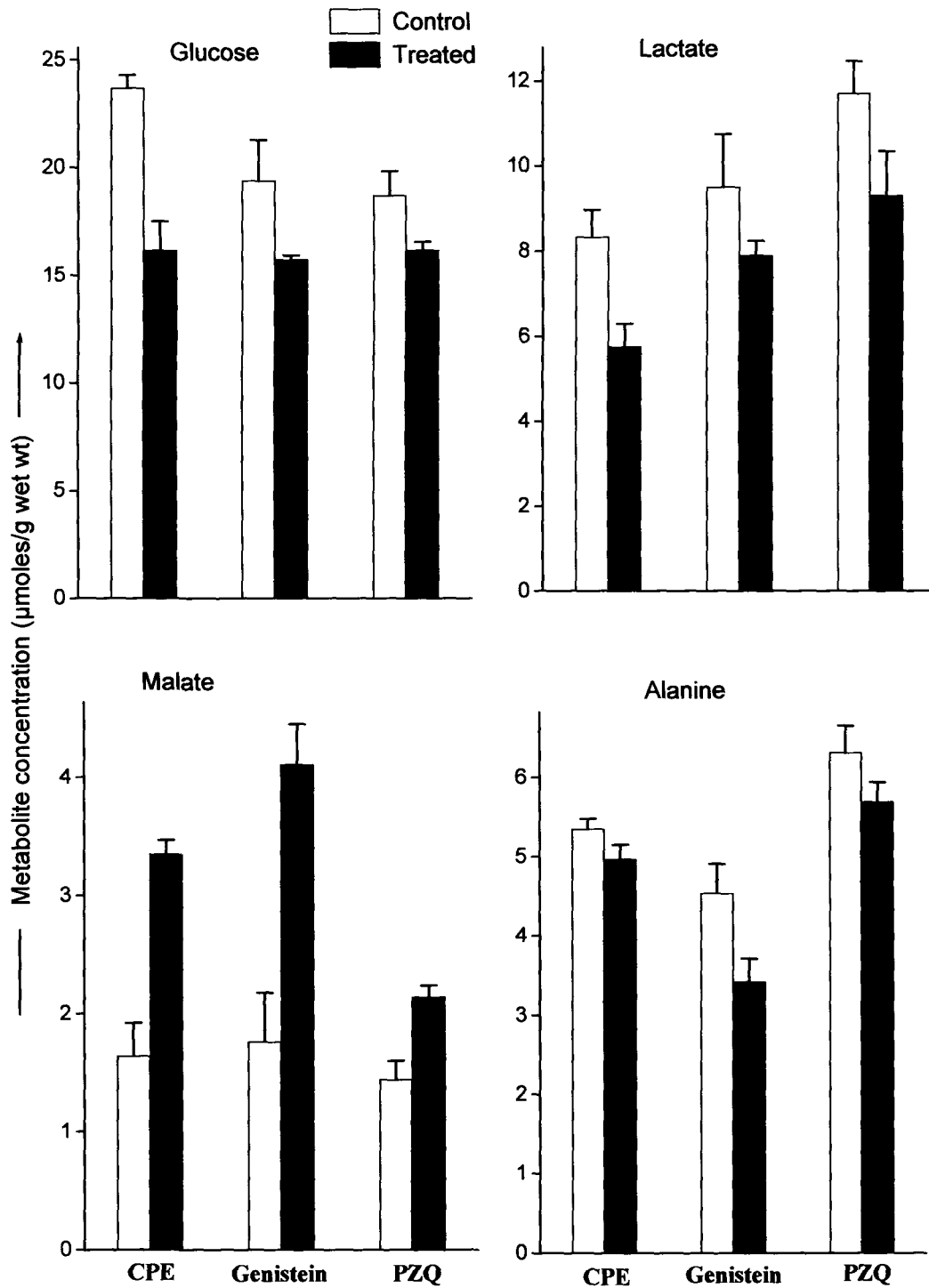
CPE - Crude-peel extract of *F. vestita*; PZQ - Praziquantel; GPase - Glycogen phosphorylase



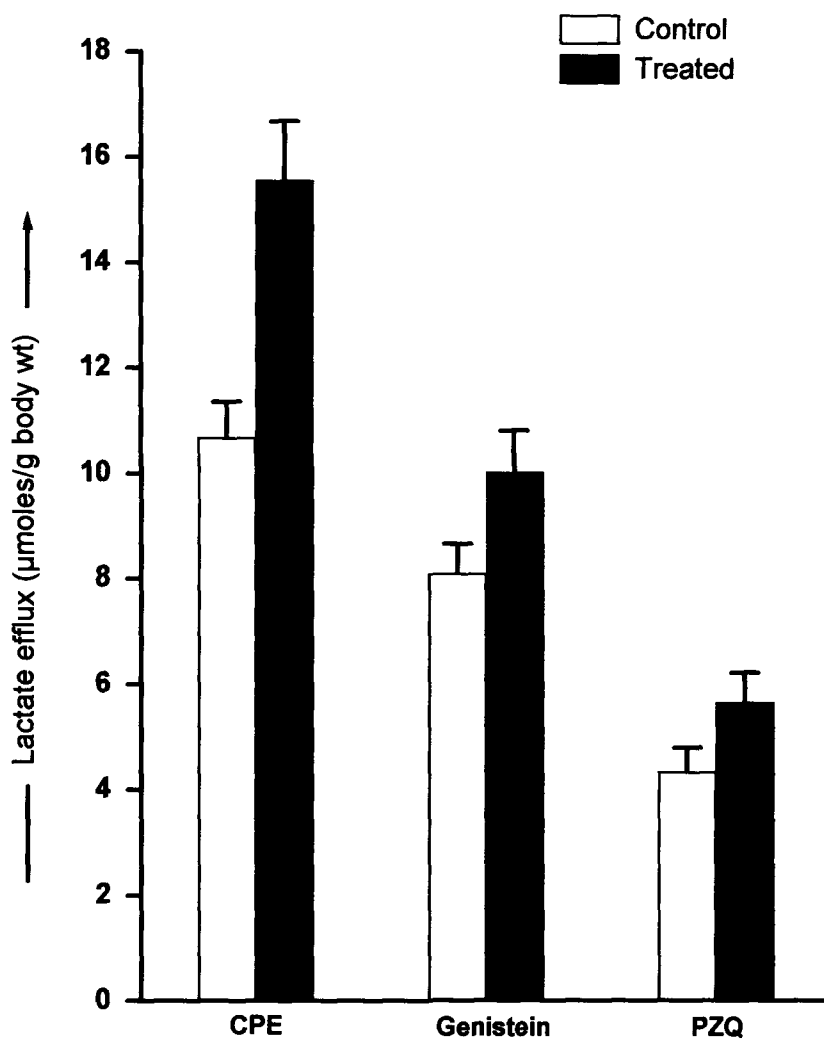
**Fig. 6.** Effects different test materials on tissue activity (units/g wet wt) and specific activity (units/mg protein) of GSase a + b (total) and GSase a (active) in *R. echinobothrida*. Values are plotted as mean  $\pm$  SEM (n = 3). CPE - Crude-peel extract of *F. vestita*; PZQ - Praziquantel; GSase - Glycogen synthase



**Fig. 7.** Physiological levels ( $\mu\text{moles/g wet wt}$ ) of different metabolites related to carbohydrate metabolism in *R. echinobothrida*. Values are plotted as mean  $\pm$  SEM ( $n = 3$ ).

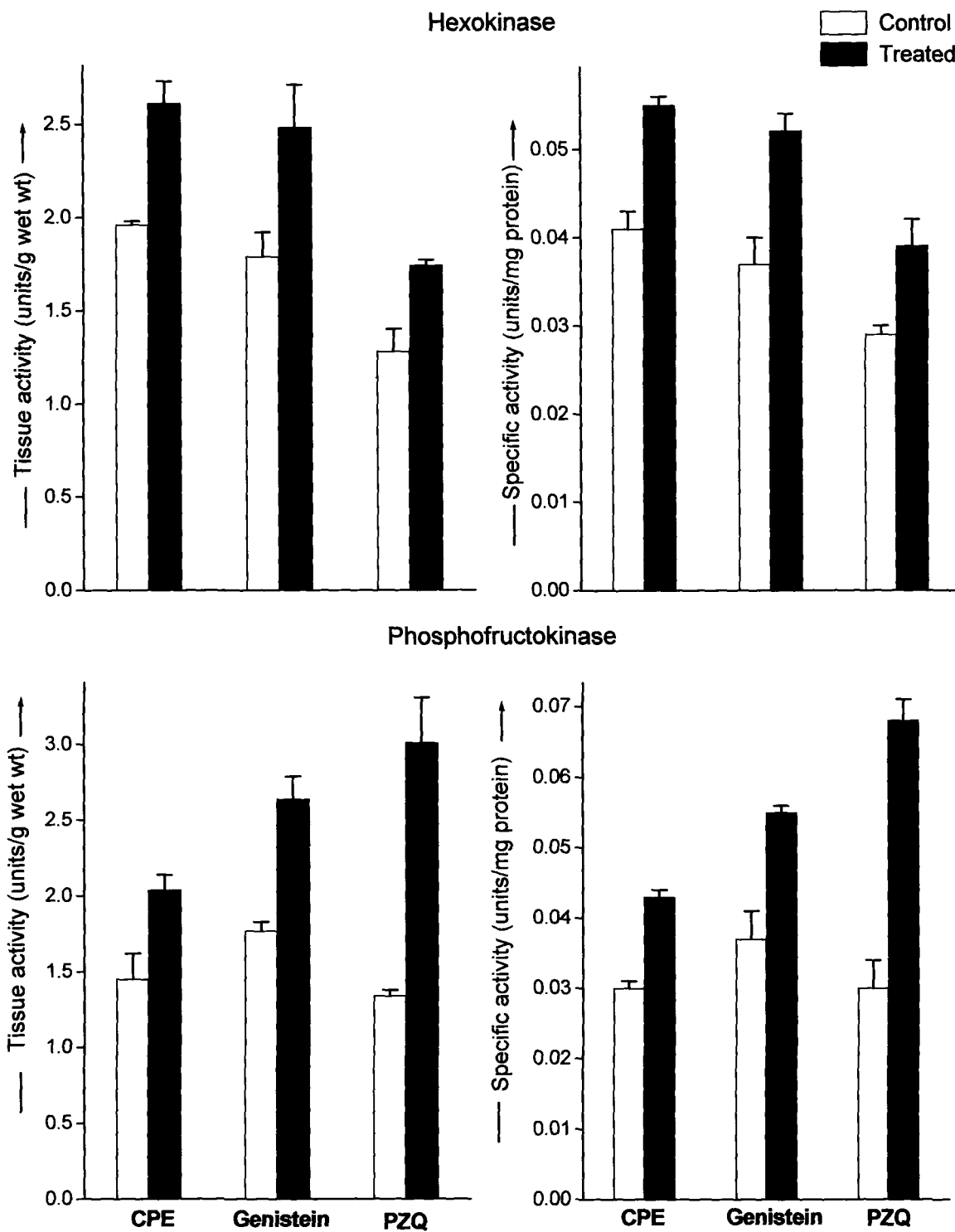


**Fig. 8.** Effects of different test materials on the concentration ( $\mu\text{moles/g wet wt}$ ) of different metabolites of glycolysis in *R. echinobothrida*. Values are plotted as mean  $\pm$  SEM ( $n = 3$ ).

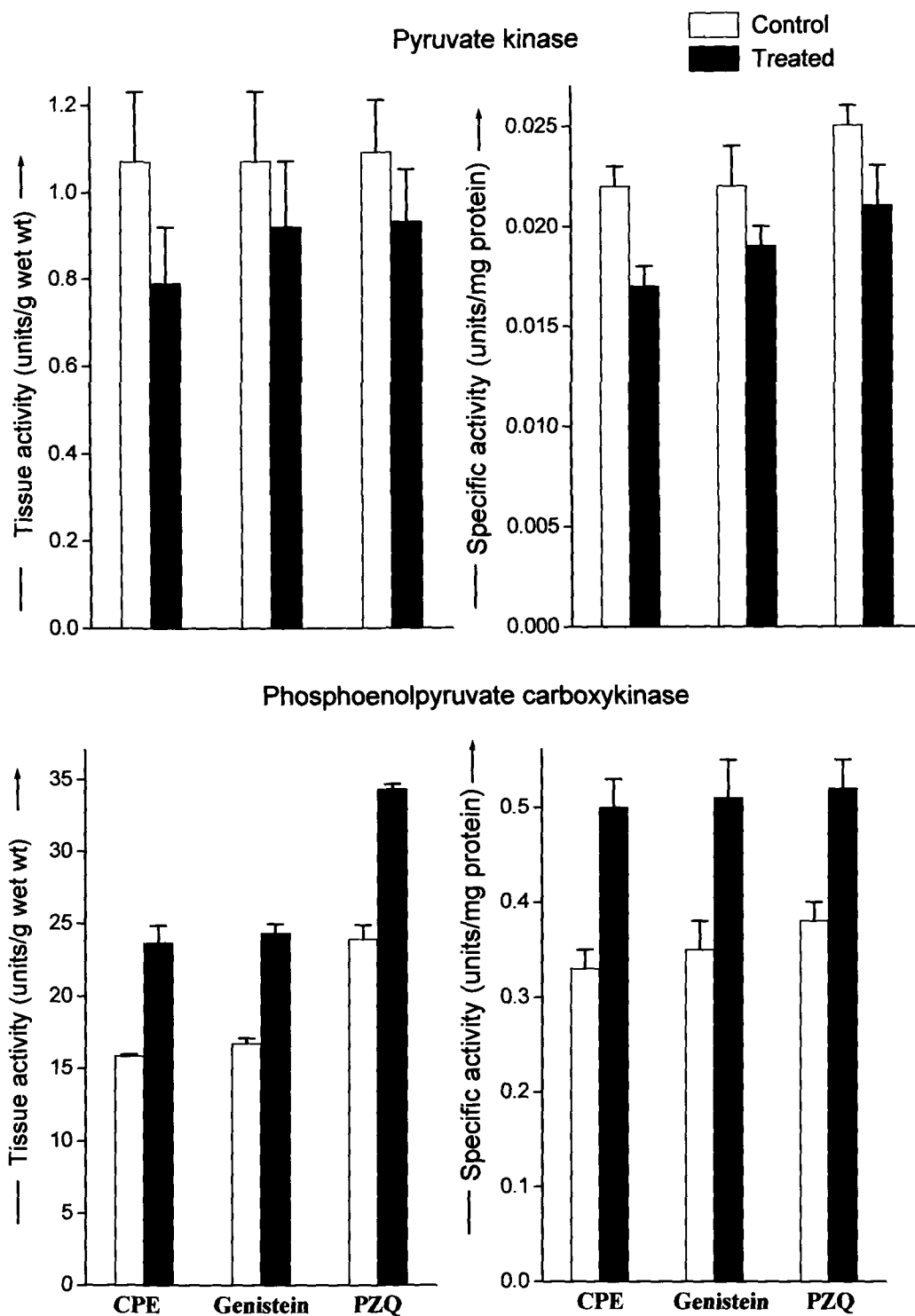


**Fig. 9.** Lactate efflux ( $\mu\text{moles/g body wt}$ ) by *R. echinobothrida* in the culture medium at paralysis time both in control and treated conditions. Values are plotted as mean  $\pm$  SEM ( $n = 3$ ).

