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Anthelmintic efficacy of ethanol extract of *Areca catechu* on the carbohydrate metabolism of *Cotylophoron cotylophorum*

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Abstract
Paramphistomosis is an important parasitic disease in sheep and goats and causes considerable economic loss to the livestock industry. Anthelmintics are commonly used against helminth parasites. The high incidence of resistance of helminth parasites to anthelmintic drugs in addition to the relative toxicity and side effects of many of these drugs urge the necessity of finding alternative safe and eco friendly agents against helminths. This applies to plant-based anthelmintics that have been used to destroy and expell the parasite from gastrointestinal tract. In the present investigation ethanol extract of *Areca catechu* on the enzymes of carbohydrate metabolism viz. pyruvate kinase, phosphoenolpyruvate carboxykinase, lactate dehydrogenase, malate dehydrogenase, fumarate reductase and succinate dehydrogenase of *Cotylophoron cotylophorum* was studied. The parasites were incubated in five different sub-lethal concentrations of ethanol extract of *Areca catechu* viz. 0.005, 0.01, 0.05, 0.1 and 0.5mg/ml for 2, 4 and 8 h. The activity of the enzymes was assayed using standard procedures. The enzyme activity was expressed in terms of protein. The data obtained were analyzed statistically. Ethanol extract of *Areca catechu* significantly inhibited the enzymes of carbohydrate metabolism and the percentage of inhibition was dose and time dependent. Present study manifested that PK, PEPCK, LDH, MDH, FR and SDH provide biochemical target for *Ac*EE which disrupt energy generation process in *C. cotylophorum*, resulting in decreased production of ATP. Consequently, the energy deprived parasite unable to sustain themselves *in situ* may be expelled from the host. The results of the present study holds a potential promise in the future use of active principles of *A. catechu* as effective anthelmintics and may help in designing assimilated solutions for the control of paramphistomosis.

Keywords: *Areca catechu*, *Cotylophoron Cotylophorum*, pyruvate kinase Phosphoenolpyruvate Carboxykinase, lactate dehydrogenase, malate dehydrogenase, fumarate reductase, succinate dehydrogenase

1. Introduction
Livestock sector plays a critical role in the welfare of India's rural population. It provides food, income, employment and many other contributions to rural development. The small ruminant, sheep is one of the most important livestock species in the global agricultural economy because of its wool and meat. Sheep skins and manure constitute important sources of earning. They play an important role in the livelihood of a large percentage of small and marginal farmers and landless labourers engaged in sheep rearing. Internal parasites cause major problem to the sheep industry. Parasitic infections are worldwide problem for both small and large–scale farmers. Helmintic infections of gastrointestinal tract and its associated organs in sheep are common and the economic losses due to these parasites are numerous, mainly due to morbidity and mortality [1]

Paramphistomes, which cause the disease paramphistomosis are parasites of ruminants which particularly affect cattle, sheep and goats. In small ruminants, parasitic infections cause slow growth rate, poor reproductive performance and death [2]. Paramphistomosis is caused by digenean trematodes, of the family Paramphistomidae, parasitizing the rumen of ruminants globally [3]. The amphistome species responsible for the majority of outbreaks of paramphistomosis in ruminants *were Cotylophoron cotylophorum, Cotylophoron jacksoni, Calicophoron calicophorum, Calicophoron raja, Calicophoron microbothrium, Calicophoron clavula, Calicophoron sukumum, Calicophoron phillerouxi and Paramphistomum cervi* [4]. *Cotylophoron cotylophorum* has a wide distribution in cattle, sheep and goats of India [5].

Chemical anthelmintics are used to treat parasitic infections in small ruminants. Due to increasing development of anthelmintic resistance and the limited availability
of synthetic drugs to the rural people as well as the high cost of such
chemical medicines, a growing interest in the ethno-veterinary approach to examine
the anthelmintic properties of plants traditionally used by local farmers in different parts of the globe is emerging [6, 7]. Plant derived drugs serve as a prototype to develop more effective and less toxic medicines [8]. Anthelmintics from natural sources could play a key role in the treatment of parasitic infections [9].

