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EFFECT OF ETHANOL EXTRACT OF *ACORUS CALAMUS* ON PYRUVATE KINASE AND PHOSPHOENOLPYRUVATE CARBOXYKINASE ACTIVITY OF *COTYLOPHORON COTYLOPHORUM*

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ABSTRACT

Cotylophoron cotylophorum is a ruminant parasite which causes the disease paramphistomosis in livestock. Immature flukes cause heavy infection resulting in foul-smelling diarrhoea, dehydration and death of the animal. Effect of ethanol extract of *Acorus calamus* on pyruvate kinase and phosphoenolpyruvate carboxykinase activity of *Cotylophoron cotylophorum* was studied in the present investigation. The parasites were exposed to various sub lethal concentrations of ethanol extract of *Acorus calamus* for 2h, 4h and 8h. Following the method of MCmanus and Smyth, pyruvate kinase and phosphoenolpyruvate carboxykinase were assayed. Maximum level of inhibition in pyruvate kinase and phosphoenolpyruvate carboxykinase activities was observed after 8h of incubation at 0.1 mg ml^{-1} concentration. Inhibition of pyruvate kinase and phosphoenolpyruvate carboxykinase may impair the phosphoenolpyruvate-succinate pathway and reduces the synthesis of ATP. Decreased generation of ATP may impair energy dependent physiological activities of the parasite and thereby eliminating the parasite from the host.

KEYWORDS: ***Cotylophoron cotylophorum,*** **Pyruvate kinase (PK), Phosphoenolpyruvate carboxykinase (PEPCK),** ***Acorus calamus.***

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INTRODUCTION

Cotylophoron cotylophorum is a gastrointestinal digenetic trematode, lives in the rumen of livestock, which cause the disease paramphistomosis. Paramphistomosis has been a neglected trematode infectious disease; however recently, it has emerged as an important cause of productivity loss and indirectly responsible for massive economic losses to the cattle farmers [1-3]. In small ruminants, parasitic infections cause slow growth rate, poor reproductive performance and death[4]. In domestic ruminants, the mortality rate due to immature paramphistomosis is very high and may go up to 80 – 90%[5]. Chemotherapeutic anthelmintic drugs have been used to control parasitic infections in cattle. Excessive use of chemo-therapeutics has resulted in the development of resistance to various synthetic drugs; hence, there is an increasing demand towards natural anthelmitics. A large number of plant products are being used to combat gastro-intestinal parasites of livestock [6-10]. Plants produce many secondary metabolites which constitute an important source of phytotherapeutic drugs[11]. Phytotherapeutic drugs are safe, non-toxic, biodegradable and do not leave residues in animal products[12]. *Acorus calamus* commonly known as 'Sweet flag' belonging to family Acoraceae, is a volatile oil containing bushy herb or medicinal plant. Traditionally *A. calamus* is used to treat loss of appetite, bronchitis, chest pain, colic, cramps, diarrheoa, digestive disorders, flatulence, gas, indigestion, nervous disorders, rheumatism and vascular disorders. The plant has a long history from various countries and has been in use for at least around 2000 years in China and India[13]. The rhizome contains active ingredients possessing antioxidant, insecticidal[14], antifungal, antibacterial[15], allelopathic and anthelmintic properties [16,17]. Considering the presence of a wide range of bioactive compounds and its broad spectrum of use *A. calamus* was selected in the present study to assess the anthelmintic efficacy against paramphistomes. Carbohydrate metabolism in parasites plays an important role in its adaptation and survival in the mammalian host. The major difference between carbohydrate catabolism of parasites and the host was acetyl-CoA, which plays an important prime role in mammals and does not play the same prime role in parasites[18], this function being taken over in parasites by phosphoenolpyruvate (PEP). PEP is converted to pyruvate by pyruvate kinase (PK) and further reduced to lactate by lactate dehydrogenase (LDH). Majority of PEP is diverted to oxaloacetate (OAA) by the action of phosphoenolpyruvate carboxykinase (PEPCK), which is further reduced to malate by malate dehydrogenase (MDH) and converted to fumarate and succinate. The major source of ATP is produced anaerobically in parasitic by the PEP-succinate pathway. Hence, in the present investigation the anthelmintic efficacy of ethanol extract of *A. calamus* (*Ac*EE) was assessed based on its effect on pyruvate kinase and phosphoenolpyruvate carboxykinase activity of *C. cotylophorum*.

