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CT OF ETHANOL EXTRACT OF ARECA CATECHU ON FUMARATE REDUCTASE AND SUCCINATE DEHYDROGENASE OF COTYLOPHORON COTYLOPHORUM

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(Received: February 02, 2016; Accepted: March 24, 2016)

ABSTRACT
Parasitic helminths represent one of the most pervasive challenges to livestock. Paramphistomosis commonly affects cattle and sheep, and is caused by various species of the paramphistomidae. Anthelmintic drugs are used to treat helminth infections. 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. The parasites depend for their energy almost entirely on carbohydrate metabolism. The pathway of carbohydrate metabolism is essentially anaerobic and involves the glycolytic and part of the reversed tricarboxylic acid (TCA) cycle. Fumarate reductase (FR) and Succinate dehydrogenase (SDH), the enzymes of TCA cycle catalyses the reduction of fumarate to succinate and oxidation of succinate to fumarate. Reduction of fumarate to succinate complex results in ATP synthesis. Hence the efficacy of ethanol extract of Areca catechu (AcEE) was assessed based on its effect on FR and SDH against Cotylophoron cotylophorum. The parasites were incubated in five different sub-lethal concentrations of AcEE for 2h, 4h and 8h. FR and SDH activity was assessed using standard procedures. The enzyme activity was expressed in terms of protein. The data obtained were analyzed statistically. Maximum level of inhibition in FR and SDH activities were observed after 8h of incubation in AcEE. Inhibition of FR and SDH activity was dose and time dependent. Inhibition of FR and SDH interferes with the terminal electron acceptor and prevents succinate formation thereby curtailing the ATP synthesis. Decreased production of ATP results in the death of the parasites. Areca catechu as a potential inhibitor of FR and SDH activity of C. cotylophorum suggests the possible application of this plant extract to combat paramphistome infection in livestock.

Keywords: Cotylophoront cotylophorum, Fumarate reductase (FR), Succinate dehydrogenase (SDH), Areca catechu.

INTRODUCTION
Helminth parasitism, especially gastrointestinal parasitism, is one of the major health problems severely limiting the animal productivity in dairy animals. (Eysker and Ploeger, 2000). Parasitic diseases cause enormous economic losses through morbidity and mortality in livestock. Paramphistomosis is caused by digenean trematodes, of the family Paramphistomidae, parasitizing the rumen of ruminants globally (Kanwal et al., 2014). Paramphistomosis has been associated with only a few species of paramphistomes, viz. Paramphistomum cervi, P. explanatum, Cotylophoron coryphorum, Gastrothylax crumenifer and Fischoederius elongates (Madzingira et al., 2002). Cotylophoron coryphorum (Fischoeder, 1901) is more prevalent in Tamil Nadu occurs in the rumen of the sheep, goat, cattle and other domestic ruminants. Immature flukes live in the small intestine of ruminants where they attach themselves to the intestinal lining with powerful suckers, whereas, adult flukes are found in the rumen or reticulum (Hassan et al., 2005).

Helminth control in small ruminants is widely based on the use of anthelmintic drugs. However, the current efficacy of these drugs has been reduced, because of resistant strain development (Bartley et al., 2003; Jabbar et al., 2006; Artho et al., 2007). Besides, most of these synthetic drugs are
highly toxic and exhibit undesired side effects in host animals (Singh and Ngachi, 1999). Furthermore, the high cost of these drugs, residual concern in food animals and environmental pollution have awaken interest in medicinal plants as an alternative source of anthelmintic drugs (Pessoa et al., 2002; Hordegen et al., 2003; Bizimenyera et al., 2006). A large number of plant products are used to treat gastro-intestinal parasites of livestock (Challam et al. 2010; Manolaraki et al. 2010).

Areca catechu is commonly called betel nut, belongs to the family Arecaceae. Areca catechu is used as anthelmintic, antiviral, antidiabetic, antioxidant, antibacterial, wound healing, hepatoprotective, antiulcerogenic, antifertility, abortifacient, anti-implantation, antivenom, anti-inflammatory and anticonvulsant drugs (Anjali and Rao, 1995; Pithayanukul et al., 2005; Priyanka et al., 2009; Azzez et al., 2007; Pithayanukul et al., 2009; Shrestha et al., 2010; Anthikat and Michael, 2011; Kafie et al., 2011). The seed contains 50-60% sugars, 15% lipids 15% condensed tannins, polyphenolics and 0.2 - 0.55% alkaloids (Reelijiro et al., 1998). Phytochemical analysis of A. catechu revealed that the presence of 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, margarini acid, myristic acid, oleic acid, philobaphenetannin and stearic acid (Senthil amuthan et al., 2012). The anthelmintic activity of Areca catechu aganist Haemonchous contortus has been reported by Andiara moraes et al (2014).

