Effect of *Punica granatum* fruit peel on glucose-6-phosphate dehydrogenase and malate dehydrogenase in amphistome *Gastrothylax indicus*

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Abstract Increasing anthelmintic resistance and the impact of conventional anthelmintics on the environment, it is important to look for alternative strategies against helminth parasite in sheep. Important lipogenic enzymes like glucose-6-phosphate dehydrogenase (G-6-PDH) and malate dehydrogenase (MDH) show subcellular distribution pattern. Activity of G-6-PDH was largely restricted to cytosolic fraction while MDH was found in both cytosolic and mitochondrial fraction in *Gastrothylax indicus*. Following in vitro treatment with ethanolic and aqueous extracts of *Punica granatum* fruit peel and commercial anthelmintic, albendazole G-6-PDH activity was decreased by 19–32 %, whereas MDH was suppressed by 24–41 %, compared to the respective control. Albendazole was quite effective when compared with negative control and both the extracts. The results indicate that phytochemicals of plant may act as potential vermifuge or vermicide.

Keywords Anthelmintic · *Punica granatum* · Glucose-6-phosphate dehydrogenase · Malate dehydrogenase · *Gastrothylax indicus*

Introduction

Gastrothylax indicus is a trematode causing amphistomiasis of domestic ruminants which is one of the most common and serious helminthic diseases characterized by acute

parasitic gastroenteritis, problem in lactation and high mortality in the host, thus resulting in considerable economic loss. It has been estimated that more than 500 million cattle world wide are at risk due to parasitic infection. Death due to immature paramphistomes is very high and may be as high as 80–90 % in domesticated ruminants (Juyal et al. 2005; Ilha et al. 2005).

Punica granatum (anar) is herbal anthelmintic drug widely used in ayurveda. Pomegranate fruit products have been used for centuries since ancient civilizations for medicinal purposes. Stomachic, inflammation, fever, bronchitis, diarrhea, dysentery, vaginitis, urinary tract infection, and, among others, malaria have been treated using various parts of pomegranate including fruit peels. The fruits of *P. granatum* (pomegranate) have been used to treat acidosis, dysentery, microbial infections, diarrhoea, helminthiasis, haemorrhage, and respiratory pathologies (Swarnakar et al. 2013). The most famous usage worldwide has been a vermifugal or taenicial agent i.e. a killer and expeller of intestinal worms (Subhedar et al. 2011). The anthelmintic activity may be chiefly due to alkaloids.

The pharmacological basis of the treatment for helminthes generally involves the interference of one or both the energy processes which cause subsequent starvation of parasitic and neuromuscular incoordination which leads to paralysis and expulsion of the parasite. Inspite of extraordinary progress which has been made over the last decade, there are many facets of traditional veterinary medicinal practices still in need of improvement. It is with this view that the present work will emphasize on elaborating the alterations induced by the in vitro incubations of ethanolic and aqueous extracts of fruit peel of *P. granatum* (EFPEPG, AFPEPG) on the lipogenic enzymes of *G. indicus*—a trematode parasite causing paramphistomiasis.

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Materials and methods

Preparation of plant crude extract

Anar (*P. granatum*) fruit was collected from in and around Chandigarh. Identification was carried out in Department of Botany, Panjab University, Chandigarh with Voucher number-8583. Fruit peel of *P. granatum* were washed thoroughly, shade dried and grounded by motor driven grinder into powder form. Both ethanolic and aqueous plant extracts were prepared according to method of Iqbal et al. (2005). Ethanolic fruit peel extract of *P. granatum* (EFPEPG) was exhaustively extracted by mixing 80 g of powdered plant material and adding approximately 300 mL of ethanol in a soxhlet apparatus. Aqueous extract was prepared by dissolving 100 g of powdered plant material mixed with 500 mL of distilled water in 1 L flask and boiled for 4–6 h in water bath. It was allowed to macerate at room temperature for 24 h and the brew was filtered through muslin gauze and Whatman filter paper No. 1.

Both ethanolic and aqueous extracts of plant material were evaporated in Rota evaporator to give crude ethanolic and aqueous extracts. The extracts were scraped off and transferred to screw capped vials at 4 °C until used.