MATERIALS AND METHODS

In vitro maintenance of C. cotylophorum

C. cotylophorum were collected from the rumen of infected sheep, slaughtered at Perambur abbatoir, Chennai. Adult live flukes were collected and washed thoroughly in physiological saline and maintained in Hedon-Fleig solution, which is the best medium for *in vitro* maintenance[19].

Preparation of solvent extracts of A. calamus

The rizhomes of *A. calamus* (Figure 1) were collected from the local country drug merchant. The rizhomes were authenticated as PCHC 1699 by the Department of Botany, Pachaiyappa's College, Chennai. The various solvent extracts were prepared following the method of Harbone[20]. The rizhomes of *A. calamus* was coarsely powdered and soaked in hexane, followed by chloroform, ethyl acetate and ethanol successively. Extracts were filtered using Whatman filter paper No.1 and concentrated by distillation using, rotary evaporator (evator). The concentrated extracts were completely dried to remove the last traces of the solvents using Lyodel Freeze Dryer (Delvac).

Sample preparation

The parasites were incubated in various concentrations these of extracts. The motility of the parasites was observed visually at a regular time interval. Based on the visual observations, five different sub-lethal concentrations of effective extract was selected for further studies. The control and drug-treated flukes were rinsed in distilled water and weighed wet and a 10% homogenate (w/v), was prepared by homogenizing the fluke in an ice-cold 0.25M sucrose solution containing 0.15M Tris-HCl (pH 7.5), using a homogenizer in an ice bath. This homogenate was centrifuged at 1000×g for 10 min and the clear supernatant was used as an enzyme source. The cytosolic and mitochondrial fractions of *C. cotylophorum* were prepared by following the method of Fry *et al*[21]. The sediment was discarded as it contains the cellular particles viz., nucleus and other heavy organelles. Protein in the sample was determined as described by Lowry *et al*[22].

Enzyme assay

Pyruvate kinase (PK, EC 2.7.1.4) and phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.32) activity were assayed following the McManus and Smyth[23]. The reaction mixture for the determination of pyruvate kinase activity contained 1 ml of 300 mM Tris-HCl buffer (pH 7.8)[24], 0.5 ml of 42 mM $MgSO_4$, 0.5 ml of 450 mM KCl, 0.3 ml of 50 mM ADP, 0.3 ml of 50 mM PEP, 0.3 ml of 2 mM NADH. 0.025 ml of 48 mM FBP, 0.025 ml of 15 units of LDH and 0.05 ml of enzyme sample. The reaction was started by the addition of the enzyme sample and the decrease in absorbance at 340 nm was recorded for 3 min at an interval of 15 sec. The enzyme activity was calculated from the millimolar coefficient of 6.22 for NADH and was expressed in n

moles NADH oxidised / min / mg protein. The assay mixture to record phosphoenolpyruvate carboxykinase activity contained 1ml of 300 mM imidazole buffer (pH 6.2)[24], 0.4 ml of 300 mM $MgSO_4$, 0.3 ml of 400 mM KCl, 0.3 ml 70 mM $NaHCO_3$, 0.3 ml of 20 mM ADP, 0.3 ml of 40 mM PEP, 0.3 ml of 2 mM NADH, 0.05 ml of MDH and 0.05 ml of enzyme sample. The reaction was started by the addition of the enzyme sample and the decrease in absorbance was read at 340 nm for 3 min at an interval of 15 sec. The enzyme activity was calculated from the millimolar coefficient of 6.22 for NADH and was expressed in n moles NADH oxidised / min / mg protein.