Areca catechu is commonly called betel nut, belongs to the family Aereaceae. Areca catechu is used as anthelmintic, antiviral, antidiabetic, antioxidant, antibacterial, wound healing, hepatoprotective, antiulcerogenic, antifertility, abortifacient, anti-implantation, antivenom, anti inflammatory and anticonvulsant drugs [10 - 12]. Phytochemical analysis of *Areca catechu* by several investigators revealed the arecaidine, arecaine, arecoline, b-carotene, b-sitosterol, capric acid, D-catechin gallic acid, guvacine, guvacoline, heneicosanic acid, isoguvacine, kryptogenin, lauric acid, leucocyanidine, leucopelargonidine, linoleic acid, margarinic acid, myristic acid, oleic acid, philobaphenetannin and stearic acid [13]. Recent studies on the ethanol extract of *A. catechu* revealed that the presence of 13 componds which include isorhamnetin, quercetin, liquiritigenin, 5,7,4'-trihydroxy-3',5' dimethoxyflavanone, (+) -catechin, resveratrol, ferulic acid, vanillic acid, 5,8-epidioxiergosta-6,22-dien-3beta-ol,stigmasta-4-en-3-one, beta-sitosterol, cycloartenol and de-O-methyllasiodiplodin [14]. Anthelmintic activity of *Areca catechu* aganist *Haemonchous contortus* was reported by Andiara moraes [15].

As carbohydrate metabolism provides the main source of energy for the survival of the parasites, the present study proposed to elucidate the effect of ethanol extract of *Areca catechu* on the key regulatory enzymes involved in carbohydrate metabolic pathway of *Cotylophoron cotylophorum.* Helminth parasites normally live on host's glucose supply. Glucose and/or glycogen constitute the main sources of energy to produce ATP for parasitic helminths [16]. During starvation it starts to utilize its glycogen store through the activation of phosphorylase, which is accompanied by a significant increase in the enzyme activity [17]. In parasitic trematode, carbohydrate is initially broken down by glycolysis. Glycolysis serves as the central pathway in carbohydrate metabolism of paramphistomes. Key regulatory enzymes involved in carbohydrate metabolism are pyruvate kinase (PK), phosphoenolpyruvatecarboxykinase (PEPCK), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), succinate dehydrogenase (SDH) and fumarate reductase (FR). Activity of these regulatory enzymes is subject to feedback inhibition or activation by one or more of the substrates in the pathway. Regulation of these key enzymes is critical for coupling the activity of the pathway to the metabolic needs of the cells.

The "usual" glycolytic pathway produces pyruvate, which in most aerobic organisms is then decarboxylated and condensed with coenzyme A to form acetyl CoA. In helminths, under anaerobic condition, PK catalyzes the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, yielding one molecule of pyruvate and one molecule of ATP. Pyruvate is further reduced to lactate by PK. Lactate is the end product of anaerobic glycolysis in the cytosol. Although adults of many parasitic flukes do excrete lactate, many have a further series of reactions that derive some additional energy [16]. LDH catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD+ [18]. Since LDH is a fairly stable enzyme, LDH has been widely used to evaluate the presence of damage and toxicity of tissue and cells [19].

When the activity of the enzyme PK is low, instead of forming pyruvate, CO2 fixation takes place and PEP is converted to oxaloacetate by the action of the enzyme PEPCK. The oxaloacetate thus formed is reduced in the cytoplasm to malate by MDH. Malate is then transported into the mitochondria via a phosphate dependent translocase. Inside the mitochondria malate undergoes a dismutation reaction; a part is oxidatively decarboxylated to pyruvate via an NAD+ linked malic enzyme and a part to fumarate which is in equilibrium with malate via the enzyme fumarase. Fumarate is reduced to succinate by FR with the generation of ATP indicating a cyclic reaction. Studies have shown that physiological stress conditions, such as infection, starvation, aestivation and anthelmintic treatment can alter its carbohydrate metabolism [20]. Keeping this in view, an attempt has been made to the assess the anthelmintic efficacy of ethanol extract of *Areca catechu* against *Cotylophoron cotylophorum* based on its effect on the enzyme involved in carbohydrate metabolism.

2. Materials and Methods

2.1 *In vitro* maintenance of *C. cotylophorum*

Parasites (*Cotylophoron cotylophorum*) (Fig. 1) were collected from the rumen of infected sheep, slaughtered at Perambur abbatoir, Chennai. Adult live flukes were collected, washed thoroughly in physiological saline and maintained in Hedon-Fleig solution, which is the best medium for *in vitro* maintenance [21]. It is prepared by dissolving 7gm of sodium chloride, 0.3gm of potassium chloride, 0.1gm of calcium chloride, 1.5gm of sodium bicarbonate, 0.5gm of disodium hydrogen phosphate, 0.3gm of magnesium sulphate and 1gm of glucose in 1000ml of distilled water.

2.2 Preparation of plant extracts

Areca catechu (Fig.2) were collected from local market at Chennai, and were authenticated in the Department of Botany, Pachaiyappa's college, Chennai and vouchered specimens are deposited in the herbarium of Pachaiyappa's College, Chennai-30. The extraction of plant materials was done following the method of Harborne [22]. *Areca catechu* were coarsely powdered and soaked serially in hexane, chloroform, ethyl acetate and ethanol. Extract was filtered using Whatman filter paper No.1.and concentrated using, rotary evaporator (EQUITIRON). The concentrated extracts were completely dried to remove the last traces of the solvents using Lyodel Freeze Dryer (DELVAC).

