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Anthelmintic efficacy of *Flemingia vestita* (Fabaceae): alterations in glucose metabolism of the cestode, *Raillietina echinobothrida*

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Abstract

The root-tuber peel of *Flemingia vestita* and its active component, genistein, were tested in respect of glucose metabolism in the cestode, *Raillietina echinobothrida*. Live *R. echinobothrida*, collected from the intestine of freshly slaughtered domestic fowl, were incubated at 39±1 °C in defined concentrations of the root-peel crude extract (5 mg/ml), genistein (0.2 mg/ml) and praziquantel (1 μg/ml) in phosphate buffered saline with 1% of dimethyl sulphoxide with simultaneous maintenance of controls. In the treated worms, there was a significant decrease in the glycogen concentration accompanied with the decrease of glucose by 14–32%, whereas the malate concentration increased by 49–134% as compared to controls. Both in controls and treated parasites, however, the pyruvate content was not measurable. While alanine and lactate contents showed a decline by 7–25% in the parasites exposed to all test materials, the lactate efflux into the incubation medium showed 37–71% increase in treatments indicating an overall increase of lactate production in comparison to controls. The results showing a decline in the glycogen and glucose contents and a significant rise in the malate content and lactate efflux under treatment conditions suggest that the energy demand in the parasites possibly got enhanced under stress, though it did not influence a switch over towards aerobic degradation of glucose in the parasites.
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Keywords: *Raillietina echinobothrida*; Intermediary metabolites; *Flemingia vestita*; Anthelmintic; Genistein; Praziquantel

1. Introduction

Traditional medicine practice among the natives in Meghalaya (Northeast India) relies on treatment with tuberous roots of an indigenously grown leguminous plant *Flemingia vestita*(Family: Fabaceae) for the purpose of eliminating intestinal worms; in case of suspected worm infections, the raw fleshy roots are consumed unpeeled. Earlier studies on the anthelmintic efficacy of the plant-derived materials have provided evidences that support and authenticate the usage of the tuberous root of this plant as vermifuge/vermicide. The root-peel extract and its major isoflavone component, genistein, caused a flaccid paralysis, damage to tegumental architecture, and alterations in the activity of enzymes associated with the tegument and coordination system both in cestodes and trematodes [1–6].

Glycogen, which is the major carbohydrate in both larval and adult cestodes, serves typically as the most important energy reserve in the parenchymatous tissue [7]. Several chemotherapeutic agents have been shown to influence glycogen metabolism in helminth parasites [8–10]. Cestodes and trematodes mainly ferment the glucose and other simple carbohydrates to meet their energy requirements [11,12]. Glucose 1-phosphate, released by the glycogenolysis, is converted by the enzyme phosphoglucomutase to glucose 6-phosphate which enters into the *type 2* fermentation, characterized by a CO_2-fixation step (by phosphoenolpyruvate carboxykinase) and malate dismutation, found in most cestodes [13]. There are conflicting reports regarding the effect of anthelmintic drugs on carbohydrate metabolism. While it has been suggested that in the cestode, *Cotugnia*

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digonopora, drugs like niclosamide, praziquantel (PZQ) and mebendazole inhibit uptake of glucose and cause increase in the production of lactic acid [14], Harder et al. [15] reported in *Schistosoma mansoni* an enhancing effect of PZQ and fluoxetine at concentrations of 0.1–10 μM and an inhibitory effect above 10 μM concentration. Likewise, in *Cysticercus fasciolaris* PZQ, mebendazole and some drug candidate compounds were shown to significantly lower the rate of glucose uptake and also to suppress the formation of lactate as a major end product of glycogen metabolism [16]. In contrast, drug-induced glycogen reduction in schistosomes was attributed to an inhibition of glycolysis rather than interference with glucose uptake [17].

In an earlier study, treatment of the cestode *R. echinobothrida* in vitro with *F. vestita* root-peel extract, genistein and PZQ was reported to cause decrease in glycogen concentration and stimulation of glycogenolysis [18]. Furthering the investigation to know the plausible mode of action of phytochemicals derived from *F. vestita* root-peel, it seemed logical to see their effect, if any, on the energy-yielding pathways, glycolysis in particular. Hence, changes in the intra-cellular glucose content and some metabolic intermediates (pyruvate and malate) and end products (alanine and lactate) of glucose metabolism formed the parameters for the present study.