Parasites and in vitro treatment

Mature *G. indicus* were collected from the rumen of sheep/goat procured from slaughter house. The worms were washed in phosphate buffered saline (PBS pH 7.2) and finally suspended in PBS. The freshly obtained live parasites were incubated in 12.5 mg of plant extracts per mL of PBS for enzyme studies. For albendazole it was 20 µg/mL. The crude aqueous extracts were diluted in PBS, whereas, crude ethanolic extracts in 1 % DMSO in PBS. Albendazole dissolved in 1 % DMSO and diluted in PBS and PBS alone served as positive and negative control respectively. These concentrations were decided on the basis of our initial experiments wherein LC-50 values were calculated for EFPEPG, AFPEPG and albendazole in *G. indicus* as reported in a previous study (Aggarwal et al. 2015).

Tissue processing and sub-cellular fractions

Treated parasites and their respective controls were retrieved from the incubation media at the time when paralysis was seen and were processed for enzymatic analysis. A 10 % homogenate of the enzymes in 0.25 M sucrose was centrifuged at 5000 rpm for 25 min at 0 °C and the resultant supernatant was used for enzyme assays.

Mitochondrial, lysosomal and cytosolic fractions were prepared by differential centrifugation of a 10 % homogenate of the parasite in 0.25 M sucrose (Sawhney and Singh 1996).

Enzyme assays

Estimations were done according to Sawhney and Singh (1996).

Estimation of G-6-PDH activity—2.6 mL of 50 mM Tris–HCl buffer (pH 7.8) containing 3.3 mM of MgCl₂ was taken in a silica cuvette, 0.1 mL of 6 mM NADP and 0.3 mL aliquot of various fractions was added to it. Equilibrated at 30 °C, adjusted the absorbance of spectrophotometer at 340 nm to zero. Started the reaction by adding 0.1 mL, 100 mM of glucose-6-phosphate, monitored the increase in A₃₄₀ and calculated the activity in terms of µmol of NADPH produced/min from the molar extinction coefficient value of 6.22×10^3 for NADPH at 340 nm.

Estimation of MDH activity—1.5 mL of assay buffer (50 mM Tris–HCl pH 8.0 with 1 mM EDTA) was taken, added 0.1 mL aliquot of various fractions, kept in water bath at 37 °C for 2–3 min, added 0.05 mL of 6 mM NADH made in 50 mM Tris–HCl pH 8.0 mixed and noted the spectrophotometer reading at 340 nm. Now started the reaction by adding 0.05 mL of 0.3 M of oxaloacetate water solution and noted the decrease in absorbance. A standard curve of NADH was prepared in assay buffer over a range of 0–0.3 µmol. Enzyme activity was µmol of malate formed/g tissue/min.

Specific activity

Specific activities of the enzymes were expressed as the units of enzymes activity per mg protein. Protein contents of different samples were estimated following Lowry et al. (1951).

Statistical analysis

Statistical analysis were carried out by employing Graph pad software 3 and data was expressed as mean ± SD for each group. The statistical significance of inter group difference of various parameters were determined by unpaired student's *t* test. The comparisons were made between the treated groups and control group of parasites.

Results

The subcellular distribution of G-6-PDH and MDH in the trematode, *G. indicus* and the effect of EFPEPG, AFPEPG and albendazole on the activity of these two enzymes are presented in Tables 1 and 2.

Table 1 Effect of EFPEPG, AFPEPG and albendazole on tissue activity (units/g wet wt/min) and specific activity (units/mg protein/min) of G-6-PDH in *G. indicus* in vitro

