**IN VITRO EFFECT OF SYZYGIUM AROMATICUM ON THE MOTILITY AND ACETYLCHOLINESTERASE OF COTYLOPHORON COTYLOPHORUM**

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**ABSTRACT**

Helminth parasites cause a wide variety of debilitating and frequently fatal diseases in animals. Paramphistomosis caused by the paramphistome *Cotylophoron cotylophorum* constitutes a major group of disease in domestic ruminants. The anthelmintic activity of *Syzygium aromaticum* on the motility and acetylcholinesterase (AChE) activity of the digentic trematode *Cotylophoron cotylophorum* was studied *in vitro*. The flukes were exposed to various concentrations of hexane, chloroform, ethyl acetate and ethanol extract of *Syzygium aromaticum* and the motility and mortality of the parasite were observed. As ethanol extract was very effective, further studies were carried out with five different sub lethal concentrations (0.005, 0.01, 0.5, 0.1 and 0.5 mg/ml) of ethanol extract of *Syzygium aromaticum* (SaEE). The electronic measurement of the motility of the treated parasites clearly indicate the direct impact of the drugs on the motility of the parasite. Maximum inhibition in the motility (86.27%) and AChE activity (86.86%) was observed in 0.5 mg/ml after 8h of exposure. Acetylcholinesterase (AChE) is an enzyme which is involved in neurotransmission. It is present in the cholinergic synapses in the central nervous system as well as in neuromuscular synapses where it rapidly hydrolyzes acetylcholine. Inhibition of AChE in the parasite results in muscular paralysis and the parasite lose its biochemical hold fast and get expelled from the host.

**Keywords**: *Cotylophoron cotylophorum, Syzygium aromaticum*, Electronic Motility Meter (EMM), Acetylcholinesterase.

**INTRODUCTION**

Paramphistomes are parasites of ruminants, which particularly affect cattle and sheep. Paramphistomosis caused by the amphistomes constitute a major group of diseases in ruminants. Several synthetic anthelmintics are available to treat helminth infection. However, large proportion of the world’s population still do not have access to, or cannot afford to pay for modern medicines, particularly in remote rural areas in poor countries. Besides, the continued usage of current anthelmintic drugs also possessed a major problem of drug resistance in several parasite species (Sangster, 2008; Alfredo et al.,

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In the World wide effort to develop worm control strategies alternative to chemotherapy, application of plant products such as phytoanthelmintics appear promising. Many of the plants that have been reported to have anthelmintic properties actually contain compounds that are directly active against parasites (Muller-Harvey and McAllan, 1992). The natural compounds derived from plants are more stable as these are mostly plant secondary metabolites synthesized over a long period of time. The natural compounds also provide greater structural diversity than synthetic ones and therefore are a source of low molecular weight structures active against a wide range of target agents and this diversity can preclude the occurrence of resistance (Asha et al., 2001).

*Syzygium aromaticum* commonly called clove, belongs to the family myrtaceae. Clove bud oil has biological activities, such as antibacterial, antifungal, insecticidal, analgesic, antispasmodic, antcarminative and antioxidant properties (Huang et al., 2002). Clove oil is also active against plant-parasitic nematodes (Pandey and Dwivedi, 2000). The major constituents in bud oil are eugenol and â-carophyllene (Srivastava et al., 2003). Pessoa et al. (2002) reported the anthelmintic activity of eugenol against *Haemonchous contortus*. In the present investigation an attempt has been made to assess the efficacy of *Syzygium aromaticum* against the paramphistome *Cotylophoron cotylophorum*. Motility is an important parameter in assessing the anthelmintic efficacy of drugs. All broad spectrum anthelmintic regardless of their mode of action drastically reduce parasite acetylcholinesterase (AChE), thus making it as a potential target (Rapson et al., 1986). The present study was undertaken to evaluate the anthelmintic efficacy of *Syzygium aromaticum* based on its effect on the motility and AChE of *Cotylophoron cotylophorum* in *vitro*.