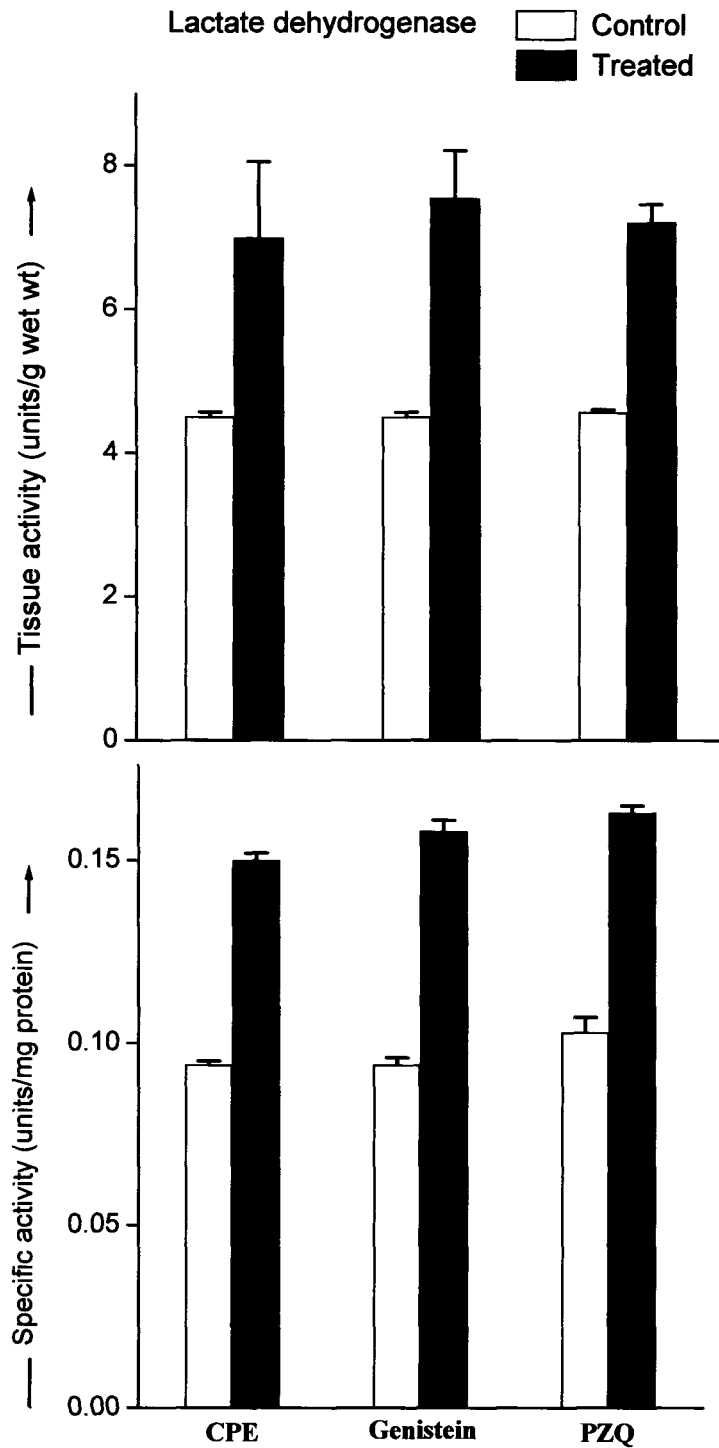
CPE - Crude-peel extract of *F. vestita*; PZQ - Praziquantel



**Fig. 10.** Effects of different test materials on tissue activity (units/g wet wt) and specific activity (units/mg protein) of hexokinase and phosphofructokinase in *R. echinobothrida*. Values are plotted as mean  $\pm$  SEM (n = 4).  
CPE - Crude-peel extract of *F. vestita*; PZQ - Praziquantel

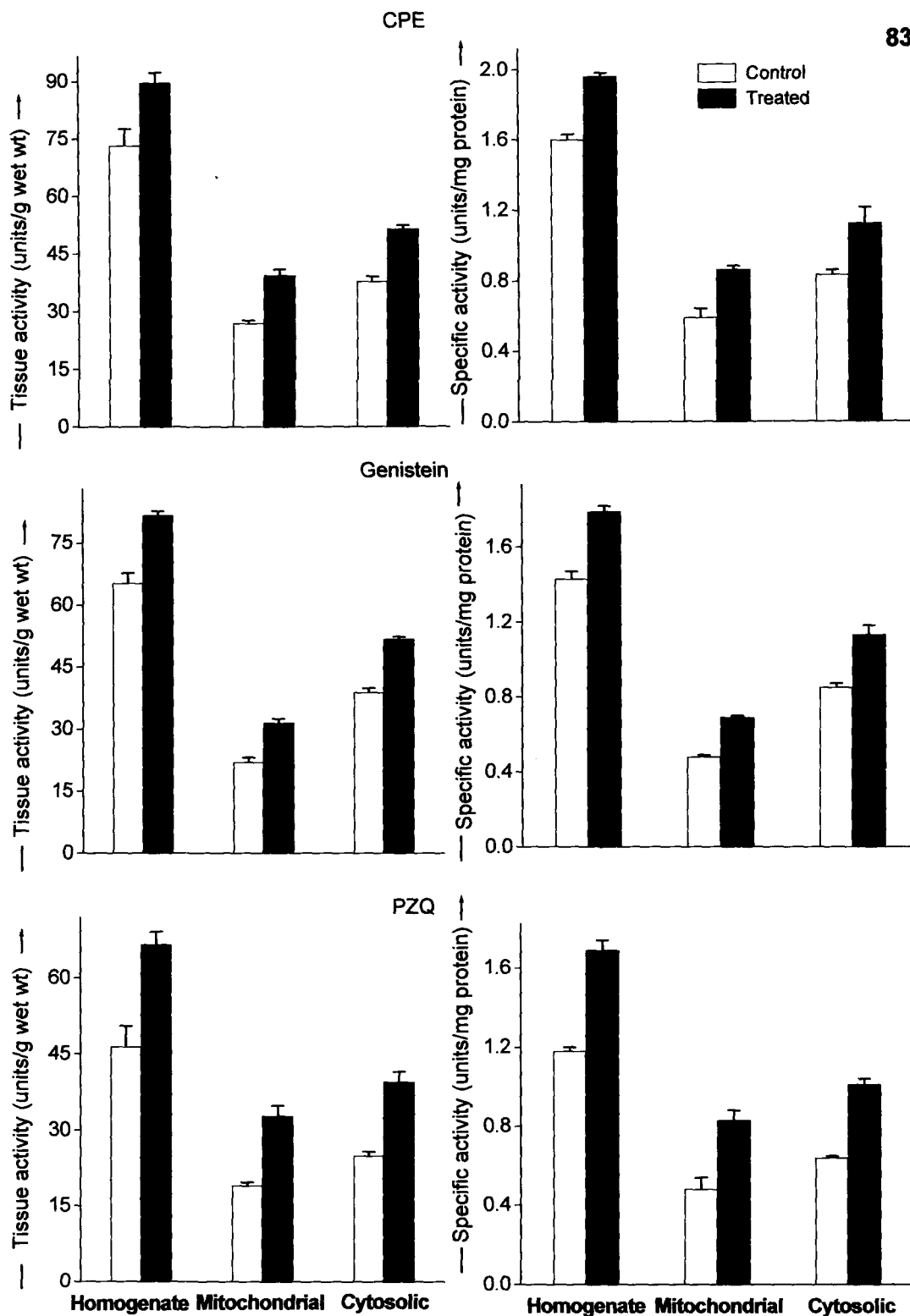


**Fig. 11.** Effects of different test materials on tissue activity (units/g wet wt) and specific activity (units/mg protein) of phosphoenolpyruvate carboxykinase and pyruvate kinase in *R. echinobothrida*. Values are plotted as mean  $\pm$  SEM ( $n = 4$ ). CPE - Crude-peel extract of *F. vestita*; PZQ - Praziquantel



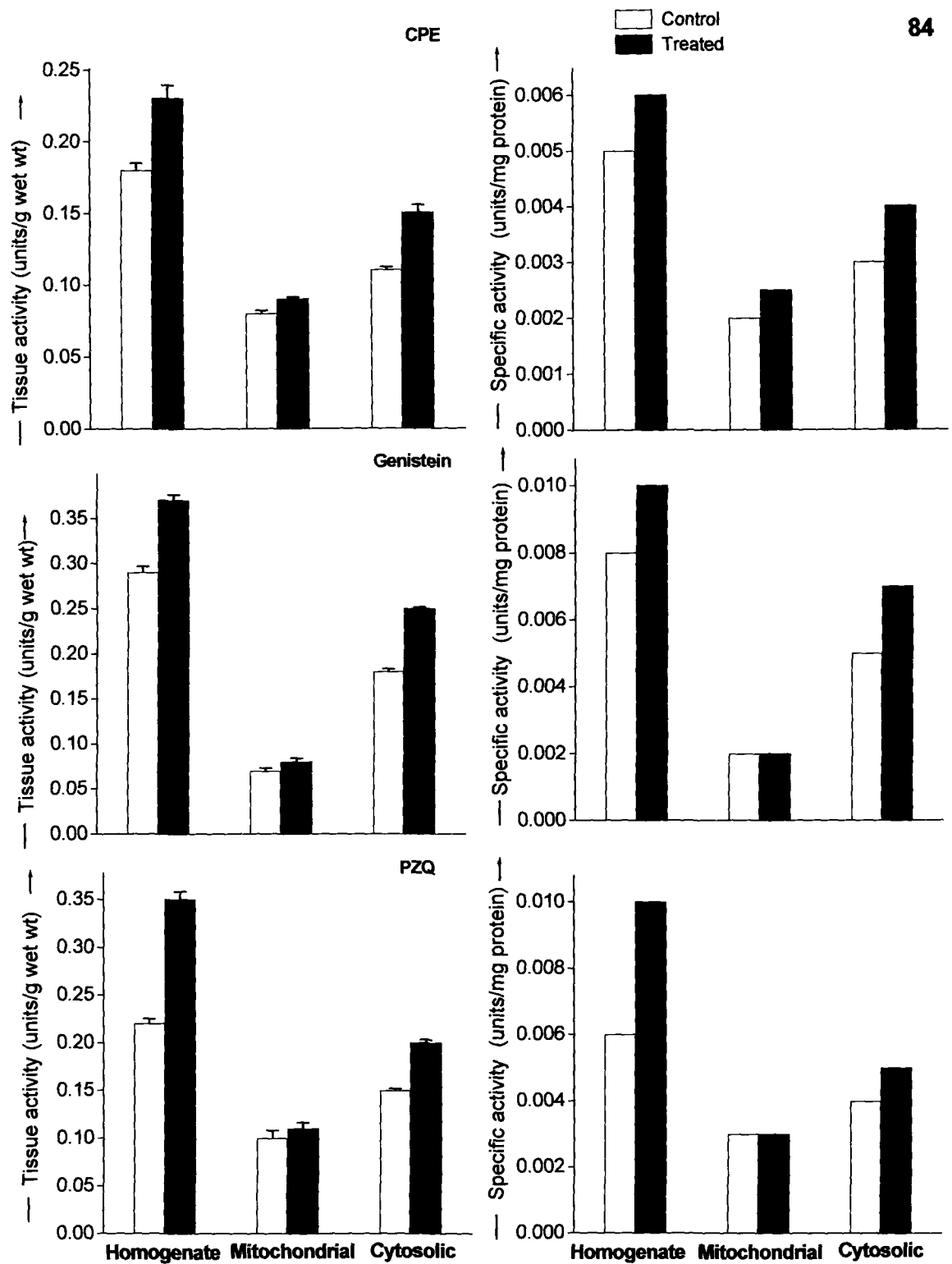
**Fig. 12.** Effects of different test materials on tissue activity (units/g wet wt) and specific activity (units/mg protein) of lactate dehydrogenase in *R. echinobothrida*. Values are plotted as mean  $\pm$  SEM (n = 4).

CPE - Crude-peel extract of *F. vestita*; PZQ - Praziquantel

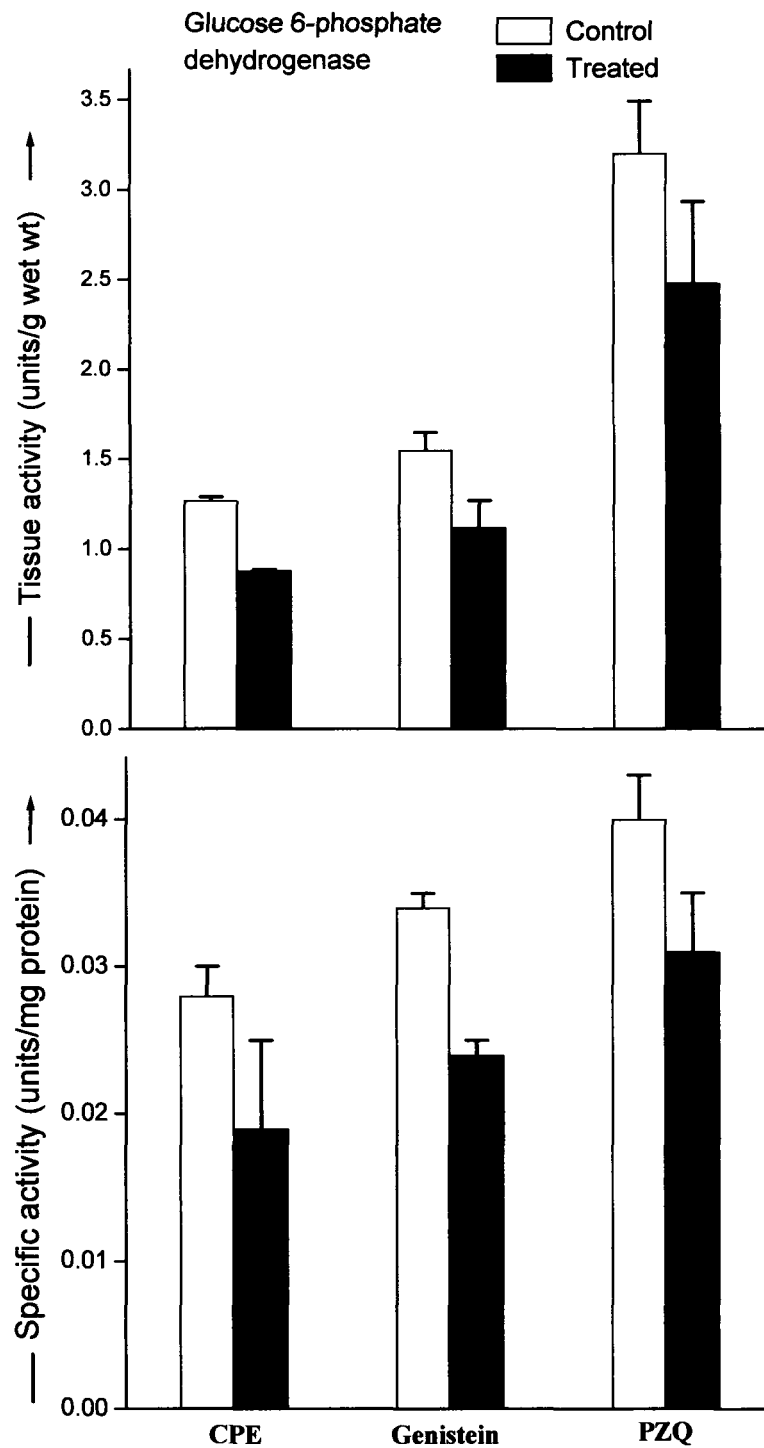


**Fig. 13.** Effects of different test materials on tissue activity (units/g wet wt) and specific activity (units/mg protein) of malate dehydrogenase in the homogenate and different sub-cellular fractions of *R. echinobothrida*. Values are plotted as mean  $\pm$  SEM (n = 4).

CPE - Crude-peel extract of *F. vestita*; PZQ - Praziquantel

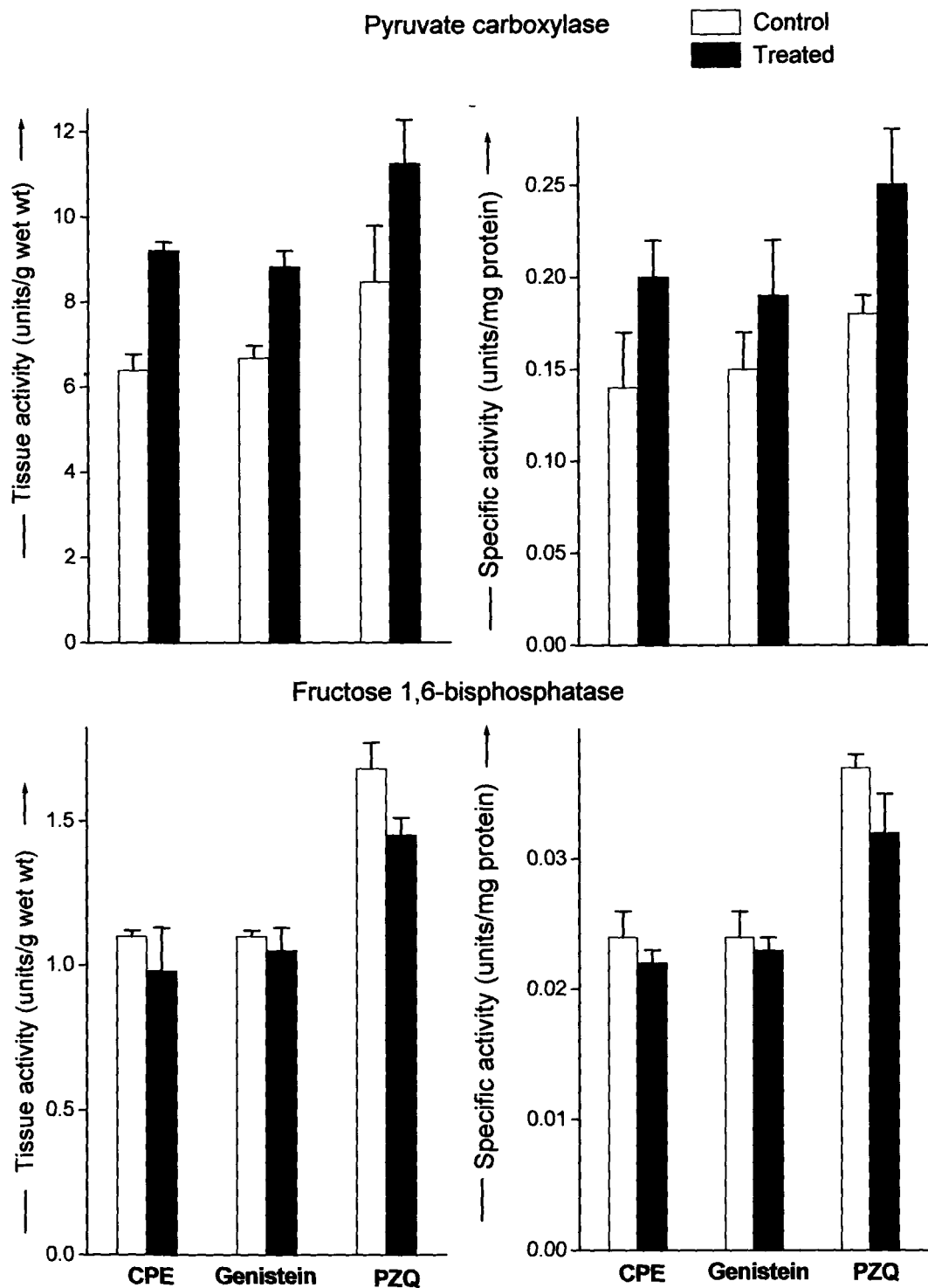


**Fig. 14.** Effects of different test materials on tissue activity (units/g wet wt) and specific activity (units/mg protein) of malic enzyme in the homogenate and different sub-cellular fractions of *R. echinobothrida*. Values are plotted as mean  $\pm$  SEM (n = 5).  
 CPE - Crude-peel extract of *F. vestita*; PZQ - Praziquantel