Control/PBS/(mg/mL) Treatment	Enzyme activity (tissue/specific)			
	G-6-PDH			
	Homogenate	Mitochondrial fraction	Lysosomal fraction	Cytosolic fraction
Control	3.28 ± 0.23	0.39 ± 0.03	0.29 ± 0.17	2.85 ± 0.18
	1.27 ± 0.12	0.15 ± 0.001 (12)	0.11 ± 0.01 (9)	1.11 ± 0.01 (87)
EFPEPG (12.5)	2.62 ± 0.20	0.32 ± 0.02	0.24 ± 0.04	2.25 ± 0.14
	1.02 ± 0.12	0.12 ± 0.01	0.09 ± 0.01	0.88 ± 0.08
	[20]*	[19]*	[19.5]	[21]*
Control	4.17 ± 0.28	0.54 ± 0.05	0.33 ± 0.01	3.50 ± 0.03
	1.63 ± 0.08	0.21 ± 0.01 (13)	0.13 ± 0.01 (8)	1.37 ± 0.02 (84)
AFPEPG (12.5)	3.35 ± 0.09	0.43 ± 0.05	0.27 ± 0.03	2.71 ± 0.12
	1.31 ± 0.11	0.17 ± 0.09	0.10 ± 0.01	1.06 ± 0.04
	[19.5]**	[20.2]	[19.3]*	[22.5]***
Control	3.93 ± 0.03	0.55 ± 0.05	0.35 ± 0.03	3.46 ± 0.03
	1.53 ± 0.01	0.21 ± 0.01 (14)	0.18 ± 0.01 (9)	1.35 ± 0.01 (88)
Albendazole (20 µg/mL)	2.71 ± 0.02	0.40 ± 0.01	0.25 ± 0.01	2.35 ± 0.02
	1.06 ± 0.01	0.16 ± 0.01	0.09 ± 0.01	0.91 ± 0.01
	[31]***	[27.2]	[28.5]	[32]***

Percentage of enzyme activity in the mitochondrial, lysosomal and cytosolic fractions compared to the activity in the homogenate is given within parentheses. Percentage decrease of enzyme activity compared to respective controls is given within square brackets

One unit of enzyme activity is the amount of enzyme catalyzing 1 µmol of NADP⁺ reduction in G-6-PDH per min at 38 °C

Values are expressed as mean ± SD. Each test was done in triplicate

*** $p < 0.0005$; ** $p < 0.005$; * $p < 0.05$

Glucose-6-phosphate dehydrogenase activity was mainly (84–88 %) localized in cytosolic fraction. G-6-PDH activity, in the cytosolic fraction was significantly reduced by 21, 22.5 and 32 %, respectively, by treatments with EFPEPG, AFPEPG and albendazole; this was followed by reduction in enzyme in tissue homogenate by 20, 19.5 and 31 % respectively.

Malate dehydrogenase activity, was noticed predominantly (58–65 %) in cytosolic fraction followed by mitochondrial (31–34 %) fraction. MDH activity was decreased by 27, 28 and 41 %, respectively in the cytosolic fraction in *G. indicus* when incubated with EFPEPG, AFPEPG and albendazole whereas, this decrease was 24.3, 24.8 and 39 % with mitochondrial fraction respectively. In tissue homogenate, MDH activity was found to be decreased by 24.5, 25.2 and 40 % respectively with various incubations in the parasite.

Negligible activity was detected for both the enzymes G-6-PDH (8–9 %) and MDH (7–10 %) in lysosomal fraction. However a significant reduction was measured for both the enzymes with various treatments in the parasite as shown in Tables 1 and 2.

Specific activities of the enzymes depicted similar alterations as the respective tissue activities of homogenate/fractions (Tables 1, 2). Albendazole was seen to be more effective than plant extracts in the present studies.

Discussion

Glucose-6-phosphate dehydrogenase activity was predominantly restricted to cytosolic fraction in the trematode parasite *G. indicus* in the present studies. Similar observations were reported by Das et al. (2004a) in a cestode *Raillietina echinobothrida*. MDH activity was found both in cytosolic and mitochondrial fraction, in the present studies, which is in agreement with the observations made by Das et al. (2004b) in *R. echinobothrida*. Aggarwal et al. (1989) also reported similar subcellular distribution for both the enzymes in an intestinal nematode parasite of fowl *Ascaridia galli*.