**MATERIALS AND METHODS**

In *vitro* maintenance of *Cotylophoron cotylophorum*: *Cotylophoron cotylophorum* were collected from the rumen of infected sheep, slaughtered at Perambur abattoir, Chennai. Adult live worms 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: The Clove buds were collected from Lakshmi stores at Chennai, and it was tested for authenticity 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 (1998). Clove buds were coarsely powdered and soaked serially in hexane, followed by chloroform, ethyl acetate and ethanol successively, and then extracts were filtered using Whatman filter paper No.1. and
In vitro effect of *Syzygium aromaticum* on the motility.....

Concentrated by distillation using rotary evaporator (EQUITRON). The concentrated extracts were completely dried to remove the last traces of the solvents using Lyodel Freeze Dryer (DELVAC). The extracts were serially diluted with Hedon-Fleig solution to obtain various concentrations (1, 3 and 5 mg/ml). Ten flukes were incubated in 25 ml of each concentration of plant extracts and the motility of the parasites was monitored at various intervals viz. 5, 15, 30 min, 1, 2, 4, 6, 8, 12 and 24 h. Simultaneously, control was also maintained in Hedon-Fleig solution without the plant extracts. The motility of the parasites was observed visually at a regular time interval. The motility response of the parasites was categorized as very active (+++), moderately active (++), slightly active (+), sluggish (+), and dead (-). Based on the visual observations five different sub-lethal concentrations (0.005, 0.01, 0.05, 0.1 and 0.5 mg/ml) of effective extract were selected for further studies.

**Quantitative measure of motility response of drug-treated worms:** The motility response of the drug-treated parasites was recorded with the aid of Electronic Micromotility Meter (EMM) as described by Veerakumari (2003). The motility of the flukes was measured at 360 nm with 10 seconds time interval for 3 min. The percentage of inhibition of motility of drug-treated flukes was calculated using the formula,

\[
\text{Percent inhibition of motility} = \frac{C - T}{C} \times 100
\]

C – Control worm
T – Drug-treated worm

**Estimation of Acetylcholinesterase:** The acetylcholinesterase (AChE, EC 3.1.1.7) activity was assayed using photometric method as described by Ellman et al. (1961). AChE in the sample hydrolyses acetylcholine, which is the substrate and forms thiocholine that will react rapidly and irreversibly with 5 thio-bis-nitrobenzoic acid. The increase in colour intensity was measured spectrophotometrically at 412 nm.

\[
\text{AChE} \\
\text{Acetylcholine} \rightarrow \text{Thiocholine + Acetate} \\
\text{thiocholine + Dinitrobenzoate} \rightarrow \text{Yellow colour}
\]

The enzyme samples were prepared by homogenizing 500 mg of control and drug-treated flukes in 1 ml of 0.1M phosphate buffer (pH 8.0). The homogenate was centrifuged at 1000 rpm for about 5 min. To 100 µl of the supernatant, 1.3 ml of 0.1 M phosphate buffer (pH 8.0) and 0.05 ml of 0.01 M 5-thio-bis-nitrobenzoic acid (DTNB) solution was added and transferred to quartz cuvette. The absorbance at 412 nm was set to zero in a UV visible double beam biospectrophotometer. 0.02 ml of 0.075 M acetylthiocholine iodide was added to the reaction mixture in the cuvette and mixed well and the absorbance was noted for 5 min at 15 seconds interval. The increase in absorbance per minute was calculated. AChE activity was calculated using the formula

\[
R = 5.74 \times 10^4 \times \frac{\Delta A}{\text{Protein content of the sample}}
\]

The protein content of the sample was estimated following the procedure of Lowry et al. (1951). The enzyme activity was expressed as number of moles of
acetylthiocholine iodide hydrolyzed D min D mg protein.

**Statistical analyses:** Statistical analyses were performed with the Statistical program for the social sciences SPSS version 16.0. The significance of drug induced inhibition in the motility and AChE activity of the parasites was assessed using analysis of variance (ANOVA) for different concentrations of ethanol extract of *Syzygium aromaticum* (SaEE).