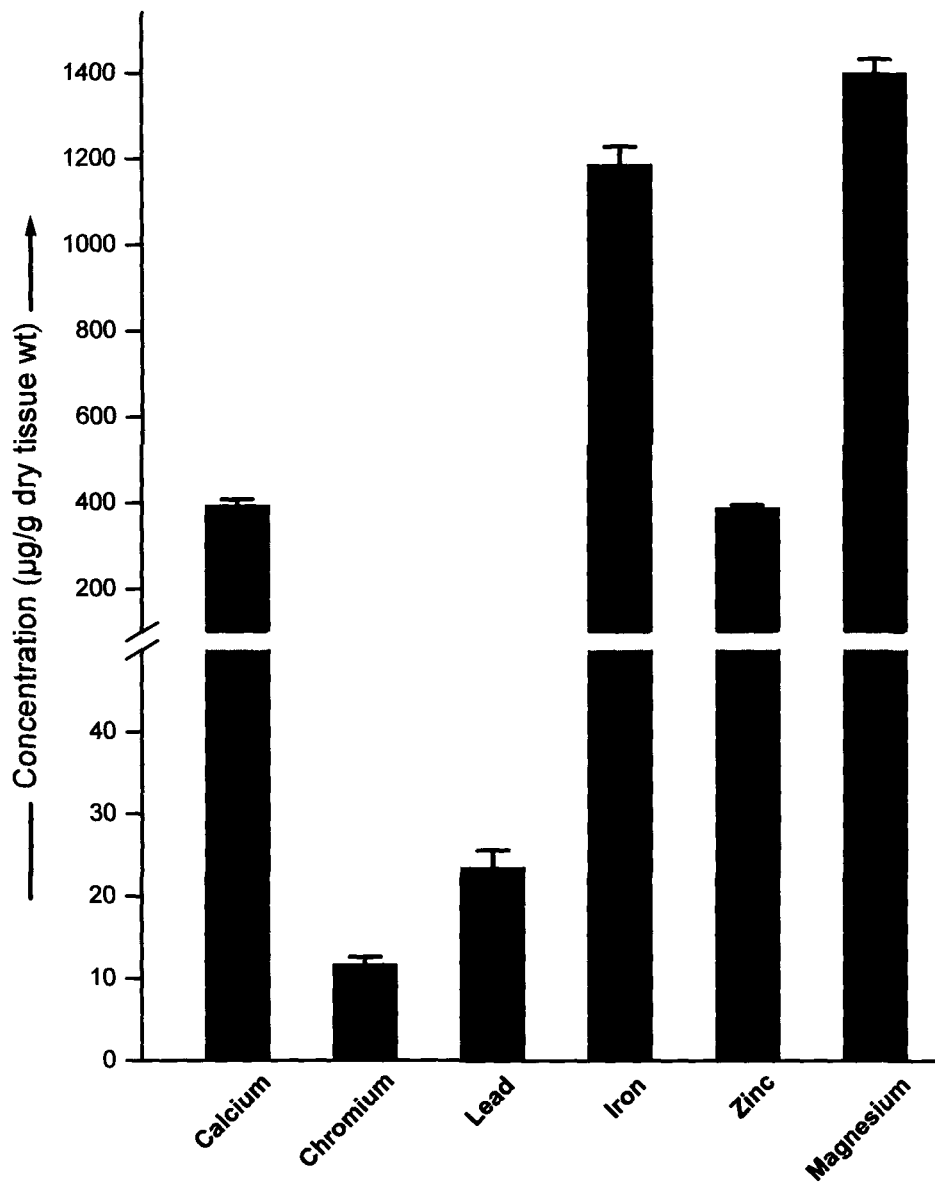


**Fig. 15.** Effects of different test materials on tissue activity (units/g wet wt) and specific activity (units/mg protein) of glucose 6-phosphate dehydrogenase in *R. echinobothrida*. Values are plotted as mean  $\pm$  SEM (n = 4).

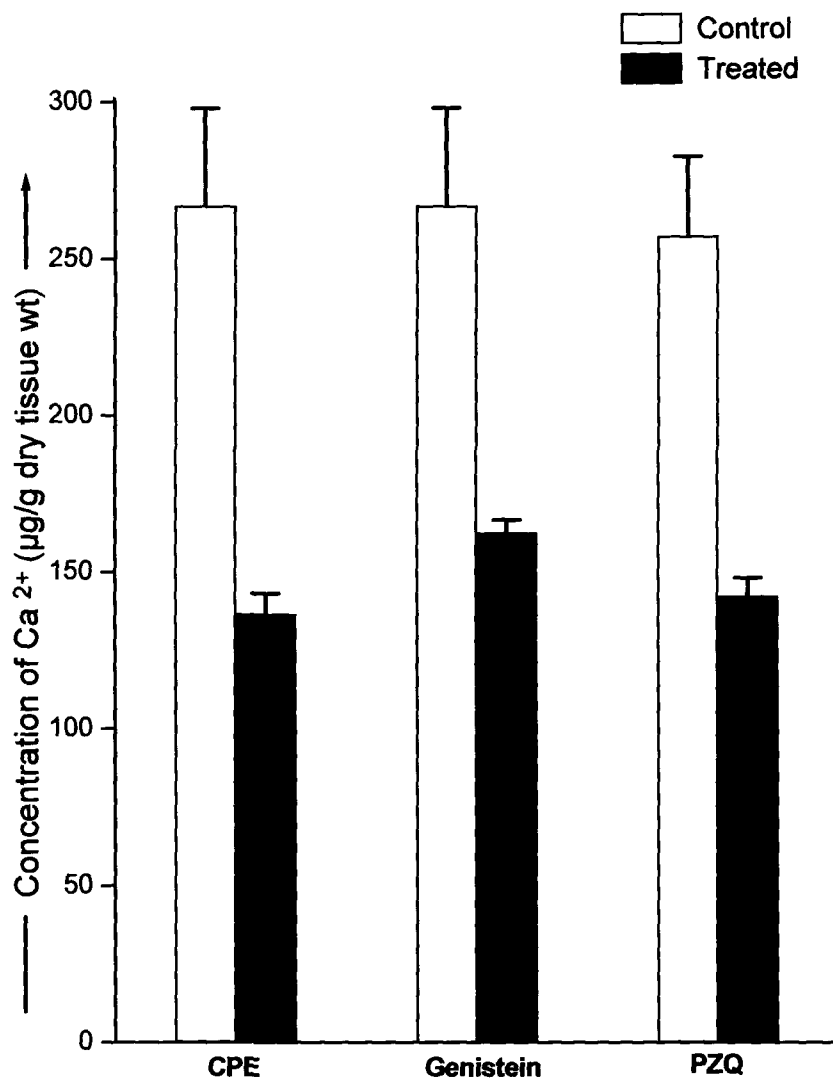
CPE - Crude-peel extract of *F. vestita*; PZQ - Praziquantel



**Fig. 16.** Effects of different test materials on tissue activity (units/g wet wt) and specific activity (units/mg protein) of pyruvate carboxylase and fructose 1,6-bisphosphatase in *R. echinobothrida*. Values are plotted as mean  $\pm$  SEM ( $n = 4$ ).  
 CPE - Crude-peel extract of *F. vestita*; PZQ - Praziquantel

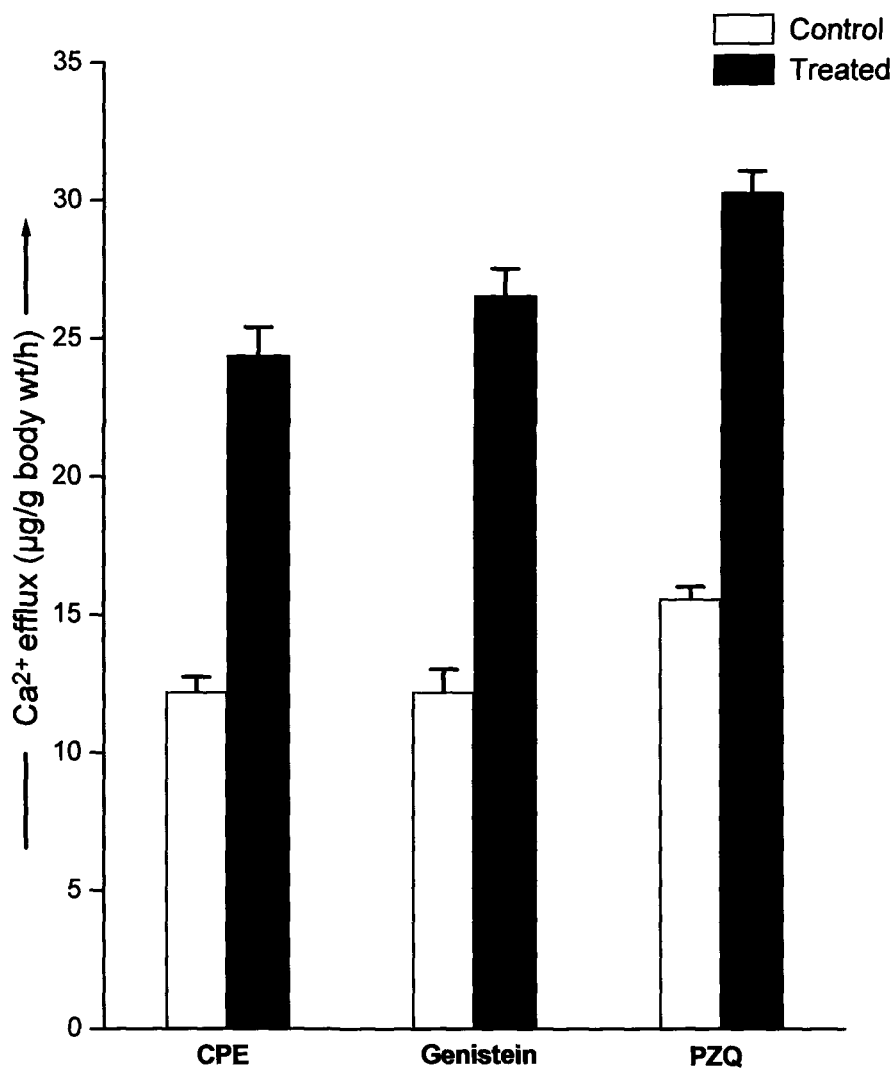


**Fig. 17.** Physiological levels of some trace elements ( $\mu\text{g/g}$  dry tissue wt) present in *R. echinobothrida*. Values are plotted as mean  $\pm$  SEM ( $n = 5$ ).



**Fig. 18.** Effects of different test materials on Ca<sup>2+</sup> level (µg/g dry tissue wt) in *R. echinobothrida* at the time of paralysis. Values are plotted as mean ± SEM (n = 4).

CPE - Crude-peel extract of *F. vestita*; PZQ - Praziquantel



**Fig. 19.** Effects of different test materials on  $\text{Ca}^{2+}$  efflux ( $\mu\text{g/g}$  dry tissue wt) by *R. echinobothrida* into the culture medium. Values are plotted as mean  $\pm$  SEM (n = 4).

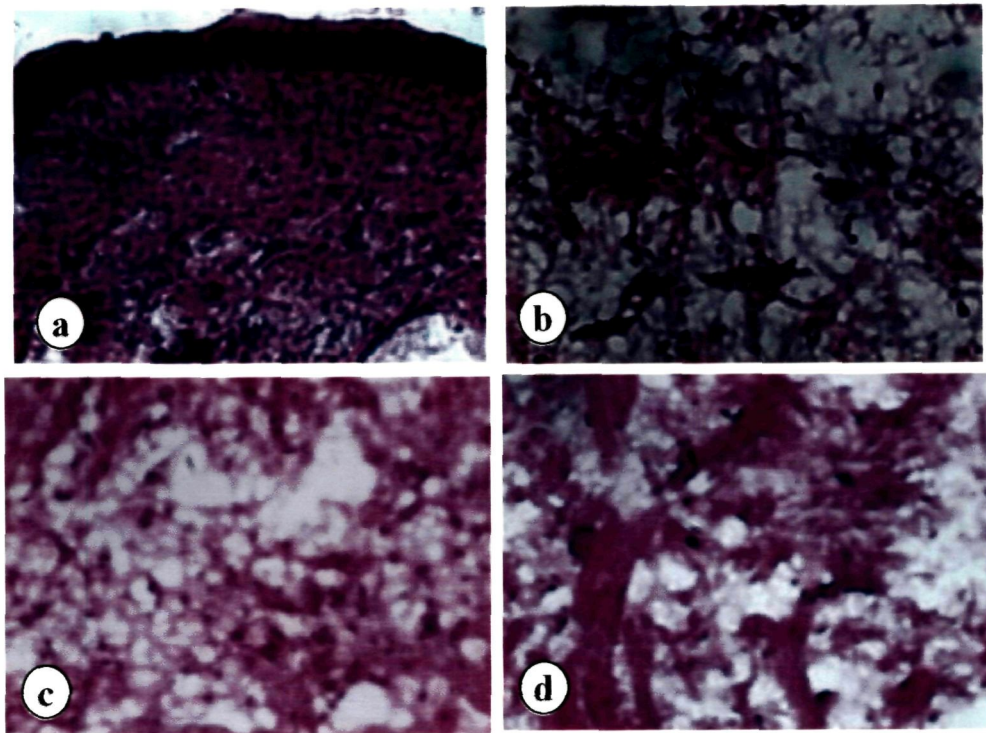
CPE - Crude-peel extract of *F. vestita*; PZQ - Praziquantel

## Plate 1



*Flemingia vestita*, whole plant

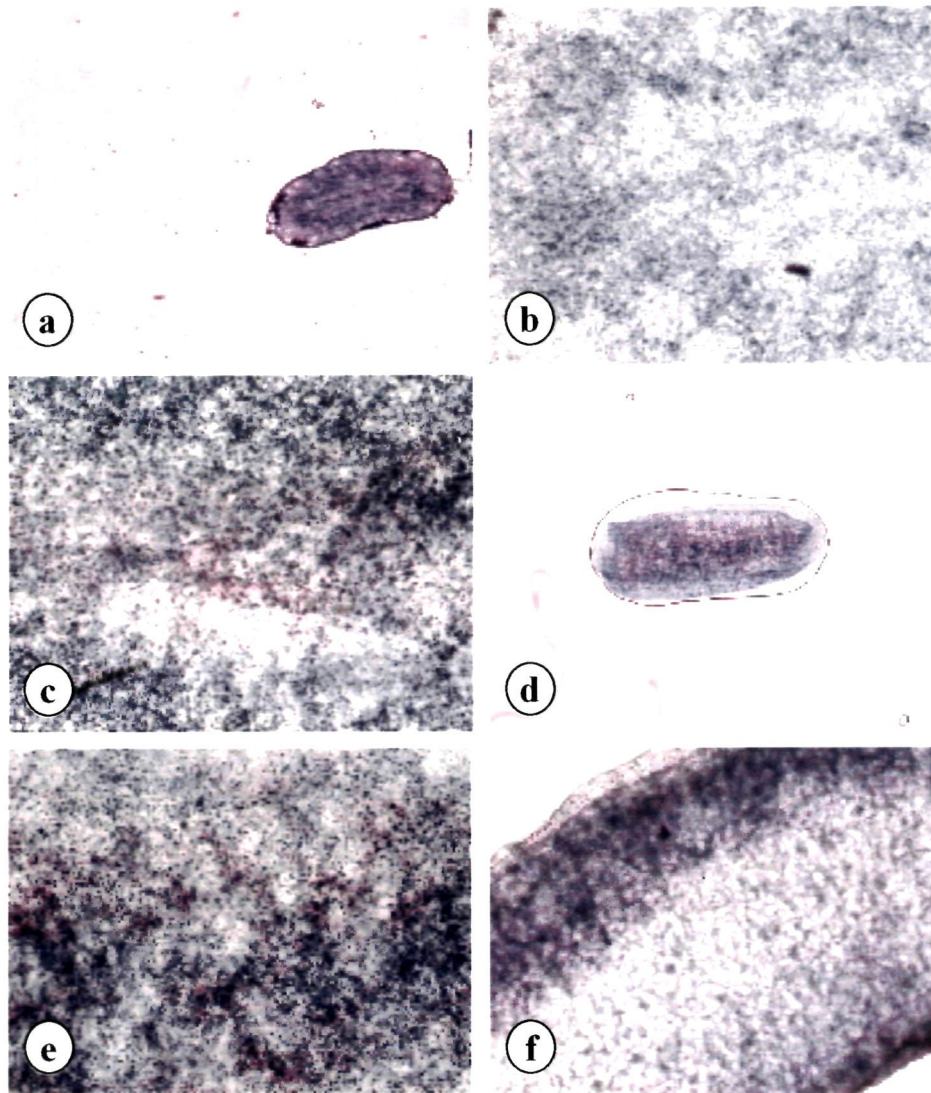
## Plate 2



**Figs. a-d. *Rallietina echinobothrida*: glycogen concentration in control and treated parasites. Photomicrographs; cross sections through mature proglottid. (Best's carmine method)**

