

Effect of *Allium sativum* on the Motility and Acetylcholinesterase of *Haemonchus contortus*

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Abstract: *Helminths are known to be a major constraint to ruminant's well-being and productive performance. Gastrointestinal helminths are widespread parasitic agents of livestock especially in small ruminants. Helminthiasis plays an important role in the small ruminant production leading to enormous economic losses in areas where extensive grazing is practiced. Haemonchosis is caused by the hematophagous parasite Haemonchus contortus. It is the most important parasites of all the gastrointestinal nematodes that constrain the survival and productivity of sheep and goats owned by rural farmers in the developing countries. In the present investigations anthelmintic effect of Allium sativum on the motility and acetylcholinesterase (AChE) of the nematode Haemonchus contortus were studied in vitro. The worms were exposed to various concentrations of hexane, chloroform, ethyl acetate, ethanol and aqueous extract of Allium sativum and the motility of the parasites were viewed. As ethanol extract was effective, further studies were conducted with various sublethal concentrations 0.005, 0.01, 0.05, 0.1 and 0.5 mg/ml. Quantitative measurement of the motility of the parasites evidently showed the direct impact of the drugs on the parasite. Maximum inhibition in the motility (88.50%) and AChE (90.25%) was remarked in 0.5 mg/ml of AsEE after 8h of exposure. AChE is concerned in muscularly harmonious combination and neurotransmission. The important role of this enzyme can stop cholinergic synaptic communication with immediate hydrolysis of the acetylcholine. Inhibition of AChE in the parasite results in paralysis of musculature and the parasite lose its biochemical holdfast and get expelled from the host. The present study clearly showed that AsEE had killed the parasites by affecting their neurotransmission.*

Keywords: *Allium sativum, Haemonchus contortus, Acetylcholinesterase.*

1. Introduction

(GI) nematodes are a major threat to sheep productivity and endanger animal welfare in developing countries. *Haemonchus* Gastrointestinal *contortus* is an important blood sucking parasite of ovines and causes an insidious drain on production [1]. The disease caused by this parasite (Haemonchosis) is prevalent wherever, sheep and goats are raised, but it exerts the greatest economic losses in temperate and tropical regions [2]. Parasitism is one of the major factors limiting sheep and goat production because they cause heavy economic losses in meat and wool production [3]. Control is achieved by use of synthetic anthelmintics in combination with grazing management. *Haemonchus contortus* has been documented to be resistant to all three families of anthelmintics namely, benzimidazole, imidathiazole and ivermectin [4, 5] and against drug with narrow spectrum of activity such as salicylanilides [6, 7, 8]. The frequent use of these anthelmintics over many years has inevitably led to the development of drug resistance to each class in parasitic nematodes. The emergence of resistance to anthelmintic drugs which is now a worldwide phenomenon [9] and the increased awareness of consumers about the food chain have stimulated investigation into alternative to commercially available anthelmintics such as medicinal plants. Many studies from various parts of the world have shown that certain plant species effectively reduce the degree of parasite infestation in ruminants (sheep) and are promising alternatives to conventional anthelmintics. Many of the plant species owe their anthelmintic effects to plant metabolites such as saponins, tannins and essential oils.

2. Background of the Study

Recent surveys in developing countries have identified many plants have the potential to be used as anthelmintics [10]. A wide range of plants and their products around the world are being explored for their possible anthelmintic effects against cestodes, trematodes [11] and nematodes [12]. Because of the economic impact of gastrointestinal parasitic nematodes in the livestock industry around the world, most of the research on plant extracts is being focused on identifying bioactive compounds from plants against nematodes. *Allium sativum* commonly called garlic belongs to the family Alliaceae. It has been used for centuries to fight infections [13]. It has traditional dietary and medicinal applications as an anti-infective agent [14]. *A. sativum* is an antioxidant [15], antibacterial, antiviral, antifungal and anti-parasitic [16,17] antihypertensive [18], anthelmintic [19] and anti-diabetic agent [20]. In veterinary practice, *A. sativum* is used for promotion of wound healing and treatment of infected wounds in calves [21]. The fresh bulb of *A. sativum* contains alliin, allicin and volatile oils. When the garlic clove is crushed, the odorless compound alliin is converted to allicin, by the enzyme allinase. Allicin gives garlic its characteristic pungent smell [22]. Volatile compounds are considered to be responsible for most of the pharmacological properties of garlic. Garlic contains at least 33 sulphur compounds, several enzymes, 17 amino acids and minerals such as selenium and germanium [23, 24, 25, 26]. In the present investigation an attempt has been made to assess the efficacy of *A. sativum* against the *H. contortus*. The most important parameter used to gauge the anthelmintic property of a plant product is the motility of the parasites *in vitro* conditions. Motility is directly related to the neuromuscular system [27] which is well developed in nematodes [28]. Plant anthelmintics target the neuromuscular system of the worms

[29]. They block cholinergic nerve transmission and act through the stereo specific binding to a receptor resulting in muscle paralysis [30]. Therefore, the present investigation was undertaken to evaluate the anthelmintic efficiency of *Allium sativum* based on its effects on the motility and AChE of *Haemonchus contortus* *in vitro*.