2. Materials and methods

2.1. Parasites

Live cestodes, *Raillietina echinobothrida*, were collected from the intestine of freshly slaughtered domestic fowl at local abattoirs and maintained in 0.9% phosphate buffered saline (PBS, pH 7.2).

2.2. Drugs and chemicals

The alcoholic crude-peel extract of *F. vestita* was obtained as reported earlier [1]. Synthetic genistein was obtained from Sigma (St. Louis, USA). Praziquantel (PZQ, Droncit), a broad-spectrum cestocide, served as the reference drug. All enzymes and co-enzymes were obtained either from Sigma or from Roche (Germany). Other chemicals used were of analytical grades and obtained from local sources.

2.3. Treatment in vitro

The freshly obtained live parasites (≈0.2 g fresh wt) were treated in vitro in 10 ml of PBS (pH 7.2) at 39±1 °C containing 5 mg/ml crude-peel extract, 0.2 mg/ml genistein or 1 μg/ml PZQ with 1% dimethylsulfoxide (DMSO), with simultaneous maintenance of controls for each treatment in PBS containing 1% DMSO. These concentrations of the test materials were used with a view to allowing sufficient time for a significant effect and in order to prolong the time for onset of paralysis. As reported in a previous study by Tandon et al. [1], the above-defined concentrations of the test materials caused paralysis in the cestode in 5.9±0.05 h, 6.7±0.04 h, and 2.9±0.05 h, respectively, while the controls survived in vitro for 72±0.05 h. For each set of treatment, the parasites were taken from a single host.

2.4. Histochemical demonstration of glycogen

Glycogen concentration was histochemically demonstrated following the Best's method as described by Pearse [19] using paraffin sections (6–7 μm thick) of the bouin-fixed material and stained with Ehrlich's haemalum and Best's carmine.

2.5. Estimation of metabolites

A 10% homogenate (w/v) of the treated parasites at the onset of paralysis and their respective controls was prepared in 50 mM Tris–HCl buffer (pH 7.4). 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×*g* for 10 min to precipitate out proteins and other macromolecules. The supernatant was decanted out and neutralised with 2 M NaOH before estimation of different metabolites.

Concentrations of the different metabolites, viz. glucose, pyruvate, alanine, malate and lactate were measured enzymatically following the method of Bergmeyer [20]. The total volume of reaction mixture for the measurement of all these parameters was always 1 ml containing suitable aliquots of the neutralized sample. The reaction mixture contained the following:

Glucose- 100 mM Triethanolamine buffer (pH 7.6), 10 mM $MgCl_2$, 0.5 mM $NADP^+$, 0.9 mM ATP, 5 units each of glucose 6-phosphate dehydrogenase and hexokinase; pyruvate -50 mM Tris–HCl buffer (pH 7.4), 0.2 mM NADH and 5 units of lactate dehydrogenase (LDH); L-lactate -0.5 mM glycine, 0.42 M hydrazine hydrate, 0.9 mM NAD^+, 5 units of LDH; malate -100 mM Tris–HCl buffer (pH 7.4), 1 mM glutamate, 1 mM NAD^+ and 10 units each of malate dehydrogenase and glutamate oxaloacetate transaminase; alanine- 100 mM 3-amino-1-propanol buffer (pH 10.0), 1 mM NAD^+, 5 units of alanine dehydrogenase.

The reaction mixture was incubated at 39±1 °C for 30 min in the case of glucose, pyruvate, alanine and malate, and for 2 h in the case of lactate for complete conversion of the different metabolites present in the processed samples. The changes in the optical density due to reduction of $NADP^+$ to NADPH (in the case of glucose) or reduction of NAD^+ to NADH (in the case of lactate, malate and alanine) or oxidation of NADH to NAD^+ (in case of pyruvate) were measured at 340 nm in a UV–Visible spectrophotometer (Beckman, Model DU640) against the respective water blanks and the concentration of the metabolite was

calculated taking 6.22×10^6 as the molar extinction coefficient value for NADH or NADPH.

2.6. Estimation of lactate efflux

For measuring the efflux of lactate, the incubation medium (10 ml) from the various treatments and their respective controls was collected at the time when the paralytic state set in the worms. The medium was centrifuged at $10\,000\times g$ for 10 min to remove the debris, if any, and the supernatant was used for estimation of lactate. The lactate efflux by the worms was expressed as μmol/g wet wt/h.