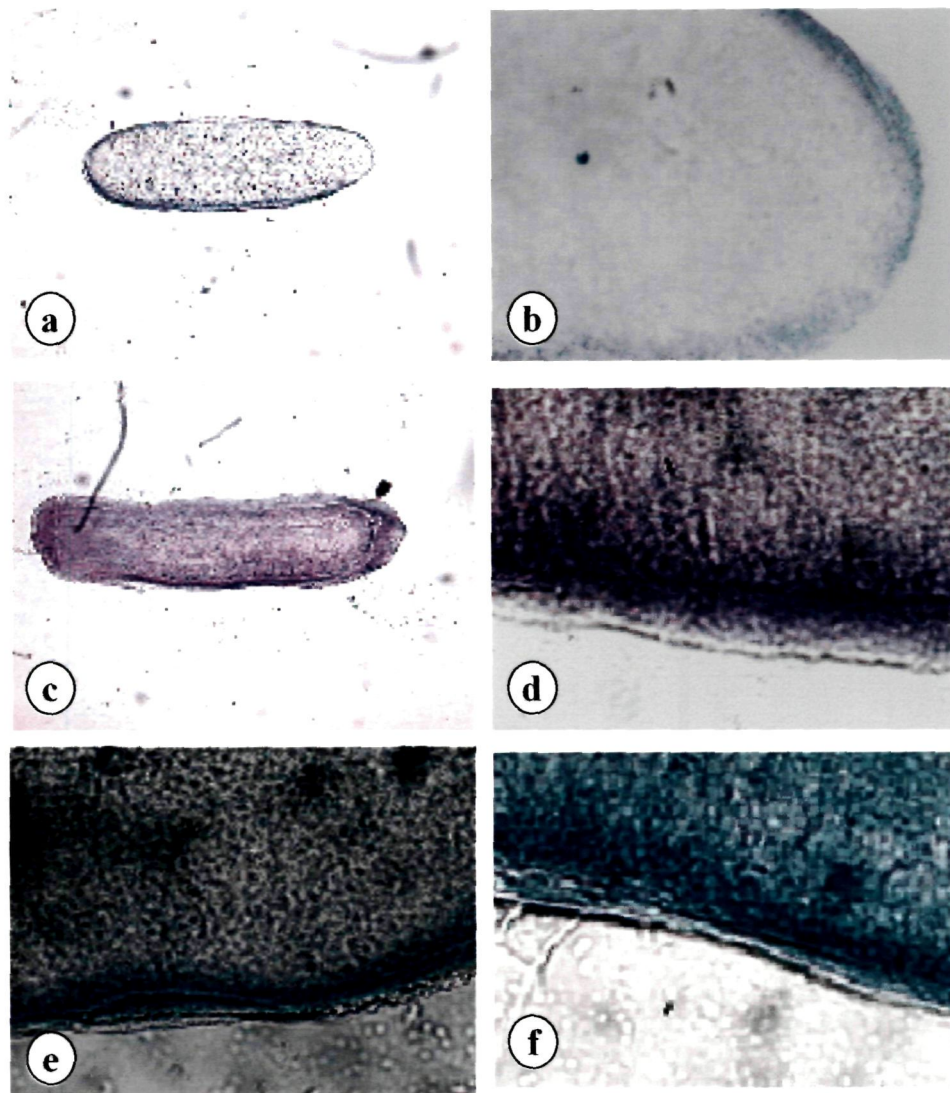
- a. Control. x 1100**
- b. Crude-peel extract. x 1100**
- c. Genistein. x 1100**
- d. PZQ. x 1100**

**Reduced stain intensity, noticeable in all treatments in comparison to control, is indicative of decline in glycogen concentration.**



- Figs. a-f. *Rallietina echinobothrida*: hexokinase (HK) activity in control and treated parasites. Photomicrographs; cross sections through mature proglottid. (Meijer's method)**
- a. Control- whole section. x 110**
  - b. Magnified view of a portion of the same. x 1100**
  - c. Crude-peel extract. x 1100**
  - d. Genistein- whole section. x 110**
  - e. Magnified view of a portion of the the same. x 1100**
  - f. PZQ. x 1100**

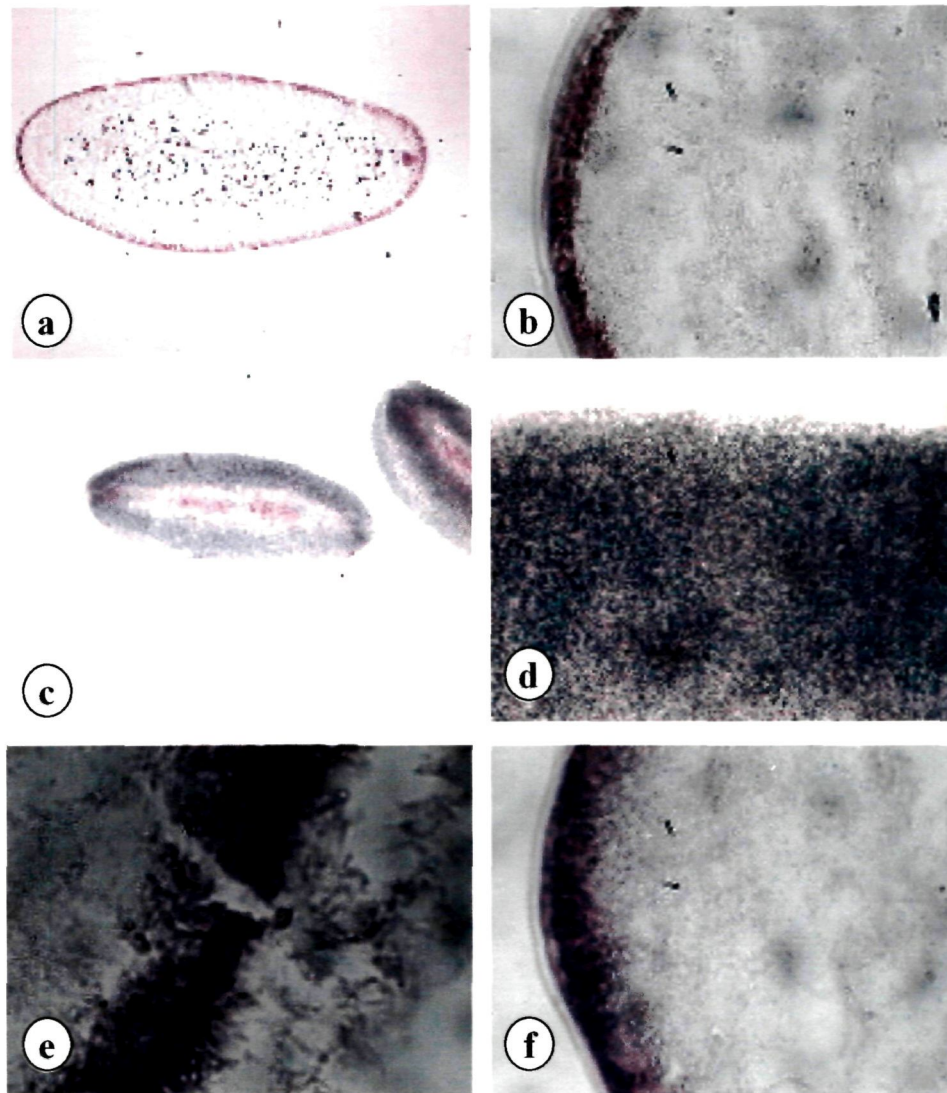
**Increased HK activity is noticeable in all treatments in comparison to control.**



**Figs. a-f. *Rallicetina echinobothrida*: lactate dehydrogenase (LDH) activity in control and treated parasites. Photomicrographs; cross sections through mature proglottid. (Jacobsen's method)**

- a. Control- whole section. x 110**
- b. Magnified view of a portion of the same. x 1100**
- c. Crude-peel extract- whole section. x 110**
- d. Magnified view of a portion of the same. x 1100**
- e. Genistein. x 1100**
- f. PZQ. x 1100**

**Increased stain intensity, indicative of increased LDH activity, is noticeable in all treatments in comparison to control.**



**Figs. a-f. *Rallietina echinobothrida*: malate dehydrogenase (MDH) activity in control and treated parasites. Photomicrographs; cross sections through mature proglottid. (after Pearse)**

- a. Control- whole section. x 110**
- b. Magnified view of a portion of the same. x 1100**
- c. Crude-peel extract- whole section. x 1100**
- d. Magnified view of a portion of the same. x 1100**
- e. Genistein. x 1100**
- f. PZQ. x 1100**

**Increased stain intensity in all treatments (c-f) indicates enhanced MDH activity in comparison to control.**

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## DISCUSSION

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Aspects of carbohydrate metabolism as indicators of anthelminticity were taken into account while ascertaining the efficacy of phytochemicals derived from *Flemingia vestita* on *Raillietina echinobothrida*, the common cyclophyllidean cestode of domestic fowl, in the present study. In view of the well-established fact that the carbohydrate reserves in cestode parasites are greatly influenced by, and fluctuate in accordance with, the nutritional status (among other things) of their host, for each set of experiments the worm material (for treatment and control) was collected from a single host and thus separate controls were maintained. The results obtained in the present study are discussed *vide infra* with reference to the chosen parameters.

### ***Glycogen and its Metabolism***

Helminth parasites are known to contain large reserves of glycogen, which acts as a major energy store. Cestodes store large quantities of glycogen in the parenchyma (Smyth and McManus, 1989). The contents of glycogen in cestodes can range up to 50% or more of dry weight (Roberts, 1983). In *R. echinobothrida*, in the present study, the glycogen content was also found to be quite high, which averaged to about 66 mg/g wet wt of tissue. The amount of glycogen stored at a given time, however, is affected by a number of environmental factors and varies with the feeding cycle and physiological status of the host, the number of parasites present (crowding effect) and the stage of development of the worms (Ramanaiah and Agarwal, 1975; Conford et al., 1983; Beg et

al., 1996). The distribution of glycogen in polyzoic cestodes is also reflected as a function of position down the strobila (Roberts, 1983). The glycogen gradient was found to be in order of mature > immature > gravid proglottids in cestodes of sheep (Tayal and Premvati, 1982). In *R. cesticillus*, the anterior halves of the strobila were found to contain higher glycogen concentration than the posterior halves and the glycogen content reduced by 45% overnight when the host was not feeding (Reid, 1942). In the present study, the glycogen level in the control parasite decreased significantly to about 40-70% within 24 h of starvation (Table 2; Fig. 4) suggesting thereby that, as true for platyhelminths, carbohydrate is the prime source of energy in this cestode too (Roberts, 1983; Bryant and Behm, 1989; Smyth and McManus, 1989). A number of anthelmintic agents have been shown to affect the glycogen concentration and metabolism in helminths (Van den Bossche, 1972; McCracken and Taylor, 1983; Bryant and Behm, 1989; Sharma et al., 1990; Jain et al., 1992; Bose et al., 1994), though several others do not cause any such changes (Criado et al., 1987). Glycogen breakdown is maximally stimulated in the trematode parasite, *S. mansoni*, when treated with PZQ in vitro (Harder et al., 1987a). While no drug-induced effect in respect to glycogen content was demonstrable in *S. mansoni* from mice dosed with Ro 15-5458, a specific inhibitor of protein kinase C (Eschete and Bennett, 1990), schistosomes recovered from artemether-treated hosts showed an increased activity of glycogen catabolism and decreased glucose uptake (Shuhua et al., 2000), thus exhibiting a helminthotoxic effect of this anti-malarial drug. Mebendazole (3.3  $\mu$ M) caused glycogen depletion and inhibited glucose uptake in *Avitellina lahorea* in vitro (Ahmad and Nizami, 1987). In the present study, the glycogen concentration in *R. echinobothrida* was found to decrease significantly by 15-

44% in the treated worms in comparison to controls (Table 2; Fig. 4). Histochemical demonstration of glycogen concentration in treated parasites in comparison to respective controls (Plate 2; Figs. a-d) also led to a similar inference.

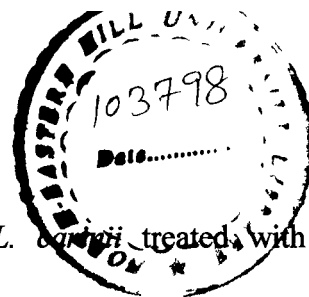
The decrease in glycogen content was accompanied by a significant increase in GPase *a* activity and decrease in GSase *a* activity in the parasite under different treated conditions (Table 3; Figs. 5 and 6). This suggests that the energy demand by the cestode was possibly enhanced under the test conditions, leading to stimulation of glycogenolysis and inhibition of glycogenesis. The regulation of GPase by various metabolites in tapeworms is presumably similar to that described in the vertebrate tissue (Roberts, 1983). The anthelmintic niridazole enhanced the activity of GPase by inhibiting the action of glycogen phosphorylase phosphatase (which dephosphorylates the active form of GPase to become inactive form) in *S. mansoni*, thus causing depletion in the glycogen reserve of the fluke (Bueding, 1970). In contrast to this, chemotherapeutics like levamisole showed a different effect on glycogen metabolism in nematodes in vitro; in *Litomosoides carinii* and *A. suum* the drug showed a decrease in the GPase activity and an increase in the activity of GSase (Komuniecki and Saz, 1982; Nelson and Saz, 1982; Donahue et al., 1983). However, even with the enhanced glycogenesis, the drug had an adverse effect by way of disrupting glycogen mobilization in the worms with diminished ATP levels due to flaccid paralysis induced by the drug. The GPase activity was found to be 25-30% higher in *H. diminuta* from amoscanate-treated hosts (Nelson and Saz, 1983). During the treatment with crude-peel extract, genistein and PZQ, the activity of GPase *a* in *R. echinobothrida* increased by 29-39%,

from which it may be suggested that the phytochemicals, as also PZQ, have a promoting role in activation of this enzyme, hence causing a decrease in the glycogen content of the parasite.

GSase and other enzymes for glycogenesis are present in cestodes as in mammals (Mied and Bueding, 1979; Barrett, 1981). GSase in *H. diminuta* is activated by inorganic phosphate, pyrophosphate and a variety of sugar phosphates including intermediates of the glycolytic pathway (Roberts, 1983). In the present study, the activity of GSase *a* in the treated worms was decreased by 36-59% in about 6 h post incubation (Table 3; Fig. 6) i.e., the time when paralysis set in. It is interesting to note that the treatment in vitro of *R. echinobothrida* with the crude-peel extract of *F. vestita*, and its active principle, genistein affected only the active form of GPase and GSase enzymes, without causing any change in their total activities. Therefore, it appears that the changes of the phosphorylation status could be one of the possible mechanisms of regulation of these two enzymes, thereby converting the existing enzyme from inactive to active form and vice versa under the influence of the various treatments. It seems unlikely that treatment with the plant-derived components for a short period of time would affect these enzymes at the transcriptional level. Alteration in  $\text{Ca}^{2+}$  concentration (as discussed vide infra) could be another means of enzyme regulation by activating the calcium-dependent protein kinases (Bollen et al., 1998).