Statistical analysis

The data obtained were analyzed statistically. Statistical analyses were performed with the Statistical program for the social sciences SPSS version 16.0. The significance of drug induced inhibition in the enzyme activity of the *C. cotylophorum* was assessed using analysis of variance (ANOVA) for different concentrations of *A. calamus*.

Figure 1
Acorus calamus

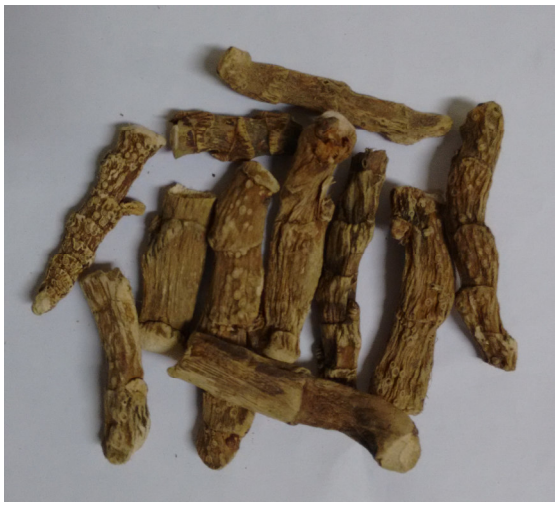

Table 1
Effect of AcEE on PK activity of C. Cotylophorum

Conc. mg ml^{-1}	Period of incubation		
	2h	4h	8h
0.001	8.78±0.31	22.11±0.02	40.07±0.02
0.005	13.32±0.06	25.96±0.07	58.12±0.04
0.01	15.00±0.03	34.68±0.15	61.05±0.05
0.05	21.80±0.05	55.30±0.07	70.39±0.08
0.1	26.63±0.03	60.18±0.04	77.70±0.05
(Mean ± S.D, n=5)			

Table 2
Effect of AcEE on PEPCK activity of C. Cotylophorum

Conc. mg ml^{-1}	Period of incubation		
	2h	4h	8h
0.001	7.89±0.07	21.07±0.05	45.31±0.05
0.005	10.01±0.07	30.08±0.05	54.32±0.09
0.01	21.13±0.05	39.05±0.07	60.07±0.06
0.05	25.45±0.05	43.70±0.06	79.18±0.02
0.1	33.13±0.04	47.66±0.08	91.80±0.03
(Mean ± S.D, n=5)			

RESULTS AND DISCUSSION

Ethanol extract of *A. calamus* (*Ac*EE) significantly inhibited both the PEPCK and PK activities in *C. cotylophorum.* The PK activity showed 77.70% of inhibition in *Ac*EE–treated flukes at 0.1 mg ml^{-1} after 8h of exposure (Table 1). Maximum level of inhibition (91.80%) in PEPCK activity was observed in flukes treated with *Ac*EE was after 8h of incubation at 0.1 mg ml^{-1} (Table 2). Thus *Ac*EE effectively inhibited the activity both of PK and PEPCK. The inhibition activity of both PK and PEPCK was found to be dependent on concentration and period of exposure. The present study revealed the inhibitory effect of ethanol extract of *A. calamus* on PK and PEPCK activity of *C. cotylophorum*. Similar inhibitory effect of medicinal plant *Allium sativum* on the PEPCK and PK activities in *Haemonchus contortus* was reported by Navaneethalakshmi and Veerakumari[25]. Swargiary *et al* reported inhibitory effect of some medicinal plants on the activity of PEPCK and PK in *Fasciola buski*[26]. Reynold stated that PK and PEPCK could act as a selective anthelmintic target for anthelmintic agents to control parasitic infections[27]. The enzymes PK and PEPCK are functionally linked. The two enzymes compete for phosphoenolpyruvate (PEP) the common substrate which directs it to aerobic (PK) and anaerobic (PEPCK) pathways[28]. Inhibition of PK results in decreased concentrations of pyruvate and PEP which ultimately result in decreased production of ATP. Similarly the inhibition of PEPCK results in decreased concentration of oxaloacetate (OAA) which reduces the production of ATP. The formation of either lactate or succinate is controlled by the competing activities of PK and PEPCK in anaerobic metabolic pathway. The carbon flow from PEP into the final products of anaerobic metabolism was directed by PK and PEPCK. Thus the enzymes compete for the substrate PEP, and their relative activities account for the PEP-lactate or acetate/PEP-succinate or propionate pathways. The succinate formation has a distinct advantage for the parasite over the LDH reaction in the anaerobic habitat of intestinal parasite, as the production of succinate is the end step in mitochondrial of metabolic pathway, which catalyses the transfer of electrons from NADH to fumarate, and fumarate serves as an end product of electron receptor. Inhibition of both PK and PEPCK activities arrests the PEP-lactate and PEP-succinate pathways. Thus the energy yielding process is impaired and deprives the parasite of its ATP production. Decreased production of ATP may lead to the elimination of parasite from the host.