2.3 Sample preparation

Adult *C. cotylophorum* were incubated in various concentration of AcEE (0.005, 0.01, 0.05, 0.1 and 0.5mg/ml) for 2, 4 and 8h. Simultaneously, control was also maintained in Hedon-Fleig solution without the plant extract. After incubation, the parasites were rinsed in distilled water. The parasites were weighed wet and a 10% (W/V) homogenate was prepared by homogenising the flukes in ice-cold 0.25 M sucrose solution containing 0.15 M Tris-HCl (pH-7.5). This

homogenate was centrifuged at 1000 rpm for 10 min. The supernatant was used as the enzyme source. The particulate and soluble fractions of *C. cotylophorum* were prepared following the method of Fry [23].

2.4. Enzyme Assay

2.4.1 Pyruvate kinase (PK)

Pyruvate kinase (PK, EC 2.7.1.4) activity in the cytosolic fraction was assayed following the method of McManus and Smyth [24]. PK catalyses the inter conversion of phosphoenolpyruvate (PEP) and pyruvate as shown below:

$$\text{PEP} + \text{ADP} \xrightleftharpoons{\text{PK}} \text{Pyruvate} + \text{ATP}$$

The reaction mixture contained 1 ml of 300 mM Tris-HCl buffer (pH 7.8) [25], 0.5 ml of 42 mM magnesium sulphate (MgSO4), 0.5 ml of 450 mM potassium chloride (KCl), 0.3 ml of 50 mM adenosine diphosphate (ADP), 0.3 ml of 50 mM PEP, 0.3 ml of 2 mM nicotinamide adenine dinucleotide reduced (NADH), 0.025 ml of 48 mM fructose biphosphate (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.

2.4.2 Phosphoenolpyruvate carboxykinase (PEPCK)

The activity of phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.32) was assayed according to the method of McManus and Smyth [24]. PEPCK catalyses the formation of oxaloacetate (OAA) from PEP.

$$\text{PEP} + \text{CO}_2 + \text{ADP} \xrightarrow{\text{PEPCK}} \text{OAA} + \text{ATP}$$

The assay mixture contained 1ml of 300 mM imidazole buffer (pH 6.2) [25], 0.4 ml of 300 mM MgSO4, 0.3 ml of 400 mM KCl, 0.3 ml of 70 mM sodium bicarbonate (NaHCO3), 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 15 units 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.

2.4.3 Lactate dehydrogenase (LDH)

The activity of lactate dehydrogenase (LDH, EC 1.1.1.27) was assayed according to the method of Yoshida and Freese [26]. LDH catalyses the oxidation of lactate and reduction of pyruvate.

$$\text{Lactate} + \text{NAD} \xrightleftharpoons{\text{LDH}} \text{Pyruvate} + \text{NADH} + \text{H}^+$$

The conversion of pyruvate to lactate occurs in anaerobic tissues and conversion of lactate to pyruvate occurs in aerobic tissues. Therefore, LDH activity can be measured spectrophotometrically either by the reduction of nicotinamide adenine dinucleotide (NAD) in the presence of lactate or by the oxidation of NADH in the presence of pyruvate. In the present study the activities in both the directions were assayed. For oxidation of lithium lactate, 0.8 ml of 60 mM phosphate buffer (pH 7.5) (Veerakumari and Munuswamy, 2000) [27], 0.1 ml of 0.5 M lithium lactate, 0.05 ml enzyme sample and 0.05 ml of 20 mM NAD were placed in 1 ml cuvette. The increase of absorbance at 340 nm was recorded for 3 min at an interval of 15 sec. For the reduction of pyruvate, 0.05 ml of enzyme sample was added to 0.8 ml of 60 mM phosphate buffer (pH 6.5) (Veerakumari and Munuswamy, 2000), 0.01 ml of 1 mM NADH, 0.01 ml of 10 mM sodium pyruvate and final volume was adjusted to 1 ml by the addition of distilled water in 1 ml cuvette. The decrease in absorbance at 340 nm was measured for 3 min at an interval of 15 sec. The enzyme activity was calculated from the millimolar coefficient of 6.22 for NAD and NADH and was expressed in n moles NAD reduced or NADH oxidised / min / mg protein.