### ***Metabolites and Enzymes of Glycolytic Pathway***

Glucose 1-phosphate, released by the stimulation of glycogenolysis by increasing the activity of GPase *a*, is converted by the enzyme phosphoglucomutase to glucose 6-phosphate to be metabolised further. Glucose and other simple carbohydrate molecules are transported by active transport, which has been demonstrated in *H. diminuta* adults (Phifer, 1960; Pappas et al., 1974; Read et al., 1974; Uglem, 1976) and larvae (Arme et al., 1973). Compared to the mammalian system, a modified form of metabolic pathways is reported to be present in cestodes (Smyth and McManus, 1989). A highly active sequence of glycolytic enzymes has been demonstrated in several cestode species, thus confirming the presence of glycolytic pathway, the main energy-yielding pathway in them (Behm and Bryant, 1975; Beis and Barrett, 1979; McManus and Smyth, 1982). Glucose and other simple carbohydrates are the main energy source for cestodes and trematodes (Roberts, 1983; Bryant and Behm, 1989). In the present study, the intracellular glucose content decreased by 36, 19 and 14% in the worms treated with the plant crude-peel extract, genistein and PZQ, respectively (Table 6; Fig. 8). This suggests that there is a need for more energy supply to the parasite under the various treatments. There was a significant decrease (20%) of glucose level in the 3 h- or 6 h- starved controls compared with the zero-time control, suggesting again that glucose is the major source of energy in the parasite. Drugs like PZQ, niclosamide and mebendazole were reported to cause changes in the catabolism of sugar towards homolactate fermentation as evidenced by the increased production of lactate in *C. digonopora* and inhibition of uptake of glucose by the parasite (Pampori et al., 1984). Inhibited glucose uptake and



increased production of lactate was also observed in *L. bangsi* treated with the antifilarial compounds, viz., 90/55 (7-oxo-1-phenyl-8, 14-dihydropyrido (3,4-b) imidazo (1,2-c) quinazolo (4,5-g) and 87/639 (6-Nitro-1-phenyl-9H-pyrido (3,4-b) indole at 0.5 and 2.0  $\mu\text{M}$  concentrations during in-vitro incubation for 2 h (Bose et al., 1994). PZQ enhanced the glucose uptake and lactate production at a concentration of 0.01  $\mu\text{M}$ , while it inhibited the effect above 10  $\mu\text{M}$  in *S. mansoni*, while the other amphiphilic drugs caused the same effect at 100-fold higher concentrations (Harder et al., 1987b). In comparison, treatment with artemether reduced the lactate content in schistosomes (Xiao et al., 1998).

The major aerobic and anaerobic end products in platyhelminth parasites are a variety of organic acids that are identified as succinate, lactate, acetate, ethanol, propionate, and malate (Rahman and Mettrick, 1982; McManus and Sterry, 1982; Barrett, 1984) and in several cases the fermentation end products are similar to aerobic (Vykhrestyuk et al., 1984). Glycogen content, glucose consumption and the production of metabolic end products by *Calicophoron ijimai* were determined under aerobic and anaerobic conditions and the major end products of fermentation were identified as lactic, acetic, propionic, isobutyric and alpha-methylbutyric acids with predominancy of propionic acid (Vykhrestyuk et al., 1984). Excretory products of carbohydrate metabolism in *H. diminuta* were also significantly altered depending on the gas phase and the presence of 5-hydroxytryptamine (Rahman et al., 1983). An isotopic study on the trematode, *F. hepatica*, showed a reduced production of propionate/acetate in the flukes treated with 0.1 mM serotonin (Matthews et al., 1986). Lactate, succinate and

acetate are the three major catabolic end products detected in *H. diminuta* (Watts and Fairbarin, 1974; Bennet et al., 1990), whereas in the pseudophyllidean *Ligula intestinalis* lactate is the main end product (Izvekova, 2000). Lactate is a product of glycolysis in the cytosol, whereas succinate and acetate are formed by reactions that take place in the mitochondria (Bennet et al., 1993). In the present study, significant levels of lactate, malate and alanine were found to be present in the *R. echinobothrida* tissue, with not much change in the concentration of these metabolites in the starved controls that were kept in vitro for 6 h or 3 h compared to zero-time control (Table 6; Fig. 8). On exposure to the crude-peel extract, genistein and PZQ, the lactate and alanine contents in the parasite tissue decreased by 20-31% and 7-25%, respectively in comparison to untreated controls, whereas the malate concentration increased by 49-134% (Table 6; Fig. 8). There was significant increase in the lactate efflux in the incubation medium, as also in the net lactate production, by the parasite treated with the crude-peel extract and PZQ (Table 7; Fig. 9). More production of lactate, as also of the malate content, in *R. echinobothrida* under treated conditions might have resulted due to anaerobiosis, as suggested in some other cestodes under rapid muscular contraction (Smyth and McManus, 1989). More net total production of lactate in the case of parasites treated with crude-peel extract compared to the genistein treated parasites could be due to the fact that the crude-peel extract of *F. vestita* root peel contains other active components such as diadzein and formononetin in addition to genistein even though in very low concentration; these components perhaps bring about higher activity of the crude-peel extract than genistein alone.

Minor change in the pH of the incubation medium containing the crude-peel extract (Table 7) is suggested to be due to more efflux of lactate. The gradual deceleration of pH changes in the incubation medium probably suggests that a balance was established between the metabolic processes of *R. echinobothrida*, accompanied by the excretion of lactate as an end product as suggested by Izvekova (2001). However, it also seems likely that this balance would be influenced by the various test materials used in the present study.

Pyruvate was not detectable in both the controls and the treated parasites, though the presence of pyruvate as an end product of carbohydrate metabolism has been reported in *L. intestinalis* and *C. digonopora* (McManus and Sterry, 1982; Pampori et al., 1984).

Glycolytic enzymes, such as HK, PFK, aldolase, G3PDH, PK, PEPCCK, LDH and ME have been reported to be present in cestodes (Arme and Pappas, 1983). Glycolysis, suggested to be the main energy-generating pathway with lactate as an end product (Omar et al., 1996), is considered to be a promising target for new drugs against trypanosomatid parasites, because this pathway plays an essential role in ATP supply (Lakhdar-Ghazal et al., 2002). Some of the enzymes related to glycolytic pathway are known to be regulated by allosteric modulators and some are by covalent modification of enzymes (Nelson and Cox, 2000).

### ***Hexokinase***

HK, that catalyzes the transfer of a phosphoryl group from ATP to glucose to form glucose 6-phosphate and ADP, is the first enzyme in the glycolytic pathway and is found in several cestodes (Smyth and McManus, 1989). Some carboline antifilarials, viz., 90/55 (7-oxo-1-phenyl-8, 14-dihydropyrido (3,4-b) imidazo (1,2-c) quinazolo (4,5-g) and 87/639 (6-Nitro-1-phenyl-9H-pyrido (3,4-b) indole at 0.5 and 2.0  $\mu\text{M}$  concentrations significantly lowered the HK activity but increased the activities of GPase, PFK and PK. These compounds also substantially inhibited glucose uptake and increased lactate concentration in *L. carinii* during in-vitro treatments (Bose et al., 1994). However, mebendazole (3.3  $\mu\text{M}$ ) had no effect on HK in *A. lahorea* but influenced the activities of some glycolytic enzymes such as phosphorylase, phosphoglucomutase and G6Pase; it also caused glycogen depletion and inhibition of glucose uptake in vitro (Ahmad and Nizami, 1987). The oxidation of external [ $^{14}\text{C}$ ]-labeled glucose at a concentration of 0.5 mM in *S. mansoni* was reported to be inhibited by 80% in the presence of 5-thioglucose (20 mM) without inhibiting the conversion of endogeneous glycogen to lactate, thus suggesting that the inhibition of glucose oxidation occurred at the entrance of glucose into the cell and/or at the hexokinase reaction possibly by inhibiting the activity of hexokinase competitively (Tielens et al., 1985). In *H. microstoma*, the specific activity of HK, PFK and PK was not stimulated by 5-hydroxytryptamine, but  $\text{CO}_2$  fixation by PEPCCK was inhibited (Rahman et al., 1982). The HK activity in the present study increased significantly by 33-39% in treatments with crude-peel extract, genistein and PZQ, respectively (Table 8; Fig. 10) as was also revealed to be so in histochemical demonstration (Plate 3; Figs. a-f). This suggests that more of glucose was utilized during

the treatments to yield more energy.

### ***Phosphofructokinase***

PFK, the pace maker enzyme of the glycolytic pathway, catalyzes the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate and its activity is regulated by a number of compounds. The most potent activators of the liver fluke PFK were found to be fructose 2,6-bisphosphate and AMP (Kamemoto et al., 1987; Kamemoto and Mansour, 1986). PFK has been investigated in adult *M. expansa* and in plerocercoids of *S. solidus* (Beis and Theophilidis, 1982). In contrast to the mammalian enzyme, PFK in *A. suum* is activated by phosphorylation (Daum et al., 1986). In *A. suum* and *F. hepatica*, phosphorylation of PFK is catalyzed by a cAMP-dependent protein kinase (Thalhofer et al., 1988; Kamemoto et al., 1989; Kamemoto and Mansour, 1986) and inhibited by PFK dephosphorylating phosphatases (Daum et al., 1992). It is supposed that phosphorylation of PFK plays an important role in the regulation of carbohydrate metabolism, particularly glycolytic pathway, in the filarial as well as the intestinal-dwelling nematodes (Kulkarni et al., 1987; Srinivasan et al., 1988). In the present study, the PFK activity increased by 125% in the PZQ-treated parasites, whereas 49 and 41% increase, respectively, was observed in the genistein- and crude-peel extract- treated parasites (Table 8; Fig. 10), indicating that the rate of glycolysis was also stimulated in the parasites under treated conditions. Although it is difficult to explain the possible mechanism(s) of stimulation of PFK in *R. echinobothrida* under all the treatments, a change in the phosphorylation status of this enzyme seems to be a plausible reason for the same.

### ***Phosphoenolpyruvate Carboxykinase***

PEPCK, a rate-limiting enzyme at the branch point of phosphoenolpyruvate, has been reported from a number of cestodes such as *M. expansa*, *H. diminuta* and *S. erinacei*, etc. (Behm and Bryant, 1975; Moon et al., 1977; Fukumoto, 1985). It is believed that *Moniliformis dubius* (Acanthocephala) is the first invertebrate animal from which the PEPCK was purified (Cornish et al., 1981). PEPCK was also demonstrated in nematodes, namely *Onchocerca volvulus*, *O. gibsoni* and *A. suum* (Walter and Albiez, 1986; Rohrer et al., 1986). In adult, *F. hepatica* PEPCK was shown to be the most important route for degradation of glucose (Tielens et al., 1987). In *Setaria digitata*, PEPCK and other glycolytic enzymes such as PK, MDH, ME, aspartate and alanine transaminases have been reported to be present in the cytosolic fraction, and the tricarboxylic acid cycle enzymes such as succinate dehydrogenase (SDH), fumarate reductase (FR), fumarase (malate dehydration), MDH (malate oxidation and oxaloacetate reduction) and ME (malate decarboxylation) in the mitochondrial fraction (Banu et al., 1991). PEPCK's main role in cestodes has been shown in degradation of glucose molecule rather than synthesis of glucose through gluconeogenic pathway (Smyth and McManus, 1989). Protoscoleces of the horse and sheep strains of *E. granulosus* and the closely related *E. multilocularis* are shown to fix carbon dioxide via PEPCK (McManus and Smyth, 1982). However, in *S. mansoni*, experiments with inhibitors of PEPCK gave no indications that this enzyme is involved in the degradation of glucose, and it was confirmed that lactate was formed from phosphoenolpyruvate via the actions of PK and LDH (Tielens et al., 1991). In *C. ijimai*, high activities of PEPCK and MDH but relatively low activities of PK and LDH have been reported (Vykhrestyuk

et al., 1984). Involvement of PEPCK in glucose break down was also evidenced in *Dipetalonema viteae* (Christie et al., 1987). In the present study, the PEPCK activity was found to be high in *R. echinobothrida*, averaging to be about 18.85 units/g wet wt. in control parasite. The activity of PEPCK was further stimulated by 49, 45 and 44% in the parasites treated with crude-peel extract, genistein and PZQ, respectively (Table 8; Fig. 11). The lower concentration of pyruvate (below the level of detection), noticed in the present study, in the parasite can be explained in the light of higher activity of PEPCK over PK in controls as well as in all treatments.

### ***Pyruvate Kinase***

PK, another potential regulatory enzyme of glycolysis, converts phosphoenolpyruvate to pyruvate, and its possible modulators has been investigated in a number of cestodes (Smyth and McManus, 1989). The PK, glyceraldehyde-phosphatedehydrogenase and phosphoglycerate kinase activities were inhibited by artemether both in male and female *S. japonicum* (Xiao et al., 1998). A marked inhibition occurred in the activity in PK, PEPCK and ATPase in the *E. granulosus* cyst wall on treating with mebendazole and albendazole, whereas PZQ had no effect, suggesting thereby that PK, PEPCK, and ATPase might be potential chemotherapeutic targets (Xiao et al., 1994; Xiao et al., 1995). Sumarmin, at a low concentration, caused a marked inhibition of most of the enzymes of phosphoenolpyruvate-succinate pathway in *S. cervi* (Hussain et al., 1990). Centperazine [developed by Central Drug Research Institute (CDRI), Lucknow, India] showed significant inhibitory action on PK, LDH, fumarase and SDH, while the CDRI compound 72/70 showed significant inhibition of

PEPCK activity; Diethylcarbamazine and levamisole, however, were found to be more or less ineffective at a lower concentration against all the enzymes of glycolytic pathway (Hussain et al., 1990). The PK activity decreased by about 14-15% in *R. echinobothrida* in treatments with genistein and PZQ, with 26% decrease in the case of crude-peel extract treatment (Table 8; Fig. 11). The lower activity of PK and the inhibitory action of the test materials corroborate the presence of very less concentration of pyruvate in the tissue of the parasite. Thus, the branch point of PEPCK/PK perhaps forms the basis of anthelmintic attack.

### ***Lactate Dehydrogenase***

LDH, which catalyzes the reversible terminal reaction in glycolysis with formation of lactate from pyruvate, has been demonstrated to be present in a number of cestodes, though the properties of the enzyme have been investigated in details only in *Hymenolepis* spp. (Burke et al., 1972). The existence of structural and kinetic differences between the LDH isoenzymes of the host and parasitic origins, as revealed in the filaroid *Molinema dessetae*, makes the latter potential chemotherapeutic targets (Marchat et al., 1996). Many anthelmintics are believed to be working via alteration in the activity of the parasite LDH. For example, benzimidazoles (mebendazole, fenbendazole and albendazole etc.) act primarily via activation of LDH, catalyzing the conversion of pyruvate to lactate (Veerakumari and Munuswamy, 2000). Parachloromercuribenzoate and iodoacetate affected the LDH activity of the digenetic trematodes, *Gigantocotyle explanatum* and *Gastrothylax crumenifer* and their host (ruminant mammals) tissues differently (Haque et al., 1990). Artmether, a derivative of

the plant, *Artemesia* and well known for its antimalarial properties, was shown to exert a potent inhibitory action on the LDH and G6PDH activities in *S. japonicum* (Xiao et al., 1999). Filarin (a drug used in Siddha medicine) and diethylcarbamazine inhibited pyruvate reduction rather than lactate oxidation in *S. digitata* by altering the activity of LDH (Banu et al., 1989). The activities of both LDH and MDH were strongly inhibited by suramin in adult *O. volvulus* (Walter and Schulz-Key, 1980). Treatments with hetrazan, levamisole and tetramisole of the adult *S. cervi* worms revealed the decreased activities of G6Pase, FBPase, LDH and ATPase (Khatoon et al., 1983). LDH was not affected significantly by levamisole, albendazole and parbendazole in *Ascaridia galli* and *Heterakis gallinae*, but levamisole completely inhibited the MDH activity in both directions in these two parasites (Sharma et al., 1987, 1989). With treatment of isatin, the alkaline phosphatase and LDH activities decreased and the acid phosphatase activity increased in the metacestodes of *E. multilocularis*, in which glucose and glycogen stores also declined significantly (Delabre-Defayolle et al., 1989). In *R. echinobothrida*, the activity of LDH increased by 67% in genistein treatment but by 55% and 58% in treatments with crude-peel extract and PZQ, respectively (Table 8; Fig. 12). Histochemically also alteration in the total LDH activity under the influence of the various treatments was demonstrable (Plate 4; Figs. a-f). The possible higher activity of LDH can be explained by the formation of pyruvate by the increased activity of cytosolic ME (by oxidative decarboxylation of malate), as the parasite contains less concentration of pyruvate. The increased net production of lactate in the parasite tissue under various treatments also corroborates it.