CONCLUSION

*Ac*EE significantly inhibited both the PEPCK and PK activities in *C. cotylophorum,* which decreases the production of ATP and ultimately results in the death of the parasite. Thus, the results of the present work suggest that *A. calamus* could be used as a potential anthelmintic phytotherapeutic drug to combat the *C. cotylophorum* infection in livestock.

REFERENCES

1. Sharma D, Katoch R, Agnihotri RK. Gastrointestinal helminths in Gaddi sheep. J Vet Parasitol. 2005 Jun;21(2):141-43.
2. Ozdal N, Gul A, Ilhan F, Deger S. Prevalence of *Paramphistomum* infection in cattle and sheep in Van Province, Turkey. Helminthologia. 2010 Feb;47(1):20-4.
3. Swarnkar G, Roat K, Sanger B, Kumawat A. Anthelminthic effect of *Trigonella foenum–graecum* on tegument of *Gastrothylax crumenifer* in cattle of Udaipur. Int J Curr Microbiol App Sci. 2014 Nov;3(5):599-06.
4. Coop RL, Kyriazakis I. Influence of host nutrition on the development and consequences of nematode parasitism in ruminants. Trends Parasitol. 2001 Jul;17(7):325-30.
5. Hassan SS, Kaur K, Joshi K, Juyal PD. Epidemiology of paramphistomosis in domestic ruminants in different districts of Punjab and other adjoining areas. J Vet Parasitol. 2005 Jun;19(1):43-6.
6. Kozan E, Kupeli E, Yesilada E. Evaluation of some plants used in Turkish folk medicine against parasitic infections for their *in vivo* anthelmintic activity. J Ethnopharmacol. 2006 Nov;108:211-16.
7. Lyndem LM, Tandon V, Das B. Anthelmintic efficacy of medicinal plants from Northeast India against hookworms: an *in vitro* study on *Ancylostoma cylanicum*. Pharmacologyonl. 2008 Jun;3:697-07.
8. Kosalge SB, Fursule RA. Investigation of *in vitro* anthelmintic activity of *Thespesia lampas* (Cav.). Asian J Pharm Clin Res. 2009 Jun;2(2):69-71.
9. Challam M, Roy B, Tandon V. Effect of *Lysimachia ramose* (Primulaceae) on helminth parasites: motility, mortality and scanning electron microscopic observations on surface topography. Vet Parasitol. 2010 Jan;169:214-18.
10. Manojdhanraj K, Veerakumari L. *In vitro* effect of *S. aromaticum* on the motility and Ache of *C.cotylophorum*. Ind J Ani Sci Res. 2014 Jun;43(3):187-94.
11. Somnath De, Akalanka Dey, Sudhakarbabu AMS, Siddabathuni A. Anthelmintic activity of methanolic extract of *Sphaeranthus amaranthoides* Burm. Int J Pharm Bio Sci. 2014 Jun;5(2):152-56.
12. Hammond JA, Fielding D, Bishop SC. Prospects for plant anthelmintics in tropical veterinary medicine. Vet Res Commun. 1997 Apr;21(3):213-28.