2.7. Statistical analysis

Data collected from three replicates were statistically analyzed and presented as mean±SEM. Comparison of the paired mean values between the experimental and respective controls were made using Student's *t*-test [21] and differences with $P<0.05$ were regarded as statistically significant.

3. Results

With exposure to the defined concentrations of the crude root-peel extract and genistein, the onset of paralytic state occurred in the parasite after ~6 h of incubation and in the case of PZQ, after ~3 h. Histochemical analysis revealed that the glycogen content in the parasite decreased significantly in all treatments as compared to the respective controls (Fig. 1). Reduced stain intensity in the cross sections through mature proglottids of the treated parasites is indicative of the decline in glycogen concentration. Table 1 shows the physiological levels of the metabolites studied related to carbohydrate metabolism in *R. echinobothrida*. Of these, the physiological level of glucose was found to be the maximum, followed by lactate, alanine and malate. Trace amounts of pyruvate, possibly present in the cestode, could not be detected with this enzymatic method. Table 2 depicts the changes in the levels of different metabolites in the parasite at paralysis time in all treatments. The level of glucose decreased significantly by 31.8% and 19%, respectively, in treatments with crude-peel extract and genistein, and only by 13.5% (not significant), when the parasite was treated with PZQ. The lactate level in the parasite tissue decreased significantly by 31%, 16.8% and 20.5%, respectively, treated with crude-peel extract, genistein and PZQ. The level of alanine decreased significantly by 24.7% in treatment with genistein, but by only 7% and 9.8%, in the case of crude-peel extract and PZQ-treated parasite, respectively. In contrast, the level of malate increased significantly by 104%, 134% and 49%, respec-

Table 1
Physiological levels of different metabolites related to carbohydrate metabolism in *R. echinobothrida* in vitro

Metabolites	Concentration (μmol/g wet wt)
Glucose	26.58±1.12
Lactate	9.35±0.56
Malate	2.01±0.25
Pyruvate	BLD
Alanine	7.45±0.91

Values are expressed as mean±SEM (n=3). BLD, below the level of detection.

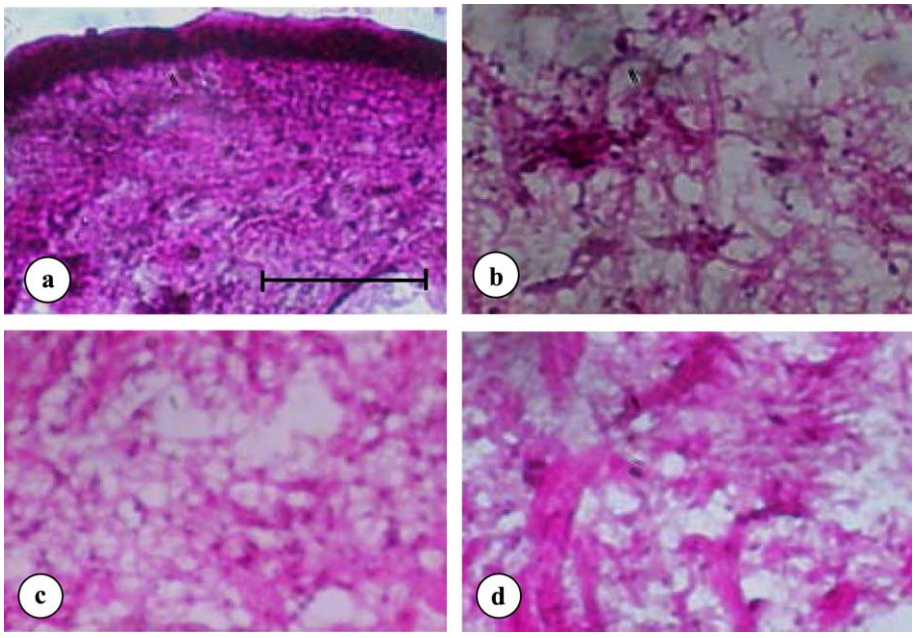

Fig. 1. *Raillietina echinobothrida*: glycogen concentration in control and treated parasites. Photomicrographs; cross sections through the region nearing tegument (a) or middle (b–d) of mature proglottid (Best's carmine method; scale bar=50 μm). (a) Control. (b) Crude-peel extract. (c) Genistein. (d) PZQ. Reduced stain intensity, noticeable in all treatments in comparison to control, is indicative of decline in glycogen concentration.

