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ANTHELMINTIC EFFICACY OF *CALOTROPIS PROCERA* : ALTERATION IN THE ACTIVITIES OF LIPOGENIC ENZYMES IN THE TREMATODE, *GASTROTHYLAX INDICUS*

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ABSTRACT
The crude ethanolic and aqueous extract of *Calotropis procera* flower and albendazole were tested against some selected lipogenic enzymes, like glucose-6-phosphate dehydrogenase (G-6-PDH) and malate dehydrogenase (MDH) in *Gastrothylax indicus.* Following exposure to these treatments, *in vitro* , G-6-PDH activity was decreased by 24-32% , whereas MDH was suppressed by 35.5-41%, compared to the respective control. Both the enzymes 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 *G. indicus.* The reference drug,albendazole showed better effect when compared with negative control and both the extracts. The results suggests that phytochemicals of plant *C. procera* have anthelmintic potential .

KEYWORDS: Albendazole, *Calotropis procera*, *Gastrothylax indicus*, Glucose-6-phosphate dehydrogenase, Malate dehydrogenase

INTRODUCTION

Helminthiasis is among the most important animal health problems, which inflicts heavy production losses. The disease is highly prevalent particularly in developing countries (Dhar et al. 1982). Chemical control of helminths coupled with improved management has been an important worm control strategy throughout the World. However, increasing problems of development of resistance in helminths (Coles 1997) against anthelmintics have led to the proposal of screening medicinal plants for their anthelmintic activities. A number of medicinal plants have been used to treat parasitic infections in man and animals (Akhtar et al. 2000 ; Iqbal et al. 2004).

There is an increasing interest concerning the plant *Calotropis procera* due to innumerous relevant biological activities found in its vegetative tissues. *C. procera*, known as Aak, is used in ethnoveterinary medicine system as an expectorant, anthelmintic, laxative, purgative, anti-inflammatory and diuretic. Despite some reports of toxicity associated with *Calotropis* feeding to animals (Badwi et al. 1998), its use in ethnoveterinary medicine is increasing based on empirical evidence in the successful treatment of different ailments. Different parts as well as latex of *C. procera* have been reported to have emetic, purgative and anthelmintic effects in traditional medicine (Jain et al. 1996). *C. procera* flowers are mostly used as an anthelmintic in small ruminants in the form of decoction and/or crude powder mixed with jaggery and administered as physic drench/balls. The crude ethanolic and aqueous extract of *C. procera* flower has been shown to act vermifugally/vermicidally against trematode, *Gastrothylax indicus* (Aggarwal et al. 2015) and also altered the activities of several enzymes that are associated with the tegument, such as acid and alkaline phosphatases, adenosine triphosphatase and glucose–6–phosphatase (Aggarwal and Bagai 2014).

Lipid metabolism of trematodes is poorly studied, possibly because it is believed that these helminths are absolutely dependent on the host for lipids necessary for membrane synthesis and egg production (Rath and Walkey 1987). 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 flower of *C. procera* (EFECP, AFECP) alongwith reference drug albendazole on the lipogenic enzymes of *Gastrothylax indicus*–a trematode parasite causing paramphistomiasis.

MATERIALS AND METHODS

Plant material

Flowers of aak (*Calotropis procera*), were collected from in and around Chandigarh. The plant material was identified in Department of Botany, Panjab University, Chandigarh with Voucher number-4830.

Preparation of extracts

Flowers of *C.procera* 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 flower

extract of *C.procera* (EFECP), was exhaustively extracted by mixing 80 gm of powdered plant material and adding approximately 300ml of ethanol in a soxhlet apparatus. Aqueous extract (AFECP) was prepared by dissolving 100gm of powdered plant material mixed with 500ml of distilled water in 1L 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 4^0C 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 pH7.2) and finally suspended in PBS. The freshly obtained live parasites were incubated in 6.25 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 postitive and negative control respectively. These concentrations were decided on the basis of our initial experiments wherein LC-50 values were calculated for EFECP, AFECP 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 0.25M sucrose was centrifuged at 5,000 rpm for 25 min at 0ºC 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 0.25M 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 50mM Tris-HCl buffer (pH 7.8) containing 3.3 mM of $MgCl_2$ was taken in a silica cuvette, 0.1 ml of 6mM NADP and 0.3 ml aliquot of various fractions was added to it. Equilibrated at 30^oC, adjusted the absorbance of spectrophotometer at 340 nm to zero. Started the reaction by adding 0.1 ml, 100mM of glucose-6-phosphate, monitored the increase in A_{340} and calculated the activity in terms of µmoles of NADPH produced/min from the molar extinction coefficient value of $6.22x\ 10^3$ for NADPH at 340 nm.

Estimation of MDH activity–1.5 ml of assay buffer (50 mM Tris–HCl pH 8.0 with 1mM EDTA) was taken, added 0.1 ml aliquot of various fractions, kept in water bath at 37^oC for 2-3 min, added 0.05 ml of 6mM 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 µmoles. Enzyme activity was µmoles 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 ± S.D. 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 EFECP, AFECP and albendazole on the activity of these two enzymes are presented in Table 1 & 2. Major activity of G-6-PDH was observed (84-88%) in cytosolic fraction . In vitro treatments of parasite with EFECP, AFECP and albendazole significantly decreased the activity of G-6-PDH by 21%, 22.5% and 32%, respectively, in the cytosolic fraction; while percentage reduction in enzyme activity in tissue homogenate was 24%, 24.5% and 31% respectively under similar conditions.