2.4.4 Malate dehydrogenase (MDH)

Malate dehydrogenase (MDH, EC 1.1.1.37) catalyses the oxidation of malate and reduction of OAA. The activity of this enzyme catalysing the malate oxidation and OAA reduction was assayed in both cytosolic and mitochondrial fractions following the procedure of Yoshida [28].

$$\text{L-Malate} + \text{NAD} \xrightleftharpoons{\text{MDH}} \text{OAA} + \text{NADH} + \text{H}^+$$

For the oxidation of malate, the reaction mixture contained 1 ml of 150 mM Tris-HCl buffer (pH 8.4 for cMDH and pH 7.2 for mMDH) [21] 0.1 ml of 100 mM sodium malate, 0.1 ml of 10 mM NAD, 1.7 ml of distilled water and 0.1 ml of enzyme sample. For MDH catalysing the reduction of OAA, the reaction mixture contained 2.5 ml of 100 mM Tris-HCl (pH 7.4 for both cMDH and mMDH) [21], 0.05 ml of 100 mM oxaloacetate, 0.05 ml of 10 mM NADH, 0.3 ml of distilled water and 0.1 ml of the enzyme sample. The activity of the enzyme catalysing oxidation and reduction reaction was measured at 340 nm for 3 min at an interval of 15 sec each. The enzyme activity was calculated from the millimolar coefficient of 6.22 for NAD and NADH and was expressed in n moles NAD reduced or NADH oxidised / min / mg protein.

2.4.5 Fumarate reductase (FR)

Fumarate reductase (FR, EC 1.3.1.6) catalyses the reduction of fumarate to succinate. The enzyme was assayed as detailed by Sanadi and Fluharty [29].

$$\text{Fumarate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{FR}} \text{Succinate} + \text{NAD}$$

The reaction mixture contained 1 ml of 150 mM Tris-HCl buffer
(pH 8.6) [21], 0.3 ml of 10 mM KCN (neutralised with 0.01 N HCl), 0.3 ml of 1 mM ethylene diamine tetra acetic acid (EDTA), 0.3 ml of 50 mM fumarate, 0.7 ml of distilled water, 0.1 ml of enzyme sample and 0.3 ml of 1.6 mM NADH in a 3 ml cuvette. After the addition of NADH, decrease in absorbance at 340 nm was measured for 3 min at an interval

of 15 sec. The enzyme activity was calculated by using the millimolar coefficient of 6.22 and expressed in n moles of NADH oxidised / min / mg protein.

2.4.6 Succinate dehydrogenase (SDH)

The activity of succinate dehydrogenase (SDH, EC 1.3.99.1) was assayed according to the method of Singer [30]. Succinate is oxidised to fumarate by the flavoprotein SDH, which contains covalently bound flavin adenine dinucleotide. This reducible co-enzyme functions as hydrogen acceptor in the following reaction.

$$\text{Succinate} + \text{E.FAD} \xrightarrow{\text{SDH}} \text{Fumarate} + \text{E.FADH}_2$$

The reduced enzyme can donate electrons to various artificial electron acceptors e.g reducible dyes. SDH assay is based on the reduction of phenazine methosulphate (PMS) by SDH. Reduced PMS is immediately reoxidised by dicholoro phenol indophenol (DCPIP). Bleaching of later dye is estimated spectrophotometrically. The reaction mixture included 0.5 ml of 300 mM phosphate buffer (pH 7.5) [21], 0.3 ml of 0.1 M succinate, 0.1 ml of enzyme, 0.3 ml of 10 mM KCN (neutralised with 0.01 N HCl), 0.1 ml of 0.75 mM calcium chloride and 1.3 ml of water. The enzyme was incubated for 5 - 7 min to permit full activation. After incubation, 0.1 ml DCPIP (0.05 %) (W/V) and 0.3 ml of PMS (0.33 %) were added to initiate the reaction and decrease in absorbance was recorded at 600 nm. The enzyme activity was calculated using millimolar extinction coefficient of 19.1 and expressed in n moles of dye reduced / min / mg protein. Total protein content was estimated following the method of Lowry [31].

2.5. Statistical Analysis

All the data obtained in the present study were statistically analysed using the statistical software SPSS version 16.0. One-way Anova using Bonferroni test was applied to find out the significant difference between the different concentrations of *Ac*EE and periods of incubation.

3. Results and Discussion

Helminth parasites had to survive in a semi-aerobic or anaerobic condition of the host body, they cannot metabolise glucose completely into O2 and H2O. In order to survive such a harsh condition, helminth parasites change their metabolic pathway to a different one and depend upon anaerobic carbohydrate metabolism to obtain energy, despite the amount of oxygen available. Consequently, glycolysis is the major energy yielding pathway in helminth parasites [32 -33]. Any drug which could cause adverse changes in the carbohydrate metabolism of the parasite, but not in the host could be a potential anthelmintic drug [34]. It is evident from the present study that *Ac*EE inhibited the activity of the enzymes involved in carbohydrate metabolism.