Table 2
Effects of root-tuber peel extract of *F. vestita*, genistein and PZQ on some intermediary metabolites of glycolysis in *R. echinobothrida* in vitro

Treatment (mg/ml)	Concentration of metabolites at paralysis time (μmol/g wet wt)				
	Glucose	Pyruvate	Lactate	Alanine	Malate
1.a. Control (in 0.9% PBS with 0.1% DMSO)	23.69±0.62	BLD	8.33±0.64	5.35±0.13	1.64±0.28
b. Extract	16.16±1.36	BLD	5.75±0.55	4.97±0.18	3.35±0.12
(5.0)	(−31.8)		(−31)	(−7)	(+104)
P	<0.05		<0.05	N.S.	<0.001
2.a. Control	19.40±1.89	BLD	9.50±1.24	4.54±0.37	1.76±0.42
b. Genistein	15.74±0.20	BLD	7.90±0.34	3.42±0.29	4.11±0.34
(0.2)	(−18.9)		(−16.8)	(−24.7)	(+134)
P	<0.05		<0.05	<0.05	<0.001
3.a. Control	18.71±1.12	BLD	11.70±0.76	6.31±0.34	1.44±0.16
b. PZQ	16.18±0.39	BLD	9.30±1.04	5.69±0.25	2.14±0.10
(0.001)	(−13.5)		(−20.5)	(−9.8)	(+49)
P	N.S.		<0.05	N.S.	<0.01

Values are expressed as mean±SEM (n=3). Percentage increase (+) and decrease (−) of metabolites compared to respective controls are given in parentheses. BLD—below the level of detection. N.S.—not significant.

tively, in the crude-peel extract-, genistein- and PZQ-treated parasites.

Table 3 and Fig. 2 show the changes in the efflux of lactate by the treated parasites. There was a significant increase in lactate efflux of about 44%, 37% and 71%, respectively, by the parasites treated with the crude-peel extract, genistein and PZQ. The net lactate production, as 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 (Fig. 2), increased by 20%, 17% and 16% in the parasite treated with the crude-peel extract, genistein and PZQ, respectively. No significant change in the pH level was observed in any of the treated conditions at the paralysis time i.e., approximately 6 h (peel extract and genistein) and 3 h (PZQ), post incubation (Table 3).

Table 3
Effects of root-tuber peel extract of *F. vestita*, genistein and PZQ on lactate efflux by *R. echinobothrida* into the culture medium and pH changes of the culture medium (PBS pH 7.2)

Treatment (mg/ml)	Lactate efflux (μmol/g wet wt/h)	pH after 1 h	pH at paralysis time
1.a. Control (in 0.9% PBS with 0.1% DMSO)	1.78±0.04	7.44±0.004	6.78±0.06
b. Extract	2.56±0.22	7.02±0.01	6.32±0.02
(5.0)	(+43.8)	(−6)	(−7)
P	<0.05	N.S.	N.S.
2.a. Control	1.35±0.15	7.45±0.01	7.10±0.10
b. Genistein	1.85±0.19	7.47±0.60	6.50±0.004
(0.2)	(+37)	(0)	(−8)
P	<0.05	N.S.	N.S.
3.a. Control	1.45±0.29	7.47±0.02	7.27±0.004
b. PZQ	2.48±0.62	7.48±0.004	7.27±0.05
(0.001)	(+71)	(0)	(0)
P	<0.01	N.S.	N.S.

Values are expressed as mean±SEM (n=3). Percentage increase (+) and decrease (−) of lactate efflux or pH changes compared to respective controls are given in parentheses.

4. Discussion

Compared to the mammalian system, a modified form of metabolic pathways is reported to be present in cestodes [7]. A highly active sequence of glycolytic enzymes has been demonstrated in several cestode species confirming the

