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Effect of Ethanol Extract of *Syzygium aromaticum* and *Trachyspermum ammi* on Pyruvate Kinase and Phosphoenol Pyruvate Carboxykinase of the Nematode, *Haemonchus contortus*

[1]S. Sathish Kumar, [2]Dr. L. Veerakumari

[1, 2] PG and Research Department of Zoology, Pachaiyappa's College, Chennai – 600 030

Abstract: ***Haemonchus contortus*** **is the most pathogenic parasite inhabiting the abomasam of sheep and goats worldwide causing the dreadful disease haemonchosis. Chemical anthelmintics are used to treat gastro intestinal nematode infection in small ruminants. However, this treatment is costly, leaving residues in animal products and drug resistance has evolved in parasites. Therefore, there is need for developing cheaper, less toxic and eco-friendly novel drugs. Medicinal plants offer an effective alternative source. In this current study, effect of ethanol extracts of** ***Syzygium aromaticum*** **(*Sa*EE) and** ***Trachyspermum ammi*** **(*Ta*EE) on the Pyruvate kinase (PK) and Phosphoenol pyruvate carboxykinase (PEPCK) of** ***H. contortus*** **was studied** ***in vitro*****. *H. contortus* were exposed to five different sub-lethal concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml) of ethanol extracts of *Sa*EE and (0.01, 0.05, 0.1, 0.5 and 1 mg/ml) of *Ta*EE for 2, 4 and 8h. Both control and drug-treated worms were assayed for PK and PEPCK. Maximum level of inhibition in both PEPCK and PK activities were observed in drug-treated worms at 0.5 mg/ml of *Sa*EE and 1mg/ml of *Ta*EE after 8h of exposure. Inhibition in the enzyme activity affects the energy generating process leads to decreased production of ATP. Energy deprivation results in the death of the worms. The present study enlightened the anthelmintic effect of ethanol extracts of *S. aromaticum* and *T. ammi* suggest that could be used as a phytotherapeutic agents to combat the *H. contortus* infection in livestock.**

Keywords: **Pyruvate kinase, Phosphoenol pyruvate carboxykinase, *Syzygium aromaticum*, *Trachyspermum ammi* and *Haemonchus contortus*.**

I. INTRODUCTION

Gastro intestinal parasites cause a wide variety of fatal diseases in animals. For rural population sheep production has remained an integral part of their life and farming system. Livestock help to improve food and nutritional balance by providing nutrient rick food productivity (Birthal and Singh, 1995). *Haemonchus contortus* is a blood sucking and serious pathogenic parasite occurring in the abomasum of small ruminants (Troll *et al*., 2003). Haemonchosis caused by *H. contortus* is characterized by anaemia, hypoproteinemia, weightloss, bottlejaw and mortality in susceptible animals. Increasing resistance, chemical residues in meat products and high cost efficiency of the synthetic drugs resulted in a growing interest in ethno-veterinary approach to examine the anthelmintic properties of plants (Zajac and Gipson, 2000; Veale, 2002). The present investigation was undertaken to assess the anthelmintic efficacy of ethanol extract of *Syzygium aromaticum* and *Trachyspermum ammi* against *Haemonchus contortus*.

Syzygium aromaticum commonly called as clove belongs to the family Myrtaceae is used traditionally in Indian ayurvedic medicine (Pulikottil and Nath, 2015; Samir *et al*., 2017).

Phytochemical screening of *S. aromaticum* revealed the presence of aldehydes, alkaloids, carbohydrates, flavonoids, glycoside, ketones, phenolic compounds, steroids, tannins, terpenoids (Pandey *et al*., 2013; Simiat *et al*., 2017). *S. aromaticum* has biological activities such as anthelminthic, anti-bacterial, antifungal, anti-inflammatory, chemopreventive, hepatoprotective, neuroprotective, analgesic, antispasmodic, anticarminative, and antioxidant properties (Huang
et al., 2002; Lagow, 2004; Chaieb *et al*., 2007; Meyer *et al*., 2008; Cortez *et al*., 2014; Vanin *et al*., 2014). Anthelmintic activity of *S. aromaticum* against *Fasciola gigantica* and *Cotylophoron cotylophorum* was reported by Kumar and Singh, (2014); Manoj Dhanraj and Veerakumari, (2014 and 2015).