### *Malate Dehydrogenase*

The presence of MDH, which converts oxaloacetate to malate, has also been demonstrated in several cestodes (Smyth and McManus, 1989). Drugs like oxyclozanide, hexachlorophene, nitroxylin, rafoxamide and diamphenethide inhibited the MDH activity in trematodes *F. gigantica*, *F. buski* and *Paramphistomum explanatum* (Probert et al., 1981). Assay of MDH, following the in-vitro treatment with cambendazole and tiabendazole, exhibited moderate inhibition of the enzyme activity in both *H. gallinae* and *A. galli*, but haloxon had little effect on any of them (Sharma et al., 1986). High levels of MDH were found in *Trichuris ovis* and the activity of soluble MDH was greater than that of the mitochondrial one (Sanchez-Moreno et al., 1989). Host and parasite MDH activities were both inhibited by a series of benzimidazoles and pyrimidine-derived compounds, some of which markedly reduced only the parasite enzyme activity, but not host enzyme activity (Sanchez-Moreno et al., 1987, 1989; Tejada et al., 1987). Diethylcarbamazine citrate did not change appreciably the activity of either mitochondrial MDH or mitochondrial ME, while filarin, a drug of herbal origin, effectively inhibited mitochondrial MDH in *S. digitata*. The leaf extracts of *Ocimum sanctum*, *Lawsonia inermis* and *Calotropis gigantea* and leaf and flower extracts of *Azadirachta indica* were, however, found to inhibit both mitochondrial MDH and mitochondrial ME (Banu et al., 1992). The MDH activity was suppressed by mebendazole, albendazole and PZQ, the inhibition rates being 34.6-61.6, 59.8 and 50.6%, respectively, but with no apparent effects on SDH and FR activities in the *E. granulosus* cyst wall (Xiao et al., 1993). The cytosolic fraction of MDH from the ovine liver *E. granulosus* protoscolices was not inhibited by p-hydroxymercuribenzoate or

fumarate but mebendazole and fructose 1,6-bisphosphate inhibited the enzyme (Vessal and Tabei, 1996). In the present study, the MDH activity in *R. echinobothrida* increased in all the treatments. The activities of mitochondrial and cytosolic MDH in *R. echinobothrida* were shown to be altered following treatments with all the test materials (Table 10; Fig. 13). The MDH activity in the tissue homogenate was found to be increased by 22, 25 and 43% in treatments with crude-peel extract, genistein and PZQ, respectively; the increase was 47, 43 and 73% in the mitochondrial fraction and 36, 33 and 58% in the cytosolic fraction. The intensity of stain of formazan particles, which is indicative of the enzyme activity, increased significantly in all the treatments in comparison to the control (Plate 5; Figs. a-f). The increase in the activity of MDH can be attributed to the increase in the concentration of malate in the tissue of the parasite.

### ***Malic Enzyme***

ME, the NAD-malic enzyme, catalyzes the divalent metal ion-dependent oxidative decarboxylation of L-malate to yield CO<sub>2</sub>, pyruvate and the reduced dinucleotide. And with reference to cestodes, it has been most investigated in *H. diminuta* (Fioravanti and Saz, 1980; Roberts, 1983). ME (NADP(H)-dependent) was demonstrated to be present in the cytosol and mitochondria of both juvenile and adult *F. hepatica* (Tielens et al., 1981, 1987). Suramin was found to inhibit the activity of ME in filarial worms. The ME activity has been evidenced in the cytoplasm and mitochondria of *Taenia crassiceps* larvae (Zenka and Prokopic, 1987) and also in the juvenile and adult *Fasciola* flukes (Tielens et al., 1989). Most of the anthelmintic compounds synthesized by the Central Drug Research Institute, were not found to inhibit ME in *H. diminuta* (Wani and

Srivastava 1994). The activity of ME in the tissue homogenate, mitochondrial and cytosolic fractions of *R. echinobothrida* was also observed; the ME activity in the tissue homogenate was found to increase by about 28% in treatments with crude-peel extract and genistein and by 25% in treatment with PZQ in comparison to controls (Table 12; Fig. 14). While there was no significant increase in the activity of ME in the mitochondrial fraction, the enzyme activity increased by 36, 39 and 33% in the cytosolic fraction with these treatments. The higher activity of cytosolic ME is likely to increase the pyruvate concentration, which is perhaps immediately converted to lactate and excreted out by the higher activity of LDH (Fig. 1).

### ***Enzymes of other Metabolic Pathways***

Scanty information is available regarding the pentose phosphate pathway in cestodes. Its main role is not to provide ATP but NADPH for fatty acid synthesis and pentoses (particularly D-ribose 5-phosphate) for nucleic acid synthesis (Smyth and McManus, 1989). A complete sequence of enzymes for pentose phosphate pathway has been demonstrated only in larval *E. granulosus* (Agosin and Aravena, 1960). However, the first two enzyme of the pathway, namely G6PDH, which involves formation of 6-phosphogluconate from glucose 6-phosphate, and 6PDH have been reported in several cestodes, both pseudophyllidean and cyclophyllidean (Körting and Barrett, 1977; McMannus and Sterry, 1982; Roberts, 1983). The presence of significant activity of 6-PDH in the nematode *O. fasciata* and G6PDH in *Angiostrongylus cantonensis* indicates that the pentose phosphate pathway might be operative in helminths (Shih and Chen,

1982; Omar et al., 1996). Histochemically, G6PDH, PFK and G3PDH were demonstrated in the hypodermal tissue, somatic muscle and reproductive organs of the adult female *O. fasciata*, indicating that both the glycolytic and pentose phosphate pathways are active in various tissues of the worm (Omar and Raoof, 1994b). Thiophenate also caused an increase in the activities of G6PDH, GDH and nonspecific esterases and a decrease in reduced nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) activity. In comparison, the activities of G6PDH, NADPH-d, cytochrome oxidase, monoamine oxidase and nonspecific esterase enzymes remained unaltered in the epithelium due to fenbendazole treatment, whereas the GDH activity got inhibited in *Haemonchus contortus*, the trichostrongylid (Kaur and Sood, 1992). The G6PDH activity in *R. echinobothrida* was found to decrease by 31, 28 and 23% in all the treatments, i.e., with the crude-peel extract of *F. vestita*, genistein and PZQ, respectively (Table 14; Fig. 15). In histochemical demonstration also the intensity of formazan particles decreased significantly in all the treatments in comparison to the control (Plate 6; Figs. a-f). Decrease in the activity of G6PDH, which is the regulatory enzyme of the pentose phosphate pathway, shows that the parasite employs its metabolic pathway primarily for the formation of reduced NADPH for energy yielding and not for the biosynthetic purpose under the treatment conditions.

The three gluconeogenic enzymes, namely PC (which is involved in anaplerotic reactions in mitochondria and gluconeogenesis), FBPase (which dephosphorylates the 6' phosphate from fructose 1,6-bisphosphate) and G6Pase (which dephosphorylates the glucose 6-phosphate) have been reported in several cestodes (Pampori et al., 1985;

Tielens et al., 1991; Omar and Raof, 1994a; Unnikrishnan and Raj, 1995). The function of this pathway is under scrutiny in cestodes. The activities of PC, FBPase and G6Pase along with PEPCK were demonstrated in homogenates of adult *S. mansoni*; all the four gluconeogenic enzymes were found to be present in the worm, though experiments with <sup>14</sup>C-labelled substrates failed to demonstrate the actual occurrence of gluconeogenesis in the parasite (Tielens et al., 1991). In the present study, the activity of PC in the cestode *R. echinobothrida* increased by 32-44% in all the treatments with the test materials (Table 14; Fig. 16).

The purified brush border membrane of the cestode *C. digonopora* showed the presence of a number of phosphohydrolases including the FBPase. The treatment of isolated brush border membrane with mebendazole, niclosamide and PZQ in vitro did not alter the activity of these enzymes, though treatment of intact worms drastically affected the integrity of the membrane (Pampori et al., 1985). The decreased activities of FBPase, LDH, G6Pase and ATPase were also evidenced in the drug-treated adult *S. cervi* worms (Khatoon et al., 1983). In the present study, the presence of FBPase was demonstrated, but there was no significant change in the activity of the enzyme under different treatments (Table 14; Fig. 16). Significant increase of activity of most of the gluconeogenic enzymes, except for FBPase, by all the test materials in *R. echinobothrida* could be an indication that the glucose production by gluconeogenic pathway was affected by all the test materials.

### ***Trace Elements***

Trace elements like calcium, copper, manganese, magnesium, lead, iron, nickel, zinc and potassium are reported to be present in some helminth parasites, viz., amphistomid trematodes (Tandon and Roy, 1994), nematode (Baruš et al., 1999; Tenora et al., 1999, 2000) and cestodes (Baruš et al., 2000; Tenora et al., 2000). In *R. echinobothrida*, in the present study, some trace elements were also detected, of which the concentration of magnesium was found to be the highest (~1400 µg) and that of chromium, the lowest (~12 µg), while manganese, cadmium and nickel were below the level of detection (Table 16; Fig. 17).

Ca<sup>2+</sup>, which is stored in calcareous corpuscles of many cestodes especially the larvae, is intimately involved in both muscle contraction and many aspects of cell movement controlled by the cytoskeleton (Bryant and Behm, 1989). Some enzymes, like GPase, GSase and protein kinases, are allosterically regulated by various modulators, where Ca<sup>2+</sup> plays a key role (Bollen et al., 1998) and also in membrane internalization (Ribeiro et al. 1998). PZQ, calcium ionophore A-23187 and the benzodiazepine Ro 11-3128 disturbed the calcium homeostasis and caused paralysis in *S. mansoni* and also in cestodes (Bryant and Behm, 1989). Though PZQ clearly affects Ca<sup>2+</sup> homeostasis in schistosomes, the mode of action of PZQ is yet unknown, and possibly it interacts with the schistosome voltage-gated Ca<sup>2+</sup> channels (Kohn et al., 2001). PZQ is reported to interact with specific Ca<sup>2+</sup>-permeable sites in the tegumental and sarcoplasmic membranes of *S. mansoni* (Blair et al., 1992). In vitro, PZQ is reported to stimulate

verapamil insensitive  $\text{Ca}^{2+}$  transport channel and to promote  $\text{Ca}^{2+}$  liberation from calcareous corpuscles in larvae of *T. pisiformis* (Martinez et al., 1992). However, Cunha and Noel (1997) discarded the mechanism of PZQ-induced contraction of *S. mansoni*. Besides muscle contraction,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  are reported to influence different enzyme activities, namely, ME, FR, NADH oxidase, SDH, fumarase and NADPH:NAD transhydrogenase in *H. diminuta* (Wani and Srivastava, 1994). In the present study, a significant amount of  $\text{Ca}^{2+}$  (350-400  $\mu\text{g/g}$  dry tissue wt) was found to be present in control parasites (Table 16). However, a significant decline in the tissue  $\text{Ca}^{2+}$ , ranging between 39-49% as compared to control, was perceptible in the parasites exposed to all the test materials (Table 17; Fig. 18), which was accompanied by more efflux of  $\text{Ca}^{2+}$  into the medium (Table 18; Fig. 19), though the total  $\text{Ca}^{2+}$  contents (in the tissue and medium) remained the same before and after the treatments.

Chemotherapeutics like PZQ are known to cause vacuolization and disruption of the surface tegument and also muscular paralysis in several parasite species (Andrews, 1978; Pax et al., 1978; Coles, 1979; Nelson and Saz, 1983; Shuhua et al., 2000). Such a change in the tegumental architecture has been attributed to the levels of  $\text{Ca}^{2+}$  concentration in the transmembranous ion influx consequent to exposure to the drug (Schmahl and Mehlhorn, 1985; Sobhon et al., 1986). In vitro, PZQ (1 mM) caused vacuolization, muscle contraction and ultimately paralysis in *S. mansoni* (Bricker et al., 1983). In adult liver flukes, *Opisthorchis viverrini*, PZQ was reported to cause depolymerization of the microtrabecular network that leads to vacuolization, swelling, blebbing, and tegumental disorganization and breakdown of myofilaments in the muscle

cells through the induction of  $\text{Ca}^{2+}$  influx (Apinhasmit and Sobhon, 1996). The prime effect of PZQ is to permit non-selective movement of  $\text{Ca}^{2+}$ , even though it is not an ionophore (Pax et al., 1978; Fetterer et al., 1980). Treatment of *R. echinobothrida* in vitro with *F. vestita* crude-peel extract and its active principle also caused alterations in tegumental organization of the parasite (Pal and Tandon, 1998a).

Considering that altered ionic influx of  $\text{Ca}^{2+}$  may account for these changes in the cestode, changes in the activity of the some key enzymes of glycogen metabolism and glucose metabolism may also be related to  $\text{Ca}^{2+}$  efflux consequent to permeability changes following exposure to the plant-derived components, which ultimately influences the energy-yielding pathways, glycolysis in particular, to derive more energy. This result is in conformity with the decline in the glucose content and a significant rise in malate and lactate content/production under treated conditions, thus suggesting that the energy demand in the parasites possibly gets enhanced under stress, but the plant-derived components do not influence a switch over towards aerobic degradation of glucose in the parasite.

### ***Conclusion***

From the results obtained, it can be hypothesized that due to the high energy demand of the parasite because of the anthelmintic stress following exposure to the plant crude-peel extract and genistein, the process of glycogenolysis and glycolysis got activated on the expense of other metabolic pathways of glucose utilization by

regulating some key enzymes of these pathways. The low activities of LDH and PK, and high activities of MDH and PEPCK suggest that anaerobic carbohydrate catabolism follows the FR pathway (Vykhrestyuk et al., 1984). In the present study, the PEPCK activity was increased and the PK activity decreased under various treatment conditions, thus suggesting that the glucose breakdown followed the PEPCK-malate pathway. Thus from the results obtained from various in-vitro experiments, it may be postulated that the phytochemicals of *F. vestita*, which seem to have tegumental interface as the primary target, also influence carbohydrate metabolism towards PEPCK-malate formation in the cestode, *R. echinobothrida*. In view of the differing primary functions of PEPCK in cestodes and their hosts, this enzyme might be selectively inhibited and thus provide an avenue for anthelmintic attack as suggested by Reynolds (1980). The phytochemicals of *F. vestita*, genistein in particular, may be acting upon PEPCK, the latter being one of the several other secondary drug targets in respect of cestode parasites.

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## SUMMARY

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The present work incorporates a study on the anthelmintic efficacy of *Flemingia vestita* Benth and Hooker (Family Fabaceae), a leguminous plant, the tuberous roots of which have a usage as vermifuge or vermicide in the indigenous traditional system of medicine in Meghalaya (Northeast India). In earlier studies, the root tuber peel extract of this plant and its major active component, genistein, were shown to cause flaccid paralysis and to be acting transtegumentally in trematode and cestode parasite. With a view to investing further the mode of action of these putatively anthelmintic phytochemicals, the crude-peel extract of *F. vestita*, genistein and the reference drug PZQ were tested in vitro against the cestode, *Raillietina echinobothrida*, in respect to the carbohydrate metabolism, the major energy yielding pathway in helminth parasites.

### Alterations in:

- \* glycogen level;
- \* activities of glycogen phosphorylase (GPase) and glycogen synthase (GSase), both active and total;
- \* physiological levels of some metabolites - glucose, lactate, pyruvate, malate and alanine;
- \* activities of some regulatory glycolytic enzymes - hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase (PK), phosphoenolpyruvate

carboxykinase (PEPCK), lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and malic enzyme (ME);

- \* activities of glucose 6-phosphate dehydrogenase (G6PDH), pyruvate carboxylase (PC) and fructose 1,6-bisphosphatase (FBPase) and
- \* physiological levels of some trace elements and changes in  $\text{Ca}^{2+}$  concentration, in particular

in treated parasites in comparison to controls form the parameters of this study. Standard techniques were used for biochemical assays and histochemical demonstrations of the mentioned parameters.

1. *R. echinobothrida* were collected in 0.9% PBS (pH 7.2) from freshly slaughtered domestic fowl and incubated at  $39 \pm 1$  °C with defined concentration of crude-peel extract (5 mg/ml), genistein (0.2 mg/ml) and PZQ (1 µg/ml) with simultaneous maintenance of controls till the onset of paralysis. Control parasites survived upto 72 h, whereas in the treated parasites paralysis set in about 5.9 h, 6.7 h and 2.9 h in case of crude root peel extract, genistein and PZQ, respectively. The treated parasites and controls were taken for various experiments.
2. After exposure to the plant crude-peel extract of *F. vestita* and genistein, the glycogen concentration was found to decrease by 15-44% in the parasites compared to their respective controls. The decrease in the physiological level of

glycogen was accompanied by an increase in the activity of GPase *a* by 29-39% and a decrease of activity of GSase *a* by 36-59% in treated parasites as compared to untreated controls, though without affecting the total activity of both the enzymes. PZQ also caused quantitative reduction in glycogen level and alterations in enzyme activities somewhat at par with the genistein treatment.

3. The glucose content in the treated worms decreased by 14-36%, whereas malate concentration increased by 49-134% as compared to controls. Both in controls and treated parasites, however, pyruvate content was not measurable, while alanine and lactate contents showed a decline by 7-31% in the parasites exposed to all test materials. Besides, the lactate content as effluxed into the incubation medium showed an increase of 9-44% in the case of treated parasites indicating an overall increase of lactate production.
4. While some enzymes of the glycolytic pathway showed an increase in their activities following exposure of the parasites to the various treatments, others showed a decline. The activities of HK, PFK, PEPCK and LDH increased by 33-39%, 41-125%, 44-49% and 55-67%, respectively and that of PK decreased by 14-26% in all treatments in comparison to the respective controls.
5. The cytosolic MDH got activated by 33-58% whereas mitochondrial MDH increased by 43-73% in the treated parasites. The MDH activity of in the tissue

homogenate was also found to be higher by 22-43% in treatments with crude-peel extract, genistein and PZQ. The ME activity in the tissue homogenate was found to be increased by 28-59% in the treated parasites; while the cytosolic ME activity showed an increase by 33-39%, there was no enhancement in mitochondrial ME activity.