The presence of lipogenic enzymes in *G. indicus* indicates that the parasite is not dependent, at least completely, on the host's lipid biosynthetic machinery and the key enzymatic steps in the intermediary metabolism are fully

Table 2 Effect of EFPEPG, AFPEPG and albendazole on tissue activity (units/g wet wt/min) and specific activity (units/mg protein/min) of MDH in *G. indicus* in vitro

Control/PBS (mg/mL) Treatment	Enzyme activity (tissue/specific)			
	MDH			
	Homogenate	Mitochondrial fraction	Lysosomal fraction	Cytosolic fraction
Control	23.38 ± 0.34	7.95 ± 0.05	1.64 ± 0.06	13.56 ± 0.24
	7.95 ± 0.03	2.70 ± 0.02	0.56 ± 0.01	4.61 ± 0.12
		(34)	(7)	(58)
EFPEPG (12.5)	17.65 ± 0.23	6.02 ± 0.12	1.23 ± 0.14	9.89 ± 0.19
	6.01 ± 0.12	2.04 ± 0.07	0.42 ± 0.04	3.36 ± 0.13
	[24.5]***	[24.3]***	[25]**	[27]***
Control	34.67 ± 0.35	10.75 ± 0.47	3.12 ± 0.14	21.15 ± 0.39
	11.79 ± 0.13	3.65 ± 0.27	1.06 ± 0.07	7.19 ± 0.17
		(31)	(9)	(61)
AFPEPG (12.5)	25.93 ± 0.52	8.08 ± 0.43	2.29 ± 0.49	15.23 ± 0.79
	9.14 ± 0.27	2.74 ± 0.29	0.78 ± 0.23	5.18 ± 0.45
	[25.2]***	[24.8]**	[26.3]*	[28]***
Control	38.63 ± 0.39	12.36 ± 0.54	3.86 ± 0.36	25.11 ± 0.23
	13.13 ± 0.15	4.20 ± 0.34	1.31 ± 0.11	8.54 ± 0.18
		(32)	(10)	(65)
Albendazole (20 µg/mL)	23.17 ± 0.28	7.54 ± 0.98	2.38 ± 0.34	14.81 ± 0.48
	7.88 ± 0.09	2.56 ± 0.48	0.81 ± 0.09	5.04 ± 0.28
	[40]***	[39]**	[38]	[41]***

Percentage of enzyme activity in the mitochondrial, lysosomal and cytosolic fractions compared to the activity in the homogenate is given within parentheses. Percentage decrease of enzyme activity compared to respective controls is given within square brackets

One unit of enzyme activity is the amount of enzyme catalyzing 1 µmol of NADH oxidation in MDH per min at 38 °C

Values are expressed as mean ± SD. Each test was done in triplicate

*** $p < 0.0005$; ** $p < 0.005$; * $p < 0.05$

operative. G-6-PDH and MDH are important lipogenic enzymes providing the reducing equivalents essentially needed for fatty acid synthesis. The presence of these enzymes assumes importance since in its usual habitat the parasite undergoes metabolism in anaerobic conditions or under stress of poor oxygen tension.

Glucose-6-phosphate dehydrogenase inhibition with plant extracts and albendazole will result in blockade of supply of reducing equivalents to the nucleotide and fatty acid biosynthesis. Inhibition of MDH, strongly suggests the arrest of carbon flux in the glycolytic pathway and generation of the necessary energy through oxidative phosphorylation. The leaf extracts of *Ocimum sanctum*, *Lawsonia inermis* and *Calotropis gigantea* and leaf and flower extracts of *Azadirachta indica* were found to inhibit MDH in *Setaria digitata*. Das et al. (2004b) reported an increase in the activity of MDH in *R. echinobothrida* with crude root peel extract of *Flemingia vestita* whereas activity G-6-PDH was significantly reduced (Das et al. 2004a). Artemether, a well known antimalarial drug derived from plant

genus *Artemesia*, exerts a potent inhibitory action on G-6-PDH activity in *Schistosoma japonicum* (Xiao et al. 2004).

Inhibition of these enzymes by ethanolic and aqueous fruit peel extract of *P. granatum* may provide an important clue regarding its mode of action in the parasite *G. indicus*. It indicates that phytochemicals of the plant may act as potential vermifuge or vermicide. In view of these observations further biochemical studies involving isolated active component(s) of this plant are warranted to confirm its anthelmintic efficacy.

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