

# Effect of isoflavone from *Flemingia vestita* (Fabaceae) on the $\text{Ca}^{2+}$ homeostasis in *Raillietina echinobothrida*, the cestode of domestic fowl

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## Abstract

The alcoholic crude root-peel extract of *Flemingia vestita* and its major isoflavone, genistein, have been shown to have a vermifugal/vermicidal effect by causing a flaccid paralysis accompanied by alterations in the structural architecture of the tegumental interface and metabolic activity in *Raillietina echinobothrida*, the cestode of domestic fowl. In the present study, the crude root-peel extract and pure genistein were tested in vitro with respect to  $\text{Ca}^{2+}$  homeostasis and the occurrence of some metal ions was detected in the parasite. Live cestodes were incubated in pre-defined concentrations of the crude root-peel extract, genistein and praziquantel (as reference drug), till the paralysis time with simultaneous maintenance of respective controls. In the parasite tissue, a significant amount of  $\text{Ca}^{2+}$  (~400  $\mu\text{g/g}$  dry tissue wt) was found to be present besides magnesium, iron, zinc, lead and chromium, whilst manganese, cadmium and nickel were below the level of detection. The  $\text{Ca}^{2+}$  concentration was decreased significantly by 39%–49%, in the parasite tissue exposed to the test materials in comparison to the respective controls. There was also an increase in  $\text{Ca}^{2+}$  efflux by 91%–160% into the culture medium under similar treatments. The changes in  $\text{Ca}^{2+}$  homeostasis may be related to the rapid muscular contraction and consequent paralysis in the parasite due to the anthelmintic stress caused by the phytochemicals of *F. vestita*. © 2005 Elsevier Ireland Ltd. All rights reserved.

**Keywords:** *Flemingia vestita*; Genistein; Anthelmintic; *Raillietina echinobothrida*;  $\text{Ca}^{2+}$  homeostasis; Cestode; Parasite

## 1. Introduction

In traditional medicine, the natives of Meghalaya conventionally use the edible root tuber of *Flemingia vestita* (Fabaceae) against intestinal helminths. The putative anthelmintic isoflavones – genistein, formononetin, pseudobaptigenin and daidzein [1] – of the alcoholic crude root-peel extract of *F. vestita* have been shown to have vermifugal/vermicidal effect against cestodes and trematodes [2]. These phytochemicals, genistein in particular, caused rapid muscular contraction followed by flaccid paralysis and alterations in the tegumental architecture (deformity, blebbing, vacuolization) in *Raillietina echinobothrida*, the cestode of domestic fowl [3]; these changes were also accompanied by alterations in the activities of several enzymes/metabolic processes in the parasite under the stress of the plant-derived components [4–8].

Praziquantel (PZQ) induced paralysis and tegumental disruption in *Schistosoma mansoni* [9,10]; these changes, having been led by a rapid, sustained muscular contraction [11], were attributed to disturbed  $\text{Ca}^{2+}$  homeostasis [12,13]. Similar changes, induced by other drugs viz. calcium ionophore A-23187 and benzodiazepine Ro 11-3128, were also observed by Blair et al. [14] and Martin et al. [15]. Recent studies have shown that PZQ interacts with  $\beta$  subunit of voltage-gated  $\text{Ca}^{2+}$  channel and responsible for changes in  $\text{Ca}^{2+}$  homeostasis in *S. mansoni* [16–19].  $\text{Ca}^{2+}$ , which is stored in the calcareous corpuscles of many cestodes, especially in the metacestode stages, is intimately involved in both muscle contraction and many aspects of cell movement controlled by the cytoskeleton [20]. Enzymes like glycogen phosphorylase, glycogen synthase and protein kinases are also allosterically regulated by various modulators, for which  $\text{Ca}^{2+}$  plays a key role [21,22].