*Ac*EE significantly inhibited both the PK and PEPCK activities in

C. cotylophorum. PK and PEPCK activity was inhibited by 28.63, 62.18, 78.60% and 39.23% 48.06%, 92.82%in *Ac*EE – treated flukes (Table 1) after 2, 4 and 8 h of incubation, at their highest concentration. Maximum level of inhibition in PK and PEPCK activity was observed in flukes treated with *Ac*EE was after 8h of incubation at 0.5 mg/ml. The inhibition of PK and PEPCK activity was directly proportional to the duration of incubation and concentration of the extracts. Similar inhibitory effects of medicinal plant *Piper betle*, *Acorus calamus* on the PEPCK and PK activities in *C. cotylophorum* were well documented [35 - 36]. Analogously, inhibitory effect of medicinal plant *Allium sativum* on the PEPCK and PK activities in *Haemonchus contortus* was reported by Navaneetha and Veerakumari [37]. Swargiary [38] reported inhibitory effect of some medicinal plants on the activity of PEPCK and PK in *Fasciola buski*. Reynold [39] stated that PK and PEPCK could act as a selective anthelmintic target for anthelmintic agents to control parasitic infections. 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) pathway. 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 [40]. Thus the enzymes compete for the substrate PEP, and their relative activities account for the PEP-lactate or acetate/PEP-succinate or propionate pathways [41]. 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 [34].

The action of PK on PEP results in the production of pyruvate. Pyruvate so formed comes under the influence of LDH, which catalyses the reduction of pyruvate to lactate and the oxidation of lactate to pyruvate. It is evident from the present investigation that *Ac*EE inhibited the LDH catalysing both the lactate oxidation and pyruvate reduction. The LDH activity catalyzing oxidation of lactate was found to be inhibited by 89.93% in flukes treated with 0.5 mg/ml of *Ac*EE (0.5 mg/ml) after 8h of exposure (Table 2), whereas the inhibition of LDH activity catalyzing pyruvate reduction was found to be 86.52% in *Ac*EE–treated flukes (Table 2) after 8 h of exposure. It is interesting to note that LDH exhibits a peculiar type of chemotherapeutic response. Inhibition of LDH activity catalyzing pyruvate reduction was found to be higher compared to LDH inhibition catalysing the oxidation of lactate. Similar findings were also reported by various workers [42 - 44]. A remarkable inhibition of LDH activity in *A. nigra*, *P. fulgens*, *C. baccans* and praziquantel treated *F. buski* was reported by Swargiary [38]. Ozcelik [45] reported the inhibitory effect of albendazole on LDH activity of *F. hepatica*. Veerakumari and Munuswamy [27] opined that accumulation of lactate due to LDH inhibition may affect the mitochondrial energy generating process which ultimately proves to be fatal to the parasite. Similarly, *Acacia concinna* inhibited the LDH catalysing both the lactate oxidation and pyruvate reduction in *C. cotylophorum* [25]. LDH is responsible for the maintenance of the cytosolic redox state of

the helminths [46] and its inhibition may be challenging for the survival of the parasite. The inhibition of lactate dehydrogenase might arrest the carbon influx in the glycolytic pathway and the generation of the necessary energy through oxidative phosphorylation [47]. Consequently, production of malate, which serves as main substrate for mitochondrial phosphorylation is reduced, which leads to reduced production of ATP [48].

Malate dehydrogenase (MDH) has been a rate–limiting enzyme in the phosphoenolpyruvate metabolism. *Ac*EE significantly inhibited the cytoplasmic MDH (cMDH) and mitochondrial MDH (mMDH) catalysing both the oxidation and reduction reactions in *C. cotylophorum*. The activity of cMDH catalyzing the oxidation of malate was inhibited by 98.27% in *Ac*EE–treated flukes at 0.5 mg/ml concentration at 0.5 mg/ml concentration (Table 3) after 8 h of incubation. Percentage inhibition of cMDH activity catalyzing the reduction of oxaloacetate was found to be 97.29% in *Ac*EE–treated flukes (Table 3). *C. cotylophorum* mMDH catalyzing the oxidation of malate was inhibited by 97.01% in *Ac*EE–treated flukes (Table 4) on incubation for 8 h at their highest concentration. Inhibition of mMdh catalyzing the reduction of oxaloacetate was 54.84, 77.35 and 94.91% in *Ac*EE–treated flukes (Table 4) after 2, 4 and 8 h of exposure. Lwin and Probert [49] and Probert [50] research work signified that MDH inhibition may subsequently inhibit fumarate reductase and reduce ATP synthesis. Furthermore, Tejada [51] studies on benzamidazole confirmed that cMDH and mMDH are the anthelmintic target sites in *A. suum, F. hepatica* and *M. expansa*. These anthelmintics may disturb the transmembrane proton gradient severely, leading to drop in cellular ATP levels [52]. The alteration of MDH and LDH activities of *T. saginata* by albendazole and niclosamide has been reported by Oztop [53]. Similar inhibitory effect of *A. concinna* on the cMDH and mMDH activity of *C. cotylophorum* was reported by Priya and Veerakumari [25]. Reduction in the MDH activity of the flukes exposed to *Ac*EE suggest that, plants act transtegumentally to target vital tegumental enzymes and interfere with the energy generating pathways depriving the parasite in acquiring ATP, thereby leading to paralysis and death [38]. The inhibition of both cMDH and mMDH observed in the present study suggests the declined production of oxaloacetate (OAA) and malate. The inhibition of MDH might subsequently result in the inhibition of FR, as OAA is essential for production of fumarate [49 - 50].