Trachyspermum ammi commonly called as omam in Tamil belongs to the family Apiaceae (Chatterjee *et al*., 2012). *T. ammi* consists of monoterpenes, sesquiterpenes, ketones, alcohols, aldehyde and esters (Nagalakshmi *et al*., 2000; Suntar *et al*., 2014). It possess antioxidant, antibacterial, antimutagenic, antiinflammatory, antifilarial, hepatoprotective and antimicrobial properties (Thangam and Dhananjayan, 2003; Dadalioglu and Evrendilek, 2004; Masih *et al*., 2012; Hassanshahian *et al*., 2014; Bajpai and Agrawal, 2015; Hassan *et al*., 2016). The anthelmintic efficacy methanol extract of *T. ammi* against *H. contortus* and *Ascaris lumbricoides* was reported by Tamurab and Iwamoto (2004); Jabbar *et al.* (2006).

Carbohydrate is the major source of energy for helminths inhabiting the alimentary tract of vertebrates. Glucose degradation involves in the formation of phosphoenol pyruvate (PEP) by the classical Emden –Meyerhof scheme, but differs from the vertebrates by the subsequent fixation of CO_2. PEP can either be carboxylated by phosphoenol pyruvate carboxykinase (PEPCK) to oxaloacetate (OAA), or dephosphorylated by pyruvate kinase (PK) to pyruvate. Hence the current study was carried out to elucidate the effect of ethanol extract of *S. aromaticum* (*Sa*EE) and *T. ammi* (*Ta*EE) on Pyruvate kinase (PK) and phosphoenol pyruvate carboxykinase (PEPCK) the key regulatory enzymes involved in energy metabolism of *H. contortus*, *in vitro*.

II. MATERIALS AND METHODS

Collection of Parasites and *in vitro* maintenance:

Adult live worms were collected from abomasum of slaughtered sheeps at Peramber slaughter house, Chennai. Worms were washed thoroughly in physiological saline and maintained in Hedon-Fleig solution (pH 7.0), which is the best medium for survival of the parasites *in vitro* (Veerakumari, 1996).

Collection of Plant material:

Buds of *S. aromaticum* and seeds of *T. ammi* were purchased from Lakshmi store. The plant materials obtained were identified and authenticated by a botanist in the department of Botany, Pachaiyappa's College, Chennai–30. The vouchered specimens are deposited at Unit of Parasitology, Pachaiyappa's College, Chennai–30.

Extract Preparation of Plant material:

S. aromaticum and *T. ammi* were cleaned, shade dried and coarsely powdered. Solvent extraction was done using cold percolation method (Harborne, 1998) by soaking in hexane, chloroform, ethyl acetate and ethanol successively for 48h. Simultaneously aqueous extract was also prepared. After 48h, the extracts were filtered by Whatman's filter paper no. 1. The solvent was removed by distillation using Evator Rotary Evaporator (EQUITIRON) and the extracts were concentrated and dried in Lyodel Freeze dryer (DELVAC). Following the procedure of Namma and Bhatnagar (1990), stock solution of 100 mg/ml concentration of both the plant extract has been prepared. Then the stock solution were serially diluted to 10, 30 and 50 mg/ml concentration for the *in vitro* maintenance of the *H. contortus*. Motility of the parasites was checked at various time intervals (5, 10, 15, 30min, 1, 2, 4, 6, 8, 12 and 24 hrs) of incubation. Based on the motility five different sub-lethal concentration of plant extracts were selected for further *in vitro* studies.

Sample Preparation:

Both control and drug-treated worms were cleansed in distilled water. Then worms were weighted wet and a 10% (W/V) homogenate was prepared by homogenizing the worms in the ice-cold 0.25 M sucrose solution containing 0.15M Tris-Hcl (pH 7.5) using tissue homogenizer in an ice-bath. Then homogenate was centrifuged at 1000 rpm for 10 minutes and the sediment containing the cellular particles viz., nucleus and other organelles was discarded. The clear supernatant was used as an enzyme source.

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Preparation of Enzyme samples:

The cytosolic fractions of *H. contortus* were prepared by the methods described by Fry *et al*. (1983). Prepared sample was centrifuged at 10000 rpm for 20 min and the supernatant thus obtained was the cytosol fraction. During the centrifugation process temperature is maintained at 4^{o}C using refrigerated ultracentrifuge (REMI C 24).