Helminth parasites have a completely different mode of life cycle compared to free living organisms to enable them to adapt inside the hosts by changing its metabolic pathways (Kita et al., 2001). Helminth parasites utilize the food from the intestinal gut of host. The metabolism depends on the feeding habits and the rich nourishment available in the gut of the host. The parasites use this nourishment for their normal development and growth. A major part of energy source utilized by the parasite is from carbohydrates (Pallewad et al., 2015).

The energy production shifts to anaerobic ones with lactic acid as the end product of glycolytic pathway instead of pyruvic acid. Glucose and/or glycogen are the main energy source of helminth parasites. Glycogen/ glucose are degraded to phosphoenolpyruvate (PEP) via the Emden-Meyerhof glycolytic pathway (Mansour, 2002). PEP can either be metabolized to pyruvate via pyruvate kinase (PK) and then to lactate in presence of lactate dehydrogenase (LDH) or via phosphoenolpyruvate carboxykinase (PEPCK) to oxaloacetate which is reduced to malate in presence of malate dehydrogenase (MDH) (Barrett, 1981). Malate permeates into the mitochondrion where it undergoes dismutation in which one-half of malate is oxidized to pyruvate by malic enzyme (ME) and the other half is dehydrated to fumarate by fumarase (FM), which is further reduced to succinate by fumarate reductase (FR). Succinate oxidized to fumarate by succinate dehydrogenase (SDH). The differences of energy transducing systems between helminth parasites and their hosts has given some indication that some of the pathways are unique to the helminth parasites and could be exploited selectively as target for anthelmintic drugs (Omura et al., 2001). Keeping in view, the importance of Fumarate reductase (FR) and Succinate dehydrogenase (SDH) in parasite metabolism, the present study was under taken to elucidate the anthelmintic potential of ethanol extract of Areca catechu (AcEE) on FR and SDH of C.catylophorum.

MATERIALS AND METHODS

In vitro maintenance of C. catylophorum

Cotylophoron catylophorum (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 (Veerakumari, 1996). 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.

Preparation of plant extracts

Areca catechu (Fig.2) were collected from Lakshmi stores at Chennai, and were authenticated in the Department of Botany, Pachaiyappa’s college, Chennai and vouched specimens are deposited in the herbarium of Pachaiyappa’s College, Chennai-30. The extraction of plant materials was done following the method of Harborne (1998). 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).

Sample preparation
Adult C. cotylophorum were incubated in various concentration of AcEE (0.005, 0.01, 0.05, 0.1 and 0.5 mg/ml) for 2, 4 and 8 h. 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 et al. (1983).

Enzyme Assay
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 (1963). The reaction mixture contained 1 ml of 150 mM Tris-HCl buffer (pH 8.6) 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.

Succinate Dehydrogenase (SDH)
The activity of succinate dehydrogenase (SDH, EC 1.3.99.1) was assayed according to the method of Singer (1974). The reaction mixture included 0.5 ml of 300 mM phosphate buffer (pH 7.5) 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.

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 AcEE.

RESULTS
AcEE significantly inhibited both the FR and SDH activities in C. cotylophorum. FR activity was found to be highly inhibited by 90.49% at 0.5 mg/ml of AcEE after 8 h exposure (Table 1). Maximum level of inhibition in SDH activity was observed in flukes treated with AcEE was 93.68 % after 8 h of incubation at 0.5 mg/ml (Table 2). Thus AcEE effectively inhibited the activity of FR and SDH. Inhibition of FR and SDH in AcEE flukes is directly proportional to concentration of the drug and period of exposure. The inhibition of both FR and SDH was statistically significant (P<0.05).