6. The key regulatory enzyme of pentose phosphate pathway, G6PDH, was found to decrease by 23-31% in various treatments. FBPase, the enzyme of gluconeogenesis, did not show any changes during the treatment conditions while the activity of PC was significantly increased by 32-44% in treated parasites in comparison to the controls.
7. Using atomic absorption spectrophotometry, some trace elements, namely, lead (~ 25 µg/g dry tissue wt), iron (~ 1200 µg/g dry tissue wt), zinc (~ 400 µg/g dry tissue wt), magnesium (~1400 µg/g dry tissue wt), calcium (~ 400 µg/g dry tissue wt), chromium (~12 µg/g dry tissue wt) were detected in the cestode while manganese, cadmium and nickel were below level of detection.
8. The calcium concentration in the parasite tissue was found to decrease by 39-49% following with the test materials though the calcium concentration in the incubation media containing the test materials was found to increase by 94-118%, indicating thereby an efflux of  $\text{Ca}^{2+}$  from the parasite under the treatment itself.

From the results obtained it can be hypothesized that due to the high energy demand of the parasites because of anthelmintic stress following exposure to the plant crude extract and genistein, the process of glycogenolysis and glycolysis got activated on the expense of other metabolic pathways of glucose utilization by regulating some key enzymes of these pathways. Changes in the homeostasis of  $\text{Ca}^{2+}$  could be one such regulating mechanism in the cestode parasites. The PEPCK/PK branch point provides an important clue for the switch over to the phosphoenolpyruvate-succinate pathway for the glucose metabolism and thus an avenue for anthelmintic attack. The phytochemicals of *F. vestita*, therefore, seem to influence the carbohydrate metabolism of the cestode, which may be a secondary target of action, the primary target being the tegumental interface of the parasite.

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## ABBREVIATIONS

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|                   |  |
|-------------------|--|
| AMP               | Adenosine 5'-monophosphate   |
| ATP               | Adenosine 5'-triphosphate  |
| cAMP              | 3',5'-cyclic adenosine monophosphate   |
| DPX               | Dibutylphthalate xylene  |
| EDTA              | Ethylenediaminetetraacetic acid  |
| EGTA              | Ethylene glycol-bis( $\beta$ -aminoethyl ether)-<br>N,N,N',N'-tetraacetic acid |
| GDP               | Guanosine 5'-diphosphate   |
| GTP               | Guanosine 5'-triphosphate  |
| NAD <sup>+</sup>  | Nicotinamide adenine dinucleotide (oxidised)                                   |
| NADH              | Nicotinamide adenine dinucleotide (reduced)                                    |
| NADP <sup>+</sup> | Nicotinamide adenine dinucleotide phosphate<br>(oxidised)                      |
| NADPH             | Nicotinamide adenine dinucleotide phosphate<br>(reduced)                       |
| NBT               | Nitro blue tetrazolium   |
| PMS               | Phenazine methosulphate  |
| PMSF              | Phenylmethanesulphonyl fluoride  |
| PVA               | Polyvinyl alcohol  |
| TRA-buffer        | Triethanol amine buffer  |

|             |  |
|-------------|--|
| Tris-buffer | Tris (hydroxymethyl) aminomethane buffer |
| UDP         | Uridine diphosphate                      |
| UDP-glucose | Uridine diphosphate glucose              |
| UTP         | Uridine triphosphate                     |
| UV          | Ultraviolet                              |

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- \* Tandon V., Lyndem L.M., Kar P.K., Pal P. and **Das B.** 2002. Anthelmintic efficacy of *Stephania glabra* (Menispermaceae): In vitro effect of crude rhizome pulp extract on intestinal parasites. *Proceedings of the 10<sup>th</sup> International Congress of Parasitology ICOPA X: Parasitology in a New World* 4-9 August, 2002, Vancouver, Canada, **PA-015: 186-187**

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Research note

## Anthelmintic efficacy of *Flemingia vestita* (Fabaceae): effect of genistein on glycogen metabolism in the cestode, *Raillietina echinobothrida*

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### Abstract

The edible root-tuber peel of *Flemingia vestita* and its major active component, genistein, have been earlier shown to have a vermifugal/vermicidal effect on cestodes in vitro by causing a flaccid paralysis and alterations in the tegumental architecture and activity of several enzymes associated with the tegumental interface of the parasite. Pursuing further investigation on the mode of action of this putative anthelmintic, the crude peel extract and pure genistein were further tested in respect of glycogen metabolism in the fowl tapeworm, *Raillietina echinobothrida*. On exposure to the plant root peel crude extract (5 mg/ml) and genistein (0.2 mg/ml), the glycogen concentration was found to decrease by 15–44%, accompanied by an increase of activity of the active form of glycogen phosphorylase (GPase *a*) by 29–39% and decrease of activity of the active form of glycogen synthase (GSase *a*) by 36–59% in treated parasites as compared to untreated controls, but without affecting the total activity (*a* + *b*) of both the enzymes. Praziquantel (1 µg/ml), the reference drug, also caused quantitative reduction in glycogen level and alterations in enzyme activities somewhat at par with the genistein treatment. These results suggest that this plant-derived component may influence the glycogen metabolism of the parasite by directing it towards utilization of glycogen.

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**Keywords:** *Raillietina echinobothrida*; Glycogen phosphorylase; Glycogen synthase; *Flemingia vestita*; Anthelmintic; Genistein; Praziquantel; Phytochemical

The edible root-tuber peel of *Flemingia vestita* (Fabaceae), an indigenous leguminous plant of Meghalaya, is conventionally used in local traditional medicine as an anthelmintic against intestinal worms. The active principles of the root-tuber peel extract were isolated by Rao and Reddy [1].

The crude extract of the root-tuber peel of *F. vestita* and its major active isoflavone component, genistein, have been shown to have a vermifugal/vermicidal effect on *Raillietina echinobothrida*, cestode of domestic fowl, in vitro by causing a flaccid paralysis accompanied by alterations in the tegumental architecture [2,3]. Alterations were also observed in the activity of several enzymes associated with the tegumental interface and nervous coordination of this cestode [4,5].

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With a view to understanding the mode of action of these plant-derived components, it seems desirable to further investigate their effect on the energy-yielding pathway in the parasite. With limited ability to metabolize lipids and amino acids, cestodes and trematodes mainly ferment glucose and other simple carbohydrate molecules to meet their energy requirements [6]. The carbohydrate occurs mainly as glycogen in both larval and adult cestodes, which serves typically as the most important energy reserve. Several chemotherapeutic agents have been shown to influence glycogen metabolism in helminths [7].

The two key regulatory enzymes in glycogen metabolism are glycogen phosphorylase (1,4- $\alpha$ -D-Glucan: orthophosphate  $\alpha$ -D-glucosyltransferase, EC 2.4.1.1, GPase) and glycogen synthase (UDP glucose: glycogen 4- $\alpha$ -glucosyltransferase, EC 2.4.1.11, GSase). The degradation and synthesis of glycogen are regulated depending upon the energy need under various physiological conditions mainly by regulating the activity of these two enzymes that are normally present in two forms—active (*a*) and inactive (*b*), which are interconverted by phosphorylation by protein kinase, and dephosphorylation by protein phosphatase [8]. The presence of GPase activity and glycogenesis in cestodes have been reported in vitro [9]. The glycogen content and the activity of these two enzymes form the parameters for the present study.

Live specimens of *R. echinobothrida* were collected from the intestine of freshly slaughtered domestic fowl at local abattoirs in 0.9% phosphate buffered saline (PBS, pH 7.2). The alcoholic crude root-tuber peel extract of *F. vestita* was obtained as reported previously [2]. Genistein was obtained from Sigma Chemicals (Code no. 6649). Praziquantel (PZQ, Droncit), a broad-spectrum cestocide, served as the reference drug. All enzymes, co-enzymes and substrates were either obtained from Sigma Chemicals (St. Louis, USA) or Sisco Research Lab (Mumbai, India). All other reagents used were of high quality and obtained from indigenous sources.

The worms ( $\approx 0.2$  g fresh wt.) were incubated in 5 ml of the medium at  $38 \pm 1$  °C with 5 mg/ml crude peel extract, 0.2 mg/ml genistein and 0.001 mg/ml PZQ dissolved in dimethylsulfoxide

(DMSO, final concentration 1% in PBS), with simultaneous maintenance of controls for each treatment kept in PBS containing 1% DMSO. The concentrations of the test materials were determined on the basis of a previous study, wherein they were shown to cause paralysis in  $5.9 \pm 0.05$ ,  $6.7 \pm 0.04$  and  $2.9 \pm 0.05$  h, respectively, while the controls survived in vitro for  $72 \pm 0.05$  h [2]. For each set of treatment cestodes were taken from a single host.

Alkali-soluble glycogen was estimated using anthrone reagent following the method of Seifter et al. [10]. For enzyme extract and assay 10% homogenate (w/v) of the treated worms and controls was prepared as per the procedure of Russel and Storey [11]. The homogenate was centrifuged at  $10\,000 \times g$  at  $0 \pm 2$  °C for 10 min and the supernatant was used for enzyme activity determinations. GPase was assayed spectrophotometrically (Beckman DU 640) following the method of Moon et al. [12]; the reaction mixture also contained 10 mM caffeine for measurement of GPase *a* alone. GSase was also assayed spectrophotometrically following the method of Passoneau and Rottenberg [13]; the reaction mixture did not contain 5 mM glucose 6-phosphate while measuring the GSase *a* activity alone. Percent GPase *a* or GSase *a* represents the ratio of the active form to the total (*a* + *b*) enzyme  $\times 100$ .

Following treatment with root peel extract and genistein, paralysis ensued after approximately 6 h of incubation, whereas in PZQ-containing medium paralysis set in after 3 h of exposure. Table 1 shows the glycogen content in the control and treated parasites. The physiological level of glycogen in the parasite was found to be quite high and averaged to approximately 66 mg/g wet wt., which decreased significantly 12 h onwards post incubation in controlled condition. The glycogen level in the parasite decreased significantly compared to the respective controls at different hours of treatment. The contents of glycogen in cestodes can range up to 50% or more of dry wt. [7]. The amount of glycogen stored at a given time, however, is affected by a number of environmental factors and varies with the feeding cycle and physiological status of the host, the number of parasites present (crowding effect) and the stage

Table 1  
Effects of root-tuber peel extract of *F. testata*, genistein and PZQ on glycogen content (mg/g wet wt.) in *R. echinobothrida* in vitro

| Treatment<br>(mg/ml)           | Concentration of glycogen <sup>a</sup> post incubation |                                    |                                    |  |  |
|--------------------------------|--|------------------------------------|------------------------------------|--|--|
|                                | 0 h  | 3 h                                | 6 h                                | 12 h                                     | 24 h                                     |
| 1a Control (in 0.9% PBS)       | 66.53 ± 4.17   | –                                  | 56.97 ± 4.41                       | 36.04 ± 2.64 <sup>1</sup>                | 20.9 ± 2.71 <sup>2</sup>                 |
| 1b Crude peel extract<br>(5.0) | –  | –                                  | 47.93 ± 2.32 <sup>1</sup><br>(–16) | 20.24 ± 2.73 <sup>b</sup><br>(–44) [–46] | 14.54 ± 2.05 <sup>1</sup><br>(–30) [–69] |
| 2a Control                     | 65.74 ± 4.44   | –                                  | 57.28 ± 4.12                       | 40.14 ± 1.62 <sup>1</sup>                | 25.89 ± 3.36 <sup>2</sup>                |
| 2b Genistein<br>(0.2)          | –  | –                                  | 48.83 ± 1.94 <sup>1</sup><br>(–15) | 26.19 ± 2.18 <sup>b</sup><br>(–35) [–39] | 18.74 ± 1.36 <sup>1</sup><br>(–28) [–61] |
| 3a Control                     | 67.56 ± 5.00   | 63.78 ± 3.59                       | 45.42 ± 2.90 <sup>1</sup>          | –  | –  |
| 3b PZQ<br>(0.001)              | –  | 51.16 ± 3.91 <sup>1</sup><br>(–20) | 32.53 ± 0.44 <sup>1</sup><br>(–28) | –  | –  |

Values are expressed as mean ± SEM ( $n=3$ ). <sup>1</sup> $P$  value significant at  $<0.05$  and  $<0.01$ , respectively in starved controls compared with the 0 h control. <sup>2</sup> $P$  value significant at  $<0.05$  and  $<0.01$ , respectively, in treatments compared with their respective controls.

<sup>a</sup> Percentage decrease (–) in glycogen content in treated worms from respective controls is given in parentheses and that to 0 h, in square brackets.

<sup>b</sup> Glycogen content in freshly recovered parasite.

of development of the worms [14]. In the present study, a significant decrease in the glycogen level in the control parasite to approximately 40–70% within 24 h of starvation suggests that, as true for platyhelminths, carbohydrate is the prime source of energy in this cestode, too [6]. Also a significant decrease (by 15–44%) was recorded in treated parasites in paralytic state. A number of anthelmintic agents have been shown to affect the glycogen concentration and metabolism in helminths [6,15], though several others do not cause any such change. While no drug-induced effect in respect to glycogen content was demonstrable in *Schistosoma mansoni* from mice dosed with Ro 15-5458 [16], schistosomes recovered from artemether-treated hosts showed an increased activity of glycogen catabolism and decreased glucose uptake [17].

As shown in Table 2, treatment of the parasite with crude peel extract, genistein and PZQ caused significant change only in the activity of the active forms of GPase and GSase, with no significant effect on the total activity of either enzyme. GPase *a*, which constituted approximately 72–74% of the total GPase activity in the control parasite, increased by 29, 39 and 30% in the parasite treated with crude peel extract and genistein for 6 h and PZQ for 3 h, respectively. However, GSase *a*

activity, which constituted approximately 56–68% of the total GSase activity, decreased by 59, 36 and 38%, respectively, in similar treatments. The decrease in glycogen content accompanied by a significant decrease in GSase *a* activity and an increase in GPase *a* activity in the parasite under different treatment conditions suggest that the energy demand by the cestode was perhaps enhanced under test conditions leading to stimulation of glycogenolysis and inhibition of glycogenesis. Chemotherapeutics like levamisole showed a decrease in GPase activity and an increase in activity of GSase in nematodes [7,18,19]. However, GPase activity was found to be 25–30% higher in *H. diminuta* from amoscanate-treated hosts [20]. An increase in the activity of GPase *a* in *R. echinobothrida* during the treatments suggests that the phytochemicals, as also PZQ, influence activation of this enzyme.

It is interesting to note that the treatment in vitro of *R. echinobothrida* with the crude peel extract of *F. testata*, and genistein affected only the active forms of GPase and GSase, without causing any change of their total activities. It appears that the changes of the phosphorylation status could be one of the possible mechanisms of regulation of these two enzymes, thereby converting the inactive to active form and vice versa.

## Table 6

Effects of root tuber peel extract of *Flemingia strobilifera* and its active principle genistein (units/g extract) of GPase and GSase of *R. echinobothrida* in vitro.

| Treatment (mg/ml)           | Enzyme activity of |                                   |                 |                                   |
|-----------------------------|--------------------|-----------------------------------|-----------------|-----------------------------------|
|                             | GPase              |                                   | GSase           |                                   |
|                             | Total (a+b)        | Active (a)                        | Total (a+b)     | Active (a)                        |
| 1a Control (in 0.9% PBS)    | 1.92±0.12          | 1.43±0.02 (74)                    | 0.73±0.04       | 0.41±0.06 (56)                    |
| 1b Crude peel extract (5.0) | 2.09±0.25 [±9]     | 1.84±0.25 (88) [±29] <sup>b</sup> | 0.65±0.03 [-10] | 0.17±0.04 (27) [-59] <sup>c</sup> |
| 2a Control                  | 2.28±0.43          | 1.66±0.31 (73)                    | 1.68±0.17       | 1.15±0.08 (68)                    |
| 2b Genistein (0.2)          | 2.49±0.27 [±9]     | 2.30±0.17 (92) [±39] <sup>a</sup> | 1.54±0.13 [-8]  | 0.74±0.14 (48) [-36] <sup>a</sup> |
| 3a Control                  | 2.42±0.53          | 1.74±0.49 (72)                    | 1.29±0.02       | 0.86±0.08 (67)                    |
| 3b PZQ (0.001)              | 2.45±0.28 [±1]     | 2.26±0.33 (92) [±30] <sup>a</sup> | 1.16±0.09 [-10] | 0.53±0.07 (46) [-38] <sup>b</sup> |

Values are expressed as mean±SEM (n=3). One unit of enzyme activity is defined as 1 µmol of NADP<sup>+</sup> reduced in case of GPase or NADH oxidized in case of GSase per minute at 38 °C.

<sup>a</sup> Percentage of GPase a and GSase a activities out of the total (a+b) is given in parentheses. Percentage increase (+) or decrease (-) of GPase a and GSase a activities in the treated worms compared to their respective controls is given in square brackets.