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 (1982). PK catalyses the inter conversion of phosphoenolpyruvate (PEP) and pyruvate as shown below:

$$PEP + ADP \xleftrightarrow{PK} Pyruvate + ATP$$

The reaction mixture contained 1 ml of 300 mM Tris–HCl buffer (pH 7.8) (Priya and Veerakumari, 2011), 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.

Phosphoenolpyruvate carboxykinase (PEPCK)

The activity of phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.32) was assayed according to the method of McManus and Smyth (1982). PEPCK catalyses the formation of oxaloacetate (OAA) from PEP.

$$PEP + CO2 + ADP \xrightarrow{PEPCK} OAA + ATP$$

The assay mixture contained 1 ml of 300 mM imidazole buffer (pH 6.2) (Priya and Veerakumari, 2011), 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. Protein content in the samples was evaluated by using the method of Lowry *et al*. (1951).

Statistical analyses were performed with the Statistical program for the social sciences SPSS version 16.0. The significance of drug induced inhibition in PK and PEPCK activity of the parasites was assessed using analysis of variance (ANOVA) for different concentrations of ethanol extracts of *S. aromaticum* and *T. ammi*. The term significant had been used to indicate differences for which $P \leq 0.05$.

III. RESULTS

The current investigation elucidate the anthelmintic efficacy of *S. aromaticum* and *T. ammi* against PK and PEPECK of *H. contortus*. The level of PK and PEPCK showed varying degrees of inhibition following incubation in *Sa*EE and *Ta*EE at different concentrations and period of exposure. Inhibition of PK activity was 51.46, 70.57 and 91.76% after 2, 4 and 8 h of incubation respectively at 0.5mg/ml of *Sa*EE (Fig. 1). Likewise, the enzyme PEPCK activity also significantly inhibited to 50.12, 83.46 and 97.20% after 2, 4 and 8h of exposure at 0.5 mg/ml of *Sa*EE (Fig. 2). Similarly, inhibition of PK was 52.86, 67.33 and 92.43% (Fig. 3) and PEPCK 51.53, 72.54 and 91.93% (Fig. 4) after 2, 4 and 8h in 1 mg/ml of *Ta*EE. Inhibition of PK and PEPCK activity in the *H. contortus* was statistically significant ($P \leq 0.05$).

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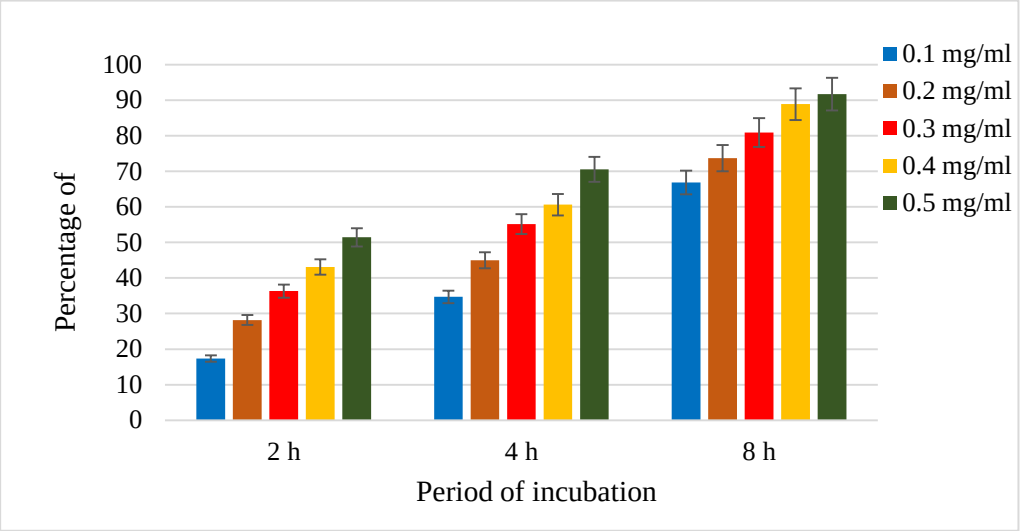