**RESULTS AND DISCUSSION**

The present study elucidated the anthelmintic efficacy of ethanol extract of *Syzygium aromaticum* (SaEE) against *Cotylophoron cotylophorum* (Table 1). The maximum level of inhibition of the motility was observed at 0.5 mg/ml after 8h of exposure. The motility of the parasite was inhibited to 20% in 0.005 mg/ml concentration after 2h of exposure. After 4 and 8h of exposure, inhibition was 33.33 and 58.02% respectively; whereas in higher concentration (0.5 mg/ml) the motility was inhibited to 43.33, 84.64 and 86.27% after 2, 4 and 8h of exposure respectively (Fig. 1).

Dose and time dependent inhibition in AChE activity was observed in drug treated parasites. Inhibition in AChE activity by SaEE at 0.005 mg/ml concentration was found to be 21.62, 33.78 and 59.15% after 2, 4 and 8h respectively. At 0.5 mg/ml concentration it was found to be 41.25, 81.48 and 86.86% after 2, 4 and 8h respectively (Fig. 2).

In the present study it has been observed that the percentage of inhibition of motility and AChE at higher concentrations (0.5 mg/ml) after 8h of exposure were 86.27% and 86.86% respectively, indicating the direct correlation between the motility and AChE.

All effective anthelmintics directly or indirectly affect the motility of the parasite. EMM is an instrument designed with spectacular advancement and automation to detect the motility response of parasites (Veerakumari, 2003). Electronic measurement of the motility of the parasite clearly indicates the direct impact of the drug on the parasite. AChE is an important enzyme involved in neuromuscular transmission found in a number of helminths (Ogilive et al., 1973). It is the normal functioning of neuromuscular and physiology important in helminth parasites to maintain their feeding site and other vital coordinating functions (Kumar and Tirupathi, 1998). The principal physiological role of AChE is believed to be the termination of transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine (Lee, 1996), and also involved with other non-enzymic functions like host parasite interaction (Nalivaeva and Turner 2001). Due to AChE inhibition there is marked increase in acetylcholine levels that causes continuous and excessive stimulation of the nervous system (Kirby et al., 2000). Inhibition of AChE causes desensitization of the muscle receptor resulting in paralysis (Opperman and Chang, 1992). Consequent to inactivation of neuromuscular co-ordination, active ingestion and movement of food through the digestive tract is arrested. The parasites thus enter a state of starvation and energy deprivation. The energy deprived worms were unable to sustain themselves *in situ* are expelled by the host.
system. Since paramphistomes exist with other helminth parasites, this study will help us to formulate or develop anthelmintic drug of natural origin with broad spectrum attitude to combat various anthelmintic infection. *Syzygium aromaticum* is highly effective against paramphistome infections of small ruminants and it is necessary to identify and isolate the possible active phytoconstituents responsible for the anthelmintic activity. It is targeted platform for pharmacological studies and development of novel anthelmintic products to fill a gap in the anthelmintic drug industry, which is facing the crisis of anthelmintic resistance to conventional anthelmintic drugs.

**Table 1**: Chronological observations on the viability and motility of *Cotylophoron cotylophorum* exposed to various extracts of *Syzygium aromaticum*

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<th>Extracts</th>
<th>Concentrations mg/ml</th>
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very active (+++), moderately active (++), slightly active (+), sluggish (+), and dead (-)

*SaHE* - Hexane extract of *Syzygium aromaticum*

*SaCE* - Choloroform extract of *Syzygium aromaticum*

*SaEAE* - Ethyl acetate extract of *Syzygium aromaticum*

*SaEE* - Ethanol extract of *Syzygium aromaticum*
**Table 2**: Chronological observations on the viability and motility of *Cotylophoron cotylophorum* exposed to sub lethal concentrations of SaEE

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very active (++++) , moderately active (+++), slightly active (++), sluggish (+), and dead (-)

**Fig. 1** *In vitro* effect of SaEE on the motility of *Cotylophoron cotylophorum*

**Fig. 2** *In vitro* effect of SaEE on the AChE of *Cotylophoron cotylophorum*
REFERENCES