*Ac*EE significantly inhibited both the FR and SDH activities in *C. cotylophorum*. Decreased FR and SDH activity was observed in the drug–treated flukes. FR and SDH inhibition was found to be 62.08, 79.64%, 90.49% and 38.98%, 68.66%, 93.68%

(Table 5) in flukes treated with 0.5 mg/ml of *Ac*EE after 2, 4 and 8 h of incubation, respectively. FR and SDH activity was found to be highly inhibited at 0.5 mg/ml of *Ac*EE after 8h exposure. *Ac*EE effectively inhibited the activity of FR and SDH. Inhibition of FR and SDH in *Ac*EE flukes is directly proportional to concentration of the drug and period of exposure. Fumarate is reduced to succinate using NADH as reducing equivalent and succinate formation is the final step of the glycolytic pathway [54]. In concurrence with the current studies Priya and Veerakumari [25] reported similar inhibition of FR in *Acacia concinna*, treated *C. cotylophorum*. Omura [55] reported, nafuredin, a novel compound isolated from *Aspergillus niger*, exhibited high selective toxicity and inhibitory effect on the FR of *H. contortus*. Antiparasitic drugs, inhibit fumarate binding to FR, slowdown the synthesis of ATP [56].

On investigating the activity of SDH in *C. cotylophorum*, a significant decrease in the enzyme activity of *Ac*EE–treated flukes was noted. Similar inhibitory effects of anthelmintics such as salicylanilide and mebendazole on the activity of SDH in *F. hepatica* was reported by Coles [57]. SDH has the ability to transfer electrons to the respiratory chain by catalysing the formation of fumarate and succinate [58]. SDH inhibition by anthelmintics could prevent the utilization of the chemical energy derived from electron transport for the net phosphorylation of ADP to ATP and deprive the parasite of its normal source of energy [59]. In addition, anthelmintics, affect tubulins bound in mitochondrial membrane of the parasites by influencing SDH–FR complex negatively, inhibit succinate metabolism and diminish ATP–synthesis [60]. Hence, SDH could potentially be an important target for anthelmintics against the gastrointestinal parasites of livestock [61]. Inhibition of FR-SDH system may not only prevent succinate formation, but also the production of ATP, the regeneration of NAD+ as well as interference with terminal electron transport. Decreased production of ATP may prove fatal to the parasites. Significant inhibition of FR and SDH system ultimately leads to reduction in ATP production. The result of the present investigation revealed that *Ac*EE impaired the enzymes involved in the PEP-succinate pathway, thereby reducing the production of ATP. Consequently, the energy deprived parasite unable to sustain themselves in situ may be expelled from the host.