Fig 1: Effect of *Sa*EE on PK activity of *H. contortus*

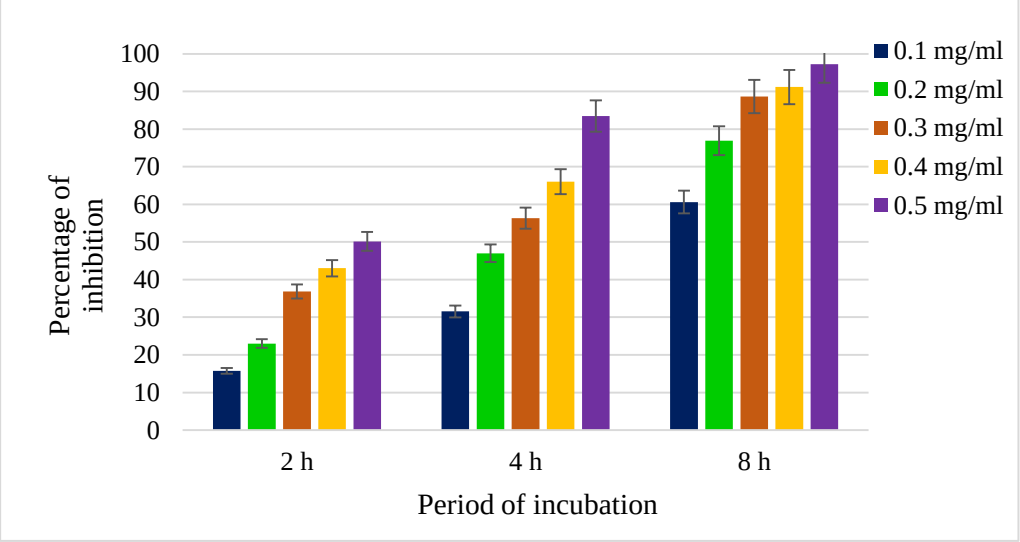

Fig 2: Effect of *Sa*EE on PEPCK activity of *H. contortus*

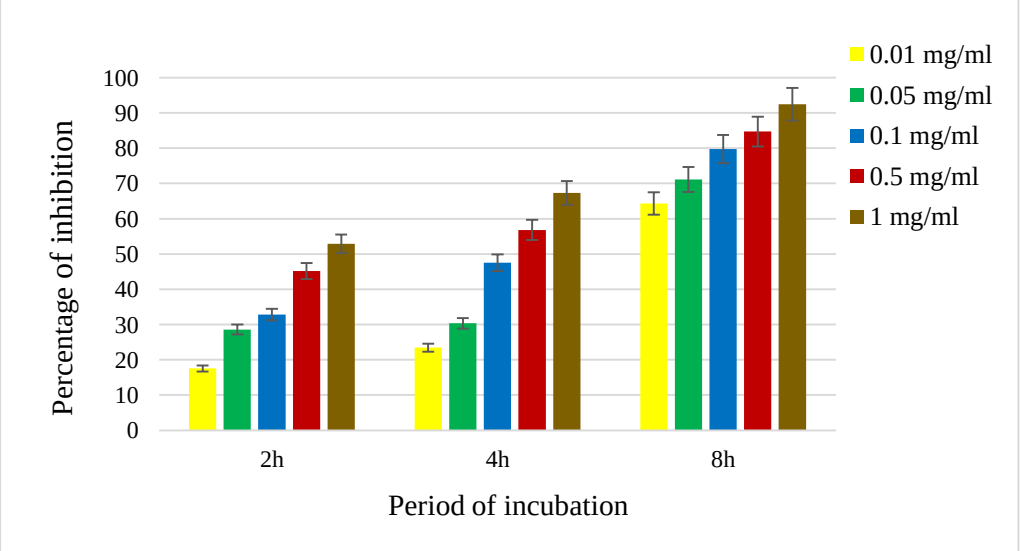

Fig 3: Effect of *Ta*EE on PK activity of *H. contortus*

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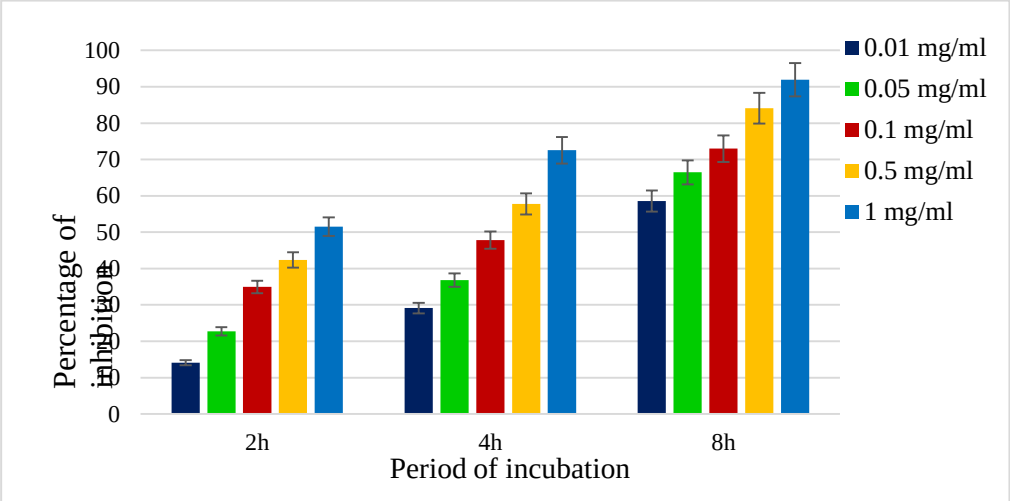

Fig 4: Effect of *Ta*EE on PEPCK activity of *H. contortus*

IV. DISCUSSION

Energy generation in parasitic helminths is based mainly on the glucose metabolism (Von Brand, 1974). Glycogen plays a vital role in providing energy to the nematodes by serving as main storage of carbohydrates (Barrett, 1981). Ward (1982) opined that PK and PEPCK are the non-equilibrium enzymes in the glycolytic pathway and the most accessible spot for metabolic regulation. PK and PEPCK plays a key role in helminth energy metabolism. They direct the flow of carbon from PEP into the end products of anerobic metabolism. The enzymes compete for the substrate PEP, and their relative activites account for the PEP- lactate or acetate/PEP-succinate or propionate pathways (Behm and Brynat, 1982). PEPCK is a vital enzyme of functional significance in helminth parasites which catalyses an inverse reaction that forms oxaloacetic acid from phosphoenolpuruvate (PEP) rather than PEP formation from oxaloacetate as in mammals (Barrett, 2009). Kohler (2006) opined that *H. contortus* larvae are converting 0 to 9% glucose into lactic acid, having a modified glycolytic pathway diverging at phosphoenolpyruvate (PEP) level towards the formation of oxaloacetate.

Renold (1980) stated that PK and PEPCK could act as a selective anthelmintic target for anthelmintic agents to control parasitic infections. Inhibition of PK and PEPCK activities treated with different anthelmintics has been reported in helminthes parasites (Roy *et al*. 2012; Swargiary *et al*. 