In the quest to find the plausible mode of action of the putative anthelmintic active principles of *F. vestita*, it seemed desirable to ascertain whether or not the changes in  $\text{Ca}^{2+}$  homeostasis are associated with the onset of paralytic state in the parasite, consequent to the exposure of the test phytochem-

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icals. Hence,  $\text{Ca}^{2+}$  in the parasite, *R. echinobothrida*, formed the parameter for the present study, while simultaneously the occurrence of some other elements was also detected.

## 2. Materials and methods

### 2.1. Parasites and treatments

Live parasites were recovered in 0.9% phosphate buffered saline (PBS, pH 7.2) from the intestine of freshly slaughtered host. The alcoholic crude root-peel extract of *F. vestita* was obtained as per the method described earlier [2]. Genistein was obtained from Sigma Chemicals (St. Louis, USA), while PZQ (Droncit), the reference drug, was from Bayer (India). For each treatment and its respective control the parasites were taken from a single host. About 2 g wet wt of the parasite (5–6 specimens) was exposed to previously defined concentrations of the test materials (5, 10, 20 and 50 mg/ml of the crude root peel extract; 0.2 and 0.5 mg/ml of genistein and 1, 5, and 10  $\mu\text{g}/\text{ml}$  of PZQ) dissolved in 1% dimethylsulfoxide (DMSO) in 10 ml of PBS at  $38 \pm 1$  °C, with simultaneous maintenance of controls for each treatment in PBS containing 1% DMSO. As determined through earlier studies, at these concentrations of the test materials the onset of paralysis in the cestode occurred at about 6, 4, 2 and 0.3 h for the crude root peel extract, 7 and 5 h for genistein and 3, 1 and 0.5 h for PZQ, post incubation, while the controls did not show any paralysis and survived for  $72 \pm 0.05$  h [2]. The mentioned doses of the test materials were thus chosen in order to ascertain the changes in the  $\text{Ca}^{2+}$  influx or efflux and to correlate the same with the earlier studies relating to alterations in the surface tegument and carbohydrate metabolism of the parasite under similar treatment conditions [3–8]. Flaccid paralysis (the reversible loss of motility due to the rapid, sustained muscular contraction) in the treated parasites was monitored by bringing back the parasites periodically (at every 15 min gap) to the warm 0.9% PBS ( $45 \pm 1$  °C, heat stimulus) to examine their motility [2].

### 2.2. Measurement of $\text{Ca}^{2+}$ in tissues

The parasites exposed to the various treatments and their respective controls were collected at the paralysis time for the measurement of  $\text{Ca}^{2+}$  [23]. However, the physiological levels of different elements were measured in the freshly collected parasites only. About ~2 g fresh tissue of the parasite was dried using a lyophilizer (Heto Lyolab 3000). Approximately 0.5 g of the powdered sample was digested in 10 ml of concentrated  $\text{HNO}_3$  in a 250 ml conical flask for overnight at 50 °C and the digested solution was kept for 1–2 h on a hot plate at 70–80 °C for complete evaporation of the acid, followed by addition of 10–20 ml deionised double-distilled water. The solution was filtered through Whatman filter paper (110 mm  $\Phi$ ) and the volume was finally made to 100 ml by adding deionised double-distilled water. The solution was used for the analysis of  $\text{Ca}^{2+}$  and other elements at 422.7 nm using a Perkin Elmer Atomic Absorption Spectrophotometer (Model 3110).

### 2.3. Measurement of $\text{Ca}^{2+}$ in culture media

The incubation media (0.9% PBS) of the treated parasites and their respective controls were also collected immediately after the paralytic state set in. The solution was centrifuged at 600  $\times g$  for 20 min to precipitate out the debris present, if any, in the incubation media. The final volume of the supernatant was made to 100 ml and taken for the analysis of effluxed  $\text{Ca}^{2+}$ . The rate of  $\text{Ca}^{2+}$  efflux into the culture medium at the time of paralysis in the various treatments is expressed as ' $\mu\text{g}$  per g dry tissue wt per hour'. The rate of  $\text{Ca}^{2+}$  efflux was calculated by dividing the amount of  $\text{Ca}^{2+}$  per gram dry tissue weight effluxed into the medium at the paralysis time by the time (h) taken for paralysis at the respective treatment condition. In the case of controls, for calculating the rate of  $\text{Ca}^{2+}$  efflux, the incubation medium was taken at 6, 6 and 3 h, i.e., the maximum time that the respective treatments (crude root-peel extract, genistein and PZQ) took to show the onset of paralysis in the parasite.