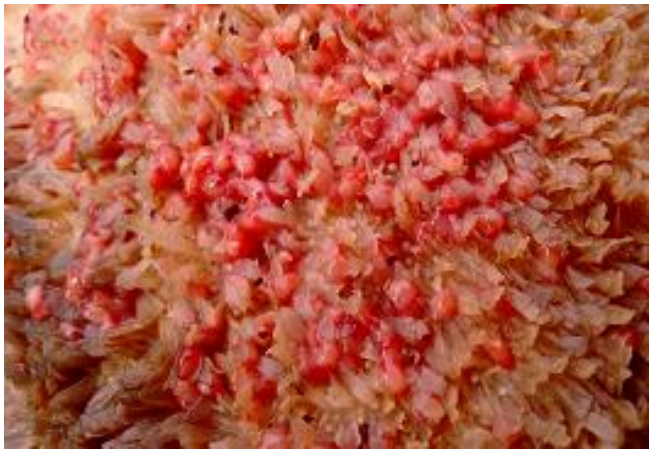

Fig 1: *Cotylophoron cotylophorum* in rumen of sheep

Fig 2: *Areca catechu*

Table 1: Effect of *Ac*EE on PK and PEPCK activity of *C. cotylophorum*

Conc. mg/ml*	% inhibition (mean ± SD of n=5) at various periods of incubation**		
	2h	4h	8h
***Ac*EE (PK)**			
0.005	9.68 ± 0.31	28.11 ± 0.02	41.07 ± 0.02
0.01	14.32 ± 0.06	29.96 ± 0.07	62.12 ± 0.04
0.05	16.00 ± 0.03	35.68 ± 0.15	64.05 ± 0.05
0.1	23.80 ± 0.05	60.30 ± 0.07	73.39 ± 0.08
0.5	28.63 ± 0.03	62.18 ± 0.04	78.60 ± 0.05
***Ac*EE (PEPCK)**			
0.005	9.69 ± 0.07	27.77 ± 0.05	46.31 ± 0.05
0.01	10.71 ± 0.07	38.88 ± 0.05	51.32 ± 0.09
0.05	23.23 ± 0.05	42.05 ± 0.07	62.57 ± 0.06
0.1	27.46 ± 0.05	44.90 ± 0.06	79.78 ± 0.02
0.5	39.23 ± 0.04	48.06 ± 0.08	92.82 ± 0.03

* Inhibitory effects of the extracts among the different concentrations of the respective plant are significantly different for each duration of incubation (P<0.05) using Bonferroni test
** Inhibitory effects of the extracts among the different hours of incubation is significantly different for each concentration of the respective plants (P<0.01) using Bonferroni test

Table 2: Effect of *Ac*EE on LDH catalyzing oxidation and reduction reaction of *C. cotylophorum*

Conc. mg/ml*	% inhibition (mean ± SD of n=5) at various periods of incubation**		
	2h	4h	8h
***Ac*EE (LDH catalyzing oxidation)**			
0.005	8.64 ± 0.02	38.82 ± 0.15	64.13 ± 0.11
0.01	15.83 ± 0.12	41.40 ± 0.05	69.46 ± 0.03
0.05	17.37 ± 0.04	43.86 ± 0.03	74.93 ± 0.05
0.1	36.53 ± 0.22	48.86 ± 0.16	77.50 ± 0.14
0.5	41.00 ± 0.06	65.34 ± 0.04	89.93 ± 0.02
***Ac*EE (LDH catalyzing reduction)**			
0.005	5.75 ± 0.05	32.91 ± 0.07	50.97 ± 0.10
0.01	25.71 ± 0.02	37.73 ± 0.15	60.63 ± 0.07
0.05	36.81 ± 0.16	40.13 ± 0.17	68.11 ± 0.08
0.1	41.45 ± 0.12	44.32 ± 0.15	79.21 ± 0.06
0.5	50.01 ± 0.11	61.32 ± 0.08	86.52 ± 0.01

* Inhibitory effects of the extracts among the different concentrations of the respective plant are significantly different for each duration of incubation (P<0.05) using Bonferroni test
** Inhibitory effects of the extracts among the different hours of incubation is significantly different for each concentration of the respective plants (P<0.01) using Bonferroni test

Table 3: Effect of *Ac*EE on cMDH catalyzing oxidation and reduction reaction of *C. cotylophorum*

Conc. mg/ml*	% inhibition (mean ± SD of n=5) at various periods of incubation**		
	2h	4h	8h
***Ac*EE (cMDH catalyzing oxidation**			
0.005	9.20 ± 0.20	34.64 ± 0.01	53.60 ± 0.02
0.01	17.01 ± 0.02	39.00 ± 0.08	58.36 ± 0.11
0.05	27.29 ± 0.14	40.43 ± 0.01	75.63 ± 0.01
0.1	32.47 ± 0.10	49.77 ± 0.12	84.96 ± 0.13
0.5	38.43 ± 0.18	63.53 ± 0.18	98.27 ± 0.07
***Ac*EE (cMDH catalyzing reduction)**			
0.005	9.50 ± 0.08	33.80 ± 0.16	66.26 ± 0.18
0.01	15.35 ± 0.01	39.81 ± 0.12	77.04 ± 0.11
0.05	17.45 ± 0.11	55.96 ± 0.02	85.48 ± 0.13
0.1	26.00 ± 0.12	64.31 ± 0.14	89.81 ± 0.15
0.5	38.59 ± 0.14	71.79 ± 0.16	97.29 ± 0.09