MDH activity, in *G. indicus* occurred both in cytosolic (58-65%) and mitochondrial (31-34%) fraction.MDH activity in both the fractions was affected in the parasite by the different treatments. It was decreased by 37%, 37.5% and 41%, respectively in the cytosolic fraction when incubated with EFECP, AFECP and albendazole whereas, this decrease was 35.5%, 36% and 39% with mitochondrial fraction respectively. In tissue homogenate, MDH activity was found to be decreased by 36%, 35% and 40% respectively with various incubations in the parasite.

Table1. Effect of EFECP, AFECP 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)			
	G6PDH			
	Homogenate	Mitochondrial fraction	Lysosomal fraction	Cytosolic fraction
Control	3.28±0.23 1.27±0.12	0.39±0.03 0.15±0.001 (12)	0.29±0.17 0.11±0.01 (9)	2.85±0.18 1.11±0.01 (87)
EFECP(6.25)	2.49±0.19 0.97±0.10 [24]*	0.29±0.03 0.11±0.01 [25.6]	0.22±0.02 0.08±0.01 [24.1]	2.11±0.19 0.82±0.03 [26]**
Control	4.17±0.28 1.63±0.08	0.54±0.05 0.21±0.01 (13)	0.33±0.01 0.13±0.01 (8)	3.50±0.03 1.37±0.02 (84)
AFECP(6.25)	3.15±0.03 1.23±0.07 [24.5]***	0.41±0.04 0.16±0.08 [24]	0.25±0.02 0.10±0.01 [24.2]	2.62±0.02 1.02±0.01 [25]***
Control	3.93±0.03 1.53±0.01	0.55±0.05 0.21±0.01 (14)	0.35±0.03 0.18±0.01 (9)	3.46±0.03 1.35±0.01 (88)
Albendazole (20 µg/ml)	2.71±0.02 1.06±0.01 [31]***	0.40±0.01 0.16±0.01 [27.2]	0.25±0.01 0.09±0.01 [28.5]	2.35±0.02 0.91±0.01 [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 1µmol of $NADP^+$ reduction in G6PDH per min at 38^0C.

Values are expressed as mean± S.D. Each test was done in triplicate. ***p<0.0005, **p<0.005, *p<0.05.

Table2. Effect of EFECP, AFECP 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±0.03	7.95±0.05 2.70±0.02 (34)	1.64±0.06 0.56±0.01 (7)	13.56±0.24 4.61±0.12 (58)
EFECP(6.25)	14.96±0.24 5.09±0.14 [36]***	5.13±0.13 1.74±0.04 [35.5]***	1.04±0.13 0.35±0.02 [36.5]	8.54±0.17 2.91±0.12 [37]***
Control	34.67±0.35 11.79±0.13	10.75±0.47 3.65±0.27 (31)	3.12±0.14 1.06±0.07 (9)	21.15±0.39 7.19±0.17 (61)
AFECP(6.25)	22.54±0.48 7.66±0.19 [35]***	6.88±0.35 2.34±0.12 [36]**	1.99±0.12 0.68±0.05 [36]	13.22±0.85 4.49±0.38 [37.5]***
Control	38.63±0.39 13.13±0.15	12.36±0.54 4.20±0.34 (32)	3.86±0.36 1.31±0.11 (10)	25.11±0.23 8.54±0.18 (65)
Albendazole (20 µg/ml)	23.17±0.28 7.88±0.09 [40]***	7.54±0.98 2.56±0.48 [39]**	2.38±0.34 0.81±0.09 [38]	14.81±0.48 5.04±0.28 [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 1µmol of NADH oxidation in MDH per min at 38^0C. Values are expressed as mean± S.D. Each test was done in triplicate. ***p<0.0005, **p<0.005, *p<0.05.

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 Table 1 & 2.

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

DISCUSSION

HMP is an important carbohydrate metabolic pathway providing NADPH for fatty acid synthesis and pentoses for nucleic acid synthesis (Smyth and McManus 1989). The presence of significant activity of G-6-PDH in *Ascaridia galli*, *Cotylophoron cotylophorum* and *Raillietina echinobothrida* indicates that HMP might be operative in helminths (Aggarwal *et al.* 1989; Aggarwal and Khera 1995; Das et al. 2004a).

G-6-PDH is the first and regulatory pacemaker enzyme of HMP. As expected, G-6-PDH activity in the present study, in the parasite, in *G. indicus* was primarily detected in the cytosolic fraction, with a minor activity showing in the mitochondrial component. Similar subcellular distribution pattern has been reported in *A. galli* and *R.* echinobothrida (Aggarwal et al. 1989; Das et al. 2004a). G-6-PDH activity was significantly decreased with plant extracts and albendazole in *in vitro* incubations of the parasite in the present study. Das et al. (2004a) reported a decrease in the activity of G-6-PDH in *R. echinobothrida* with crude root peel extract of *Flemingia vestita*. 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). These results suggest that, under stress conditions, NADPH synthesis may get inhibited due to lowered activity of this key regulatory enzyme of HMP in the parasite.

The presence of MDH, which converts oxaloacetate to malate, has also been reported in several helminths. 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 the enzyme in an intestinal nematode parasite of fowl *A. galli.* 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* (Banu et al. 1992). Das et al. (2004b) reported an increase in the activity of MDH in *R. echinobothrida* with crude root peel extract of *Flemingia vestita.* Inhibition of MDH,by plant extracts and albendazole in the present study, strongly suggests the arrest of carbon flux in the glycolytic pathway and generation of the necessary energy through oxidative phosphorylation. In conclusion, evidence is provided in *G. indicus*, ethanolic and aqueous extract of *Calotropis procera* flower inhibit the lipogenic enzymes, may provide an important clue regarding its mode of action in the parasite, indicating the test plant to be a potential vermifuge or vermicide. However, its clinical efficacy is to be explored by in vivo studies and further investigation should be directed towards identification and purification of active ingredients.

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