### 2.4. Statistical analysis

Data from 4–5 replicates were statistically analyzed and expressed as mean  $\pm$  SEM and  $P < 0.05$  was regarded as statistically significant. The paired mean values between the experimental and respective controls were compared using Student's *t*-test [24].

## 3. Results

The presence of some elements and their concentration in quantitative terms in the parasite tissue is depicted in Table 1. Out of the elements detected, the concentration of magnesium was found to be the highest (~1400  $\mu\text{g}$ ) and that of chromium, the lowest (~12  $\mu\text{g}$ ), while manganese, cadmium and nickel were below the level of detection. Since  $\text{Ca}^{2+}$  has been implicated in bringing about trans-tegumental permeability changes in the parasite's interface [20], the status of  $\text{Ca}^{2+}$  as under the influence of the various test materials was investigated further. A significant amount of  $\text{Ca}^{2+}$  (350–400  $\mu\text{g}/\text{g}$  dry tissue wt) was found to be present in control parasites. However, a decline in the  $\text{Ca}^{2+}$  concentration in the tissue was recorded in the parasite exposed to all the test materials (Table 2). The  $\text{Ca}^{2+}$  concentration decreased significantly by 47%–

Table 1  
Levels of elements ( $\mu\text{g}/\text{g}$  dry tissue wt) present in *R. echinobothrida*

Elements	Concentration
Calcium	394 $\pm$ 14
Chromium	12 $\pm$ 1
Manganese	BLD
Cadmium	BLD
Lead	24 $\pm$ 2
Nickel	BLD
Iron	1185 $\pm$ 43
Zinc	387 $\pm$ 7
Magnesium	1396 $\pm$ 33

Values are expressed as mean  $\pm$  SEM ( $n=5$ ).

BLD – Below the level of detection.

Table 2

Effects of crude root-peel extract of *F. vestita*, genistein and PZQ on the concentration of  $\text{Ca}^{2+}$  ( $\mu\text{g/g}$  dry tissue wt) in the parasite tissue and  $\text{Ca}^{2+}$  efflux ( $\mu\text{g/g}$  dry tissue wt) by *R. echinobothrida* into the incubation medium at paralysis time

Treatment (onset of paralysis, time in h)	$\text{Ca}^{2+}$ concentration (parasite's tissue)	$\text{Ca}^{2+}$ efflux (incubation medium)
1.a. Control*	266.7 $\pm$ 31.3	73.2 $\pm$ 2.6
1.b. Crude root-peel extract		
5.0 mg/ml (5.9 $\pm$ 0.5 h)	136.6 $\pm$ 6.5 (-49) <sup>a</sup>	149.4 $\pm$ 3.3 (+100) <sup>b</sup>
10.0 mg/ml (4.08 $\pm$ 0.1 h)	139.5 $\pm$ 2.7 (-48) <sup>a</sup>	139.9 $\pm$ 4.3 (+91) <sup>b</sup>
20.0 mg/ml (2.03 $\pm$ 0.08 h)	141.0 $\pm$ 4.2 (-47) <sup>a</sup>	151.1 $\pm$ 3.7 (+106) <sup>b</sup>
50.0 mg/ml (0.3 $\pm$ 0.01 h)	138.8 $\pm$ 3.6 (-48) <sup>a</sup>	146.0 $\pm$ 2.7 (+99) <sup>b</sup>
2.a. Control*	266.7 $\pm$ 31.3	73.2 $\pm$ 2.6
2.b. Genistein		
0.2 mg/ml (6.7 $\pm$ 0.04 h)	162.5 $\pm$ 4.1 (-39) <sup>a</sup>	159.4 $\pm$ 2.4 (+118) <sup>b</sup>
0.5 mg/ml (4.4 $\pm$ 0.07 h)	159.8 $\pm$ 3.1 (-40) <sup>a</sup>	163.6 $\pm$ 5.3 (+123) <sup>b</sup>
3.a. Control*	256.9 $\pm$ 25.4	46.8 $\pm$ 1.2
3.b. PZQ		
1 $\mu\text{g/ml}$ (2.9 $\pm$ 0.05 h)	142.1 $\pm$ 5.9 (-45) <sup>a</sup>	90.9 $\pm$ 1.6 (+94) <sup>b</sup>
5 $\mu\text{g/ml}$ (0.89 $\pm$ 0.04 h)	139.9 $\pm$ 4.2 (-46) <sup>a</sup>	110.3 $\pm$ 2.3 (+136) <sup>c</sup>
10 $\mu\text{g/ml}$ (0.47 $\pm$ 0.07 h)	136.2 $\pm$ 3.8 (-47) <sup>a</sup>	121.7 $\pm$ 3.6 (+160) <sup>c</sup>