3. Materials and Methods

3.1 Collection and *in vitro* maintenance of *H. contortus*

Adult female *H. contortus* were collected from the abomasum of sheep, slaughtered at Perambur slaughter house, Chennai. The worms were washed in physiological saline and maintained in Hedon-Fleig solution (pH 7.0) at 37°, which is the best medium for *in vitro* maintenance [31].

3.2 Preparation of plant extracts

The bulbs of *Allium sativum* was collected made into paste and soaked serially in hexane, chloroform, ethyl acetate, ethanol and water in an aspirator bottle and extracted by cold percolation method after 48 h [32]. The filtrate was collected by passing the mixture through Whatman filter paper No.1 and concentrated by using Rotary Evaporator (EQUITRON). The concentrated extracts were dried to remove the solvents using Lyodel freeze Dryer (DELVAC, Chennai).

3.3 Gross visual observation on the motility of the worms

Sixty milliliters of Hedon-Fleig solution containing various concentrations (10, 30 and 50 mg/ml) of the aqueous and solvent extracts were individually distributed in 100 ml of conical flasks. Ten live and active parasites were incubated in various extracts. The activity of the worms was checked at various intervals (5, 15, 30 min, 1, 2, 4, 6, 8, 12, and 24 h) of exposure. Simultaneously, control was also maintained in Hedon-Fleig solution without the plant extracts. Based on the motility of the worms, the observations were categorized as active (++++), moderately active (+++), slightly active (++) , sluggish (+) and dead (-). The worms with no movement were regarded as dead. Based on the motility of the parasite for 8 h of exposure, five different sub-lethal concentrations were selected (0.005, 0.01, 0.05, 0.1 and 0.5 mg/ml) for further studies.

3.4 Quantitative measure of motility response of drug-treated worms using Electronic Micromotility Meter (EMM)

The parasites were incubated for 2, 4 and 8 h in the various sub-lethal concentrations of the plant extracts for further *in vitro* studies. The motility response of drug-treated parasites was recorded with the aid of Electronic Micromotility Meter (EMM) [33]. The motility of the worms was measured at 360nm with 10 seconds time interval for 3 min. The percentage of inhibition in the motility of the parasites was calculated following the formula,

$$\% \text{ inhibition of motility} = \frac{C - T}{C} \times 100$$

Where, C - Deviation of voltage signal in the control worm and T - Deviation of voltage signal in the worm treated with plant extracts.

3.5 Estimation of Acetylcholinesterase

The acetylcholinesterase (AChE) (EC 3.1.1.7) activity was tested using photometric method as described by Ellman *et al* [34]. Acetylthiocholine is a substrate hydrolyzed by AChE in the sample and forms thiocholine which will react rapidly and irreversibly with 5-thio-bis-nitrobenzoic acid (DTNB) producing a yellow anion of 5-thio-2-nitrobenzoic acid. The increase in color intensity is measured spectrophotometrically at 412 nm.

AChE

Acetylthiocholine —————> Thiocholine + acetate
 Thiocholine + di thionitrobenzoate ———> Yellow color

The sample for the enzyme test was prepared by homogenizing 100 mg of control and treated parasites in 1 ml of 0.1 M phosphate buffer (pH 8.0). The homogenate was centrifuged at 1000 rpm for 5 min. To 0.1 ml of the supernatant, 1.3 ml of 0.1 M phosphate buffer (pH 8.0), 0.05 ml of 0.01 M DTNB and 0.02 ml of 0.075 M acetylthiocholine iodide were added and mixed well. The reaction mixture was transferred to a cuvette and the absorbance was measured at 412 nm in a UV-Visible double beam biospectrophotometer. The absorbance was noted for 3min at an interval of 15 sec and the change in absorbance per minute (ΔA) was calculated. AChE activity was calculated using the formula.

$$R = 5.74 \times 10^{-4} X \frac{\Delta A}{\text{Protein content of the sample}}$$

ΔA – Increase in absorbance per minute.

The protein content of the sample was estimated following the procedure of Lowry *et al* [35]. The enzyme activity was expressed as number of moles of acetylthiocholine iodide hydrolyzed/ min/ mg protein.

3.6 Statistical Analyses

The experimental results were expressed as mean \pm standard deviation. Each value is expressed as mean of triplicate experiments. Statistical analyses were performed by ANOVA using SPSS version 20 for different concentration of ethanol extract of *A. sativum*.