13. Immanuel Selvaraj C, Mythili Avadhani MN, Rajasekharan PE, Tharachand C. The sweetness and bitterness of sweet flag [*Acorus calamus* L.] – a review. Res J Pharma Bio and Chem Sci. 2013 Jun;4(2):598-10.
14. Balakumbahan R, Rajamani K, Kumanan K. *Acorus calamus*: an overview. J Med Plants Res. 2010 Oct;4(25):2740-745.
15. Srividya AR, Aishwaria SN, Vishnuvarthan VJ. Evaluation of antioxidant, antimicrobial and cytotoxicity activity of hydroethanolic extract and its fractions of *Acorus calamus* Linn. Inter J Pharma Res Sch. 2014 Jun;3(1):114-25.
16. Tariq K, Tantry M. Preliminary studies on plants with anthelmintic properties in Kashmir-the North-West temperate himalayan region of India. Chin Med. 2012 Jun;3(2):106-12.
17. Prashanta D, Ranjib G, Sankari D, Tejendra B. *In-vitro* anthelmintic activity of *Acorus calamus* leaves. Asian J Pharm Clin Res. 2013 May;6(3):135-37.
18. Lloyd GM, Barrett J. *Fasciola hepatica*: Inhibition of phosphoenolpyruvate carboxy-kinase, and end product formation by quinolinic acid and 3-mercaptopicolinic acid. Exptl Parasitol. 1998 Oct; 56:259-65.
19. Veerakumari L. *In vitro* studies on the effect of some anthelmintics on *Cotylophoron cotylophorum* (Fischoeder, 1901) (Digenea: paramphistomidae). A structural and biochemical analysis [Ph. D. thesis]. Chennai: University of Madras; 1996.
20. Harbone J. Phytochemical methods: a guide to modern techniques of plant analysis. Nature: Springer;1998.
21. Fry M, Bazil C, Jenkins DC. A comparison of mitochondrial electron transport in the intestional parasitic nematodes *Nippostrongylus brasiliensis* and *Ascardia galli*. Comp Biochem Physiol. 1983 Oct;75B:451-53.
22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. J Biol Chem. 1951 Jun;193:265-75.
23. McManus DP, Smyth JD, Intermediary carbohydrate metabolism in protoscoleces of *Echinococcus granulosus* (horse and sheep strains) and *E. multiocularis*. Parasitol. 1982 Apr;84:351-66.
24. Priya P, Veerakumari L. *In vitro* effect of *Acacia concinna* on the key enzymes of carbohydrate metabolism of *Cotylophoron cotylophorum* (Fischoeder, 1901). Biomed. 2011 Jul;31(3):329–33.
25. Navaneetha LK, Veerakumari L. Effect of *Allium sativum* on the phosphoenolpyruvate carboxykinase and pyruvate kinase activity of *Haemonchus contortus in vitro.* Pharmacogn Mag. 2009 May;5(20):430–32.
26. Swargiary A, Roy B, Giri BR, Rohang B. A comparative study on the anthelmintic efficacy of some medicinal plants of North–East India: alteration in the glycolytic enzymes of *Fasciola buski*, a giant intestinal fluke. Asian Pac J Trop Med. 2013 Aug; 412–20.
27. Reynold CH. Phosphoenolpyruvate carboxykinase from the rat and from the tapeworm, *Hymenolepis diminuta*. Comp Biochem Physiol. 1980 Mar; 65B:481–87.
28. Bueding E, Saz HJ. Pyruvate kinase and phosphoenolpyruvate carboxy-kinase activities of *Ascaris* muscle, *Hymenolepis diminuta*and *Schistosoma mansoni*. Comp Biochem Physiol. 1968 Apr; 24:511–18.