Values are expressed as mean $\pm$ SEM ( $n=5$ ).

Percentage increase (+) and decrease (-) of  $\text{Ca}^{2+}$  concentration or  $\text{Ca}^{2+}$  efflux compared to the respective controls are given in parentheses.

<sup>a</sup>, <sup>b</sup>, <sup>c</sup>:  $P$  value significant at  $>0.05$ ,  $>0.01$  and  $>0.001$ , respectively, in treatments compared with their respective controls.

\* Controls in 0.9% PBS survived for 72 $\pm$ 0.05 h [2]; however, the control parasites and control incubation media were taken for measurements at 6, 6 and 3 h in the case of crude root-peel extract, genistein and PZQ, respectively, in order to compare with the respective treatment conditions.

49%, 39%–40% and 45%–47%, respectively, in treatments with the crude root-peel extract, genistein and PZQ. In corroboration with this decrease in the  $\text{Ca}^{2+}$  concentration in the tissue as influenced by these treatments, the  $\text{Ca}^{2+}$

concentration in the incubation medium was found to be increased by 91%–106%, 118%–123% and 94%–160%, respectively, indicating thereby an increased efflux of  $\text{Ca}^{2+}$  into the medium (Table 2). The rate of  $\text{Ca}^{2+}$  efflux into the medium has also been shown in Fig. 1.

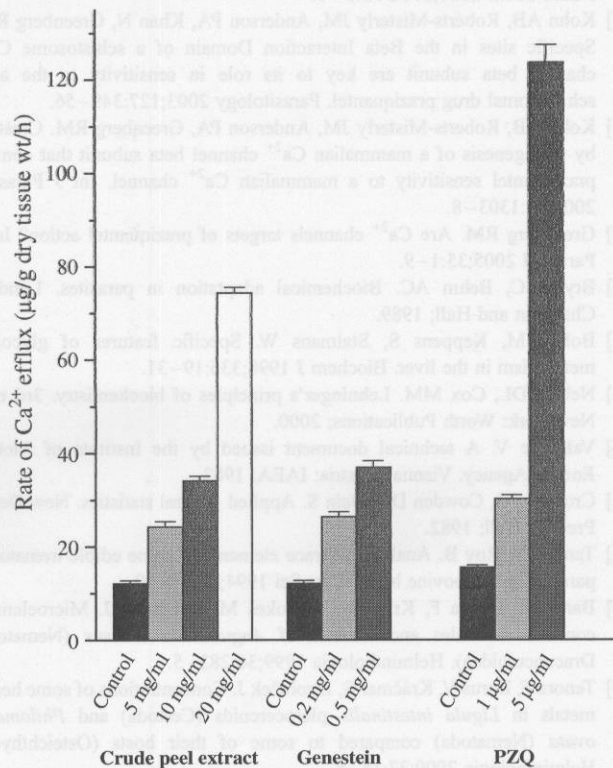


Fig. 1. Effects of crude root-peel extract of *F. vestita*, genistein and PZQ on the rate of  $\text{Ca}^{2+}$  efflux ( $\mu\text{g/g}$  dry tissue wt/h) by *R. echinobothrida* into the incubation medium at paralysis time. The rate of  $\text{Ca}^{2+}$  efflux was calculated by dividing the amount of  $\text{Ca}^{2+}$  per g dry tissue wt effluxed into the medium at the paralysis time by the time (h) taken for paralysis at the respective treatment condition.