2013). The inhibitory effect of ethanol extract of *Acorus calamus* and ethyl acetate extract of *Piper betle* on PK and PEPCK activity of *Cotylophoron cotylophorum* was reported by Jeya and Veerakumari (2016) and Lokesh and Veerakumari (2016). Similarly inhibitory effect of *Allium sativum* on the PEPCK and PK activites in *H. contortus* was reported by Navaneethalakshmi and Veerakumari (2009).

Inhibition of PK activity results in reduced engenderment of pyruvate. Withal, the inhibition of PEPCK apprehends the PEP-succinate pathway and diverts the PEP towards the formation of pyruvate. This results in reduced production of malate, which accommodates as the main substrate for mitochondrial phosphorylation (Navaneetha and Veerakumari, 2009; Lakshmi and Veerakumari, 2012). Due to this, both PK and PEPCK arrests the PEP-succinate/PEP-lactate pathways. Thus the energy yielding process is impaired and deprives the worms of its ATP production. Decreased level of ATP production leads to the elimination of *H. contortus* from the host abomasum of the sheep.

V. CONCLUSION

Gastrointestinal parasites cause devastating effects on sheep and goats. *H. contortus* developing resistance to chemical anthelmintics including benzimidazoles (BZs), imidazothiazoles and macrocyclic lactone has been a world-wide scenario causing massive threat to small ruminant industry. This leads to the development of plant based ethano-veterinary remedies, without causing any adverse effect in host. Furthermore, this research reveals that *Sa*EE and *Ta*EE significantly inhibit both PK and PEPCK activities. By this desirable results we suggest that these plants could be used as a potential anthelmintic phytotherapeutic drug to combat the nematode, *H. contortus*. This paves a way for holistic investigation of plants with anthelmintic efficacy and fame their potential exploitation in farming systems.

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