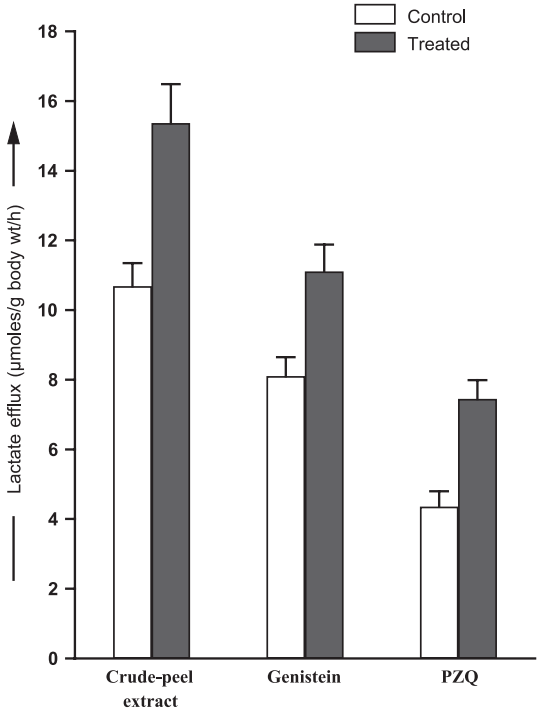

Fig. 2. Lactate efflux by *R. echinobothrida* in the culture medium at paralysis time both in control and treated conditions. Values are plotted as mean±SEM (n=3). The total efflux was calculated for 6 h in the case of crude-peel extract and genistein and for 3 h in case of PZQ.

presence of glycolytic pathway, in them [22–24]. Glucose and other simple carbohydrates are the main energy source for cestodes and trematodes [11,12], while glycogen serves as the most important energy reserve in cestodes [7]. In an earlier study pertaining to the efficacy of *F. vestita*-derived components, it was found that at the paralysis time the glycogen concentration in *R. echinobothrida* decreased by 19–44% in different treatments by stimulating glycogenolytic enzymes, particularly the active form of glycogen phosphorylase [18]. In the present study, a reduced stain intensity of glycogen in the cross sections through mature proglottids of the parasite was visibly discernible in treatments with all test materials. The glycogen concentration and its metabolism in helminths are known to be affected by a number of anthelmintic agents [12,16,25–28]. The intracellular glucose content decreased by 31.8%, 19% and 13.5% in the worms treated with the plant crude peel-extract, genistein and PZQ, respectively. This suggests that there is a need for more energy supply to the parasite under stress (caused by various treatments). The changes in the glucose content in the cestodes largely vary depending on the feeding stage of the host [11]. Drugs like PZQ, niclosamide and mebendazole reportedly cause change in the catabolism of sugar towards homolytic fermentation as evidenced by the increased production of lactic acid in *C. digonopora* [14].

The major aerobic and anaerobic end products in platyhelminth parasites are a variety of organic acids, identified as succinate, lactate, acetate, ethanol, propionate, and malate [29–31] and in several cases the fermentation end products are similar to aerobic [32]. Lactate, succinate and acetate are the three major catabolic end products detected in *Hymenolepis diminuta* [33,34], whereas in the pseudophyllidean *Ligula intestinalis* it is lactate [35]. In the present study, significant levels of lactate, malate and alanine were found to be present in *R. echinobothrida* tissue. On exposure to the plant-derived test material, genistein and PZQ, the lactate and alanine contents in the parasite tissue decreased (by 17–31% and 7–25%, respectively), whereas the malate concentration increased (by 49–134%). There was a significant increase in the net lactate production (16–20%), as also in lactate efflux (by 37–71%) in the incubation medium by the parasite at paralysis time treated with the plant extract, genistein and PZQ. More net production of lactate in the crude-peel extract-treated parasites compared to the genistein-treated ones could be due to the fact that the crude-peel extract of *F. vestita* contains other active components such as diadzein and formononetin, besides genistein, even though in very low concentration [36]; these components perhaps bring about higher activity of the crude-peel extract than genistein alone. More production of lactate, as also of malate content, in *R. echinobothrida* under treated conditions might have resulted due to anaerobiosis, as suggested in some other cestodes under rapid muscular contraction [7]. Little change in the pH of the incubation medium containing the crude extract is suggested to be due to more efflux of lactate; lactate is a product of glycolysis in the cytosol, whereas succinate and acetate are formed by reactions that take place in the mitochondria [37]. The gradual deceleration of pH changes in the incubation medium probably suggest that a balance is established between the metabolic processes of *R. echinobothrida*, accompanied by excretion of lactate as an end product [38]. However, it seems that this balance is influenced by the various test materials used in the present study. Pyruvate was also not detectable in both controls and treated parasites, though pyruvate as an end product of carbohydrate metabolism has been reported in *L. intestinalis* and *C. digonopora* [30,14].

The results showing a decline in the glycogen and glucose content and significant rise in malate and lactate content/production under treatment conditions suggest that energy demand in the parasite possibly gets enhanced under stress but the plant-derived components do not influence a switch over towards aerobic degradation of glucose in the cestode.

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