<sup>b</sup> P<0.05

<sup>c</sup> P<0.01

under the various treatments. Chemotherapeutics like PZQ are known to cause vacuolization and disruption of the surface tegument and also muscular paralysis in several parasite species [17,21]. Treatment of *R. echinobothrida* in vitro with *F. strobilifera* crude peel extract and its active principle also causes alterations in tegumental organization of the parasite [3]. Considering that altered influx of Ca<sup>2+</sup> may account for these changes in the treated cestode, alteration in the activity of the enzymes of glycogen metabolism may also be related to Ca<sup>2+</sup> influx, which needs to be further investigated.

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disulphide while cysteine derivatives (S-methyl, S-ethyl and S-allyl cysteine), which had an effect dependent on interference of cysteine uptake, were the least effective. These data suggest that individual garlic components may affect *Giardia* by different mechanisms and thus encourages their potential use as an alternative therapy, even in drug-resistant giardiasis.

•• SO2-4-B

ANTHELMINTIC EFFICACY OF *FLEMINGIA VESTITA* (FABACEAE) ALTERATIONS IN GLUCOSE METABOLISM OF THE CESTODE, *RAILLIETINA ECHINOBOOTHIRIDA*

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The edible root tuber of *Flemingia vestita*, an indigenous leguminous plant of Northeast India, has long been used as an anthelmintic against intestinal worms including cestodes in local traditional medicine. The root-tuber peel of *Flemingia vestita* and its active component, genistein (4', 5, 7-trihydroxyisoflavone) have been shown to have a vermifugal/vermicidal effect on cestode parasites *in vitro* by causing a flaccid paralysis accompanied by alterations in the tegumental architecture and activity of several tegumental enzymes of *Raillietina echinobothrida*. Pursuing further the investigation, the crude root-peel extract and pure genistein were tested in respect of glucose metabolism in the parasite. Changes in the intra-cellular glucose content and the metabolic intermediates pyruvate and malate, and the end products alanine and lactate of glucose metabolism formed the parameters for the present study. Live *R. echinobothrida* were collected from the intestine of freshly slaughtered domestic fowl in phosphate buffered saline (PBS) and incubated at 39±1°C in defined concentrations of the root peel crude extract (5mg/ml) and genistein (0.2mg/ml) in PBS with 1% of dimethyl sulphoxide (DMSO) with simultaneous maintenance of controls. Praziquantel (0.001mg/ml) (PZQ) a broad spectrum of anthelmintic, served as reference drug. The glucose content and intermediate metabolites and the end products of glucose metabolism were estimated biochemically immediately after paralysis set in the treated worms. Following treatment with root peel extract and genistein, paralysis ensued in the parasites after 6 h of incubation, whereas in Praziquantel-containing medium paralytic state set in after 3 h of exposure. In the treated worms the glucose content decreased by 25-55% and the malate concentration increased ~5-10 folds as compared to controls. Both in controls and treated parasites, however, pyruvate content was not measurable while alanine and lactate contents showed a decline by 6.5-24%, respectively in the parasites exposed to all test materials. Besides, the lactate content as effluxed into the incubation medium showed 22-45% increase in treatments in comparison to controls. The results showing a decline in glucose content and a significant rise in malate content under treatment conditions suggest that the plant derived components influence a switch over towards aerobic degradation of glucose in the parasites.

•• SO2-4-C

EXPERIMENTAL STUDY ON A NOVEL COMPOUND EXTRACTED FROM TCM FOR TREATMENT OF ALVEOLAR ECHINOCOCCOSIS (ALVEOLOCCOSIS)

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To evaluate the efficacy of a novel compound derived from TCM for treatment of murine alveolar echinococcosis. Two prerequisites were prepared. The first was to extract a compound from TCM by means of a serial procedure including amalgamation of TCM drugs, boiling, cold condensation using a reflux instrument, and evaporation by mild heating, finally forming deep brown powder. The second was to establish animal model of alveolar echinococcosis. A bit of alveococcos tissue derived from murine alveolar echinococcosis was transplanted into peritoneal cavity of *Mus musculus* Kuming Strain. The infected duration after inoculation were classified into 1 week infection and 10 weeks infection, which were again divided into treated group and control group. Estimation of alveococcus inhibitory rate and electron microscopic observation were served as main methods for assessment of the efficacy. The infected mice of treated groups were intra-gastrically administered once per day with TCM extractive powder at doses of 20 mg/kg for three months continuously. All the animals were killed and examined half a month after the end of TCM treatment. Total wet of alveococcus from 9 mice of treated group after 1 week infection was 37.76g (X ± S 4.196 ± 2.090g) being obviously lower than that from 10 mice of control group (121.294g X ± S 12.129 ± 4.305g). So the inhibitory rate of alveococcus was 65.7% (P < 0.01). Similarly total wet weight of alveococcus from 7 mice of treated group after 10 weeks infection was 4.362g (X ± S 6.19 ± 0.1207g), being apparently lower than that from 6 mice of control group (17.85g, X ± S 2.926 ± 3.275g). The inhibitory rate of alveococcus was 80.6% (P < 0.01). The ultra-structural appearances of alveococcus showed an obvious difference between treated group and control group. TCM extractive powder for treatment of murine alveococcosis is considered to be a hopeful anti-echinococcus compound which will be significant for further study.

•• SO2-4-D

PURIFICATION OF RECOMBINANT TRYPANOSOME ALTERNATIVE OXIDASE

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African trypanosoma is a parasite which causes African sleeping sickness of human and nagana disease of cattle. Because at present drugs against trypanosomes have strong side effect and are not effective for chronic patients, a new effective drug is awaited now. We found that ascofuranone isolated from *Ascochyta visiae* inhibits specifically mitochondrial respiration of blood stream form of *Trypanosoma brucei brucei* at very low concentration. In addition, we found that injection of ascofuranone into infected mice made blood stream form of *T. brucei brucei* die out. It has been thought that the target of ascofuranone is cyanide resistant terminal oxidase located at mitochondrion of blood stream form of *T. b. brucei*. This enzyme is referred to as TAO (trypanosome alternative oxidase), and functions ubiquinol oxidase unlike cytochrome c oxidase of mitochondrion of mammal. Because mammals don't have TAO that is essential for reoxidation of reducing equivalent generated during glycolysis, TAO is expected as a candidate of target for chemotherapy against trypanosomes. It is also reported that TAO is highly homologous to alternative oxidase of mitochondria of plants, but biochemical analysis has not been carried out due to

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•• PA-011

EVALUATION OF LONG ACTING MALATHION PAINT FORMULATION (SLOW RELEASE EMULSIFIED SUSPENSION) IN THE CONTROL OF SANDIY IN KALAZAR AREA OF BIHAR

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A pilot trial for the control of *Paragentes* through malathion, SRES (Slow Release Emulsified Suspension) paint formulation, was carried out in an endemic village, Gulmahiyabagh, taken as test village in comparison to DDT sprayed village, Rakabganj, taken as control village. Organophosphorus insecticide spraying of 5% Malathion in the dose of 2 gm/m<sup>2</sup> was applied in 204 houses in test village and DDT in recommended dose was applied in 233 house in control village. Resurgence of *Paragentes* was observed in test village nearly after 21-23 months of spray where as in control village after 4-6 months. Taking operational cost into consideration, Malathion, SRES, paint spraying seems to be less expensive as compared to DDT which require four round of spray in two successive years to maintain the same residual efficacy in comparison of one round of malathion, SRES. It is a safe insecticide with no toxicity on human beings exposed to it (unpublished data). This study indicates that the malathion, SRES (5%) paint formulation is more effective, lesser toxic and more cost-effective for containment of vector population as compared to DDT spray. This project was funded by WHO and IDR.

•• PA-012

NEW COMPOUNDS IN CONTROL OF PARASITES

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In many groups of parasitoses, there exists an absolute need for new chemotherapeutic drugs. In some cases (e.g. *Cryptosporidium* species, microsporia) there was never existing a convenient chemotherapy, in others (e.g. *Giardia*) new need arose due to the possible transmissions from animals to man. In helminths a broad series of drug resistance became established during the last years. In insects and ticks a need for broad acting repellents and safe insecticides/acaricides is highly desirable since these ectoparasites are vectors of many parasites leading to diseases or even death in humans and animals. Some new classes of such compounds will be presented and their properties discussed.

•• PA-013

SUCCESSFUL TREATMENT OF PRIMARY AMEBIC MENINGOENCEPHALITIS IN IRAN

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The primary amebic meningoencephalitis (PAM) is rare and fatal disease caused by free living amoeba called *Naegleria*. So far about 180 cases were reported from all-around the world and just 7 cases were survived. This is the first report of primary amebic Meningoencephalitis from Iran and the 8th survived case in the world. The patient was an eighteen months old boy referred to the

hospital in August 2001 with the symptoms of nausea, vomiting, fever, drowsiness and convulsion. Examination of CSF indicated the 12000/mm<sup>3</sup> WBC with the majority of 83% neutrophil, 238 mg/dl protein and glucose of 34 mg/dl. Blood sugar was 100 mg/dl. Gram stain and culture of CSF for bacteria was negative. Microscopic examination of wet smear from CSF revealed the motile amoeba with pseudopodia. In Giemsa staining, the trophozoites were seen among the Leukocytes and red cells. Treatment of patient with Amphotericin B, rifampin and chloramphenicol for 6 weeks responded well and the patient survived remarkably. The patient was followed for 3 months and no sign of sequelae was observed. Although the PAM is fatal, but should be differentiated from the bacterial meningitis and history of patient in contact with freshwater should be considered and the treatment should be applied urgently.

•• PA-014

EARLY APPEARANCE AND TREATMENT OF GOAT WARBLER IN IRSIND, KANDH AND KHAND AREA OF RAKHI MUNI, DERA GHAZI KHAN, PAKISTAN

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The objective of this study was to find out the early date of appearance of goat warbles and to control goat warbles *Prichoaliskama silenus Brauer* at L2 stage in the body of animal with the use of ivermectin. Prevalence of goat warbles (*Prichoaliskama silenus Brauer*) was carried out on eighteen flocks (n = 281) in three villages at Rakhi Muni, Dera Ghazi Khan. Study was conducted during 2nd week of August, 1999-2000. The nodules of goat warbles larvae were palpated on the back of infected animals through hand palpation method and infested animals were given the treatment with ivermectin injection. The occurrence of goat warbles at Irsind, Kandh and Khand was 65%, 36% and 30% respectively. The ivermectin injection at dose rate of 200 mg/kg through intramuscular was effective against L2 and L3 stages of goat warbles. The occurrence of goat warbles as early as 2nd week of August was recorded for the first time in Pakistan and the ivermectin proved a very effective warbicide.

•• PA-015

ANTHELMINTIC EFFICACY OF *STEPHANIA GLABRA* (MUSCIPERMACEAE) IN VITRO EFFECT OF CRUDE RHIZOME PULP EXTRACT ON INTESTINAL PARASITES

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In the traditional medicinal practices in Meghalaya (Northeast India) *Stephania glabra* has a usage as anthelmintic against intestinal worm infections, for which aqueous concoction of the powder of the dried rhizome is used. In order to ascertain the anthelmintic efficacy of this putative medicinal plant, the alcoholic crude extract of the pulp of the edible rhizome was tested against the various helminth parasites - nematode *Haemonchus gallinarum*, *Ascariida galli* (parasites in the intestine of domestic fowl); *Ancylostoma ceylanicum* (from hamster) and *Ascaris suum* (from pig); cestode *Rattinetma echinobothrida* (from domestic fowl) and trematode *Tasciopsis buski* (from pig) in the dosages of 25

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mg/ml, 50 mg/ml and 100 mg/ml in 0.9% phosphate buffered saline (PBS) at 38±1°C. The controls kept in PBS survived for average 22 h (trematode), 72 h (cestode) and 120-352 h (nematodes). The cestode and trematode parasites showed a pronounced effect of the treatment. A dose dependent gradual decline in physical motility was observable in *R. echinobothrida* and *F. buski*, with a reversible flaccid paralysis setting in at a mean time of 2 h 23 min and 1 h 45 min, and death at 3 h 29 min and 2 h 15 min, respectively for the former and 3 h 30 min, 2 h 34 min and 4 h 11 min, 3 h 22 min for the latter, following incubation in dosages of both 50 mg/ml and 100 mg/ml. In contrast much less effect was revealed in respect of nematodes, the mean paralysis and death times of *H. gallinaum*, *A. ceylanicum*, and *A. galli* in treatment with 50 mg/ml crude extract were 10 h 20 min and 14 h 26 min, 8 h 45 min and 11 h 10 min, and 10 h 11 min and 12 h 55 min, respectively. *A. suum*, the large nematode showed a feeble effect, with the onset of paralysis occurring at 31 h 3 min and death, at 37 h 18 min in incubation with the same dose. The phytochemical(s) in the pulp of *S. glabra* seems to be effective against soft-bodied plathelminth parasites that have a tegumental interface and has little or no effect on cuticle-covered nematodes. The active principle of the rhizome pulp needs to be identified.

**•• PA-016**

POLY-INFILCTION WITH *SCHISTOSOMA HAEMATOBIMUM* AND *SCHISTOSOMA MANSONI* INVADING SPINAL CORD, BLADDER AND COLON IN A FRENCH TOURIST

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**Observation** A 31-year-old traveller coming back from a one year trip in South Africa presented with a symptomatic myelopathy polyradiculopathy, amyotrophy and sphincteric disturbance. Spinal magnetic resonance imaging revealed a granuloma of the conus. Eosinophilia in the serum and the cerebro-spinal fluid (CSF) incited us to detect *Schistosoma* antibodies in the serum and the CSF. Serological testing by Indirect immunofluorescence showed positive titers both in the serum (1/320) and the CSF (1/40). Beside, *Schistosoma haematobium* eggs were isolated from urine and *Schistosoma mansoni* eggs were detected in the rectal biopsy. The patient was treated with Praziquantel 40 mg/kg of body weight twice a day at 4 hour interval, associated with corticotherapy. After treatment, symptoms slowly improved over months.

**Discussion** Schistosomiasis due to *S. mansoni* or *S. haematobium* of the spinal cord has been described in South African patients. Here, we report a multisystem infection involving spinal cord, bladder and colon due to both *S. mansoni* and *S. haematobium*. It occurred in a French tourist after an eight-month trip in South Africa. Intensive and short-term exposure in a nonimmune patient can thus result in a poly-parasitic and multisystem schistosomiasis.

**•• PA-017**

THE SOUTHERNMOST TRANSMISSION FOCUS OF SCHISTOSOMIASIS IN SOUTH AMERICA AND THE ROLE OF SENSITIVE COPROSCOPIC METHODS FOR DETECTION OF HUMAN INFILCTION

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In 1997, *Biomphalaria glabrata* was identified for the first time, in the Brazil's southernmost State, Rio Grande do Sul and one year later, snails were found infested in a locality named Esteio, next to Porto Alegre. Two ponds and one abandoned and closed channel, next to the Sinos River, were identified as transmission foci. Approximately 7,000 people live next to these sites, but there is not a widespread use of natural water bodies neither in leisure nor in work activities, by the population. A sero-epidemiological and copro-parasitological survey is presently underway and has been repeated every six months. Three slides are prepared (Kato-Katz method) and the rest of the fecal sample is dissolved in water and sedimented (Hoffmann method). The resulting sediment is sequentially processed by the Ritchie method (ether formalin) and the final sediment is totally examined under the microscope. 11 out of 1558 investigated individuals have so far been found infected in the area. 9 out of 11 were negative by Kato-Katz presenting less than 1 egg/g feces as estimated by examination of the whole sample. These results stress the need for optimization of diagnostic methods in areas of recent introduction, where low prevalence and parasitic burden appears to be the rule. Serology as a screening procedure is currently been evaluated in a study in the area. Four of the individuals were infected while collecting *Biomphalaria* sp. to use as a fishing bait. The locality of Esteio became the southern boundary of occurrence both to *B. glabrata* and *Schistosoma mansoni* in the Americas what should stand as a serious alert for the potential expansion of this endemic parasitic disease to Uruguay and Argentina. Financial support: FUNDASA/SI S/SMS, FAPIRGS, CNPq, PUCRS.

**•• PA-018**

DETECTION OF HISTOLYTICA G. LAMBLLIA AND CRYPTOSPORIDIUM COPRO-ANTIGENS IN STOOL SAMPLES

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A double antibody sandwich ELISA technique using a chromatography purified antisera against *E. histolytica*, *G. lamblia* and *Cryptosporidium* antigens was applied to detect copro-antigens of the corresponding parasites in 90 patients. All positive cases were diagnosed by parasitological examination and proved to have the infection solely. Beside the 90 positive cases, 40 age-matched controls were included in the study, of which 20 individuals were infected with other parasites but not *Cryptosporidium*, *E. histolytica* or *G. lamblia* (acted as an infected control group) and the other 20 individuals with no intestinal parasites (normal control group). The assay could detect 100% of those infected with both of *G. lamblia* and *E. histolytica* and 96.6% (29/30) of patients with *Cryptosporidium* infection. False positive

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