DISCUSSION
Helminth parasites have to sustain in a semi-aerobic or anaerobic condition of the host body, they cannot metabolize glucose completely into O2 and H2O. In order to survive such a hard condition, helminth parasites alter their metabolic pathway to a different one and rely upon anaerobic carbohydrate metabolism to acquire energy, in contempt of the amount of oxygen available. Consequently, glycolysis is the major energy yielding pathway in helminth parasites (Roy et al. 2012; Bueding, 1970; Schulman et al., 1982; Donahue et al., 1983) reported the impact of anthelmintics on the carbohydrate metabolism of helminth parasites. A fine understanding on different carbohydrate metabolic reactions forms solid basis for choosing suitable targets for new chemotherapeutic agents. In the present investigation AcEE significantly inhibited the FR and SDH activity of Cotylophoron cotylophorum. Anthelmintic drugs inhibit or accelerate the enzymes involved in the carbohydrate metabolic pathway (Veerakumari and Munuswamy, 1999; Veerakumari and Priya, 2011; Manoj
Table 1: In vitro effect of AcEE on FR activity of C. cotylophorum

<table>
<thead>
<tr>
<th>Conc. mg/ml*</th>
<th>% inhibition (mean ± SD of n=5) at various periods of incubation**</th>
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<tr>
<td></td>
<td>2h</td>
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<tr>
<td>AcEE</td>
<td></td>
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<tr>
<td>0.005</td>
<td>22.22 ± 0.01</td>
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<tr>
<td>0.01</td>
<td>40.82 ± 0.04</td>
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<td>0.05</td>
<td>56.45 ± 0.15</td>
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<tr>
<td>0.1</td>
<td>59.36 ± 0.04</td>
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<tr>
<td>0.5</td>
<td>62.08 ± 0.16</td>
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</table>

* 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: In vitro effect of AcEE on SDH activity of C. cotylophorum

<table>
<thead>
<tr>
<th>Conc. mg/ml*</th>
<th>% inhibition (mean ± SD of n=5) at various periods of incubation**</th>
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<tr>
<td></td>
<td>2h</td>
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<tr>
<td>AcEE</td>
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<tr>
<td>0.005</td>
<td>7.30 ± 0.05</td>
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<td>0.01</td>
<td>14.00 ± 0.14</td>
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<tr>
<td>0.05</td>
<td>30.95 ± 0.04</td>
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<tr>
<td>0.1</td>
<td>33.29 ± 0.16</td>
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<td>0.5</td>
<td>38.98 ± 0.18</td>
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* 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

Figure 1: Cotylophorion cotylophorum in rumen of sheep

Figure 2: Areca catechu
Anthelmintics are known to inhibit the synthesis of mitochondrial ATP in parasites (Veerakumari, 1996). This is particularly important as the energy metabolism differs significantly from those of the host. Inhibitory effect of AcEE on FR activity of C. cotylophorum was apparent in the present analysis. Fumarate is reduced to succinate using NADH as reducing equivalent and succinate formation is the final step of the glycolytic pathway (Maule and Marks, 2006). In concurrence with the current studies, Priya and Veerakumari (2011) reported similar inhibition of FR in Acacia concinna–treated C. cotylophorum. Omura et al. (2001) 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 body constituents, curtail the energy production in the parasites (Kumari, 2006), uncouple oxidative phosphorylation, hamper ATP production (Lemke et al., 2013) and present an excellent biochemical target in the treatment of helminthic infections (Turrens, 2012). On investigating the activity of SDH in C. cotylophorum, a significant decrease in the enzyme activity of AcEE–treated flukes was noted. Similarly, salicylanilide and mebendazole inhibited the activity of SDH in F. hepatica and T. spiralis (Coles 1974; Boczon 1976). 

SDH has the ability to transfer electrons to the respiratory chain by catalysing the formation of fumarate and succinate (Parvathi and Aruna, 2012). 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 (Skuce and Fairweather, 1990). 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 (Swan, 1999). Hence, SDH could potentially be an important target for anthelmintics against the gastrointestinal parasites of livestock (Chen et al., 2001). 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. This investigation evidently asserted the significant inhibition of FR and SDH system ultimately leads to reduction in ATP production in flukes treated with higher concentration of AcEE at 8h exposure.

FR and SDH is the vulnerable drug target for ethanol extract of Areca catechu. Significant hindrance in the activities of FR and SDH was noticed in all the treated parasites. Inhibition of FR and SDH obstructs the terminal electron acceptor and averts succinate formation there by curtailing the ATP synthesis. Decreased production of ATP results in death of the parasites. This study has elucidated the anthelmintic efficacy of Areca catechu against C. cotylophorum. Further identification of effective phytochemicals is necessary for the development of potential phytotherapeutic drug to treat paramphistomosis in livestock.

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