* Inhibitory effects of the extracts among the different concentrations of the respective plant are significantly different for each duration of incubation (P<0.05) using Bonferroni test
** Inhibitory effects of the extracts among the different hours of incubation is significantly different for each concentration of the respective plants (P<0.01) using Bonferroni test

Table 4: Effect of *Ac*EE mMDH catalyzing oxidation and reduction reaction of *C. cotylophorum*

Conc. mg/ml*	% inhibition (mean ± SD of n=5) at various periods of incubation**		
	2h	4h	8h
AcEE (mMDH catalyzing oxidation)			
0.005	14.14 ± 0.02	38.65 ± 0.14	51.69 ± 0.06
0.01	34.49 ± 0.07	41.27 ± 0.07	71.70 ± 0.12
0.05	37.69 ± 0.12	44.37 ± 0.05	80.42 ± 0.08
0.1	41.52 ± 0.08	51.69 ± 0.16	89.66 ± 0.05
0.5	45.75 ± 0.17	58.52 ± 0.12	97.01 ± 0.11
AcEE (mMDH catalyzing reduction)			
0.005	20.51 ± 0.08	49.05 ± 0.13	62.54 ± 0.16
0.01	34.41 ± 0.11	62.10 ± 0.01	73.80 ± 0.02
0.05	39.78 ± 0.07	65.72 ± 0.16	82.70 ± 0.11
0.1	43.79 ± 0.15	74.63 ± 0.02	87.79 ± 0.10
0.5	54.84 ± 0.22	77.35 ± 0.28	94.91 ± 0.13

* Inhibitory effects of the extracts among the different concentrations of the respective plant are significantly different for each duration of incubation (P<0.05) using Bonferroni test
** Inhibitory effects of the extracts among the different hours of incubation is significantly different for each concentration of the respective plants (P<0.01) using Bonferroni test

Table 5: Effect of *Ac*EE on FR activity of *C. cotylophorum*

Conc. mg/ml*	% inhibition (mean ± SD of n=5) at various periods of incubation**		
	2h	4h	8h
***Ac*EE (FR)**			
0.005	22.22 ± 0.01	57.87 ± 0.04	69.36 ± 0.21
0.01	40.82 ± 0.04	65.60 ± 0.05	78.55 ± 0.15
0.05	56.45 ± 0.15	71.64 ± 0.06	81.69 ± 0.13
0.1	59.36 ± 0.04	75.20 ± 0.21	85.66 ± 0.03
0.5	62.08 ± 0.16	79.64 ± 0.15	90.49 ± 0.11
***Ac*EE (SDH)**			
0.005	7.30 ± 0.05	33.72 ± 0.03	61.28 ± 0.05
0.01	14.00 ± 0.14	37.14 ± 0.12	67.39 ± 0.14
0.05	30.95 ± 0.05	44.20 ± 0.11	70.73 ± 0.12
0.1	33.29 ± 0.16	55.42 ± 0.14	83.60 ± 0.23
0.5	38.98 ± 0.18	68.66 ± 0.12	93.68 ± 0.19

* Inhibitory effects of the extracts among the different concentrations of the respective plant are significantly different for each duration of incubation (P<0.05) using Bonferroni test
** Inhibitory effects of the extracts among the different hours of incubation is significantly different for each concentration of the respective plants (P<0.01) using Bonferroni test

4. Conclusion

The present study elucidated the anthelmintic effect of *Ac*EE on *C. cotylophorum*. *Ac*EE inhibited the activity of the enzymes involved in the carbohydrate metabolism such as pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), fumarate reductase (FR) and succinate dehydrogenase (SDH). The inhibition of PK activity results in reduced production of pyruvate. The inhibition of PEPCK apprehends the PEP-succinate pathway and switches the PEP towards the formation of pyruvate. This results in reduced production of malate, which serves as the main substrate for mitochondrial phosphorylation. Inhibition in the LDH activity catalysing both the oxidation of lactate to pyruvate and reduction of pyruvate to lactate was observed. Further, significant inhibition of MDH, FR and SDH was also observed. The inhibition of the enzymes of carbohydrate metabolism affects the energy generation process of the parasites and leads to reduced production of ATP. Decreased

production of energy ultimately results in the death of the parasites. *Ac*EE possesses a remarkable anthelmintic activity against *C. cotylophorum*. Plants having multiple mode of action are very promising as they are eco-friendly, do not leave residues on animal products and development of resistance could be minimized. The discovery and development of noval substances for helminth control is greatly needed and has promoted studies of traditionally used anthelmintic plants, which are generally considered to be very important source of bioactive substances. Hence, *Ac*EE could be suggested as an alternative drug to control paramphistomosis in livestock. This study paves the way for designing integrated solutions for the control of ruminal paramphistomosis.

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