#### 4. Discussion

The presence of elements like calcium, copper, manganese, magnesium, lead, iron, nickel, zinc and potassium has been reported in some helminth parasites, viz., amphistomid trematodes [25], nematodes and cestodes [26–28]. In the present study, elements like calcium, chromium, lead, iron, zinc and magnesium were detected in the parasite, *R. echinobothrida*. However, some of the elements like manganese, cadmium and nickel were below the level of detection. Though  $\text{Mg}^{2+}$  is present in higher concentration in the parasite,  $\text{Ca}^{2+}$  is known to play a key role during rapid sustained muscular contraction [22].

In vitro, PZQ caused vacuolization, muscle contraction and ultimately paralysis in *S. mansoni* and these changes were attributed to an increased  $\text{Ca}^{2+}$  influx by the drug [29]. However,  $\text{Mg}^{2+}$  competitively blocked the  $\text{Ca}^{2+}$  influx by blocking the  $\text{Ca}^{2+}$  permeable sites, which are targets of PZQ under elevated  $\text{Mg}^{2+}/\text{Ca}^{2+}$  ratio leading to flaccid paralysis [30]. Besides *Schistosoma* spp, PZQ was also found to be effective against other trematodes and cestodes [31]. In cestodes the similar effect, i.e., paralysis, caused by PZQ was attributed to the increased  $\text{Ca}^{2+}$  efflux [32,33]. In the adult liver flukes, *Opisthorchis viverrini*, PZQ was reported to cause depolymerization of the microtrabecular network that led to vacuolization, swelling, blebbing, tegumental disorganization and breakdown of myofilaments in the muscle cells through the induction of  $\text{Ca}^{2+}$  influx [34]. Besides PZQ, other chemotherapeutics are also known to cause vacuolization and disruption of the surface tegument and also muscular paralysis in several

parasite species [35,36]. 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 [37,38]. In the present study, the crude root-peel extract, genestein and PZQ caused a significant decline ( $p < 0.05$ ) in  $\text{Ca}^{2+}$  concentration (39%–49%) in the parasite tissue in comparison to the respective controls. In corroboration with the decrease of  $\text{Ca}^{2+}$  concentration in the tissue, there was a significant increase of  $\text{Ca}^{2+}$  concentration (91%–160%) in the incubation medium, indicating thereby an increased efflux of  $\text{Ca}^{2+}$  into the medium during the treatments. Though the concentration of  $\text{Ca}^{2+}$  in the medium remained the same at the paralysis time at different dosages of treatments, the rate of  $\text{Ca}^{2+}$  efflux into the medium was greatly varied (Fig. 1). The dose-dependant increased  $\text{Ca}^{2+}$  efflux indicates that the crude root-peel extract, genestein and PZQ bring out the  $\text{Ca}^{2+}$  efflux in the parasite during the rapid sustained muscular contraction followed by paralysis. This is in conformity with the observations of Prichard et al. [32], who found a comparable decrease in the tissue  $\text{Ca}^{2+}$  levels in *Hymenolepis diminuta* treated with PZQ.

The test phytochemicals in the present study, genistein in particular, seem to affect the  $\text{Ca}^{2+}$  homeostasis (by way of altering the  $\text{Ca}^{2+}$  flux into or through the parasite's tegumental interface) that, in turn, leads to such detrimental changes in the parasite [2]. Perhaps the changes in the  $\text{Ca}^{2+}$  homeostasis in the parasite subsequently also lead to changes in the activities of several enzymes/metabolic processes [5–8] in the parasite under the high energy demand because of the anthelmintic stress caused by the plant-derived components.

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