4. Results and Discussion

The present investigation enlightened the anthelmintic efficiency of ethanol extract of *A. sativum* (AsEE) on the viability and motility against *H. contortus* (Table 1). The ethanol extract of *A. sativum* (AsEE) was found to be more effective against *H. contortus*. Therefore five sub-lethal concentrations 0.005, 0.05, 0.01, 0.1 and 0.5 mg/ml of AsEE was selected for further *in vitro* studies and the motility response of parasites at sub-lethal concentrations were recorded in the table 2. The percentage of inhibition on motility by AsEE are 52.50%, 78% and 88.50%, after 2, 4

and 8 h exposure at higher sub-lethal concentration 0.5 mg/ml (Table 3). Dose and time dependent inhibition in AChE activity was observed in drug treated parasites. Inhibition in AChE activity by AsEE at 0.5 mg/ml was found to be 50%, 82.43% and 90.25% after 2, 4 and 8 h respectively (Table 4). The present study revealed the destructive effect of AsEE on the motility and AChE of *H. contortus*. All effective anthelmintics directly or indirectly affect the motility of the parasite. EMM is an instrument fabricated with spectacular advancements to record the motility of the parasites. Electronic measurement of the motility of the parasite clearly shows the direct impact of the drug on the parasite.

AChE is an enzyme broadly spread in many species, including parasites. The presence of esterase has been showed in many helminth parasites [36, 37, 38]. Many roles have been proposed for AChE, including a 'biological hold fast', inhibition of mucus secretion and modulation of host immune response. Although few other roles have also been applied to AChE, the primary function of this enzyme is to limit the cholinergic synaptic transmission, by rapid hydrolysis of the neurotransmitter acetylcholine (ACh) [39, 40]. AChE is thus proved to be a functional protein, involved in multifaceted activities, which can serve as a suitable candidate for diagnostic purposes. The principal role of AChE is termination of transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter, acetylcholine [41].

The inhibition of AChE results in the accumulation of endogenous acetylcholine, thereby disrupt the neuromuscular transmission. According to Kaur *et al* [42] and Opperman *et al* [43], drug-induced interruption of the neuromuscular transmission leads to paralysis of musculature. Consequent to inactivation of neuromuscular coordination, active

swallowing and movement of food through the digestive tract is arrested. The parasites thus entered starvation and energy deprivation. The energy-deprived worms unable to sustain themselves *in situ* are expelled by the host. AChE is thus proved to be a functional antigen, involved in multifaceted activities, and can therefore serve as a suitable candidate for both diagnostic purposes, vaccine development, and drug design. This study will help us to develop anthelmintic drug of natural origin with attitude to combat various anthelmintic infection. *A. sativum* is highly effective against helminths infection 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 drug products to fill a gap in the anthelmintic drug industry, which is facing the crisis of anthelmintic resistance to conventional anthelmintic drugs.

5. Conclusion

The present studies showed that ethanol extract of *A. sativum* have anthelmintic activities against *Haemonchus contortus*. From these observations, it can be assumed that the bulbs of *A. sativum* are the good sources of traditional medicines for helminth infections and further studies would be attempted for isolation, purification and identification of potential compounds from plant extract.

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Table 1: Gross visual observation on the motility of *H. contortus* treated with various extracts of *A. sativum*

Extracts	Conc. mg/ml	5min	15min	30min	1h	2h	4h	6h	8h	12h	24h
Control		++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
AsHE	10	++++	++++	++++	+++	+++	++	++	++	-	-
	30	++++	+++	+++	+++	+++	++	+	+	+	-
	50	+++	+++	+++	++	++	+	+	-	-	-
AsCE	10	++++	++++	++++	++++	+++	+++	++	+	-	-
	30	+++	+++	+++	+++	+++	++	+	+	-	-
	50	++	++	+	+	-	-	-	-	-	-
AsEaE	10	++++	++++	++++	++++	++++	+++	++	+	+	-
	30	+++	+++	+++	+++	++	+	+	+	-	-
	50	+++	++	++	++	+	-	-	-	-	-
AsEE	10	+++	++	+	+	-	-	-	-	-	-
	30	++	+	+	-	-	-	-	-	-	-
	50	++	+	+	-	-	-	-	-	-	-
AsAE	10	++++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	30	+++	+++	+++	+++	++	++	++	+	-	-
	50	+++	+++	+++	++	++	+	+	+	-	-

++++: Very active; +++: Moderately active; ++: Slightly active; +: Sluggish; -: Dead

AsHE – *A. sativum* hexane extract; AsCE – *A. sativum* chloroform extract; AsEaE – *A. sativum* ethyl acetate extract; AsEE – *A. sativum* ethanol extract; AsAE – *A. sativum* aqueous extract.

Table 2: Gross visual observation on the motility of *H. contortus* treated with sub-lethal concentrations of ethanol extract of *A. sativum*

Extracts	Conc. mg/ml	5min	15min	30min	1h	2h	4h	6h	8h	12h	24h
Control		++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
ASEE	0.005	++++	++++	++++	+++	++++	+++	+++	+++	+++	++
	0.01	++++	++++	++++	++++	++++	+++	+++	++	++	+
	0.05	++++	++++	++++	++++	+++	+++	++	++	++	+
	0.1	++++	++++	++++	+++	+++	++	++	+	+	-
	0.5	++++	++++	++++	+++	++	++	++	+	+	-

++++: Very active; +++: Moderately active; ++: Slightly active; +: Sluggish; -: Dead

Table 3: Effect of ethanol extract of *A. sativum* on the motility of *H. contortus*

Conc. mg/ml*	inhibition (mean \pm SD of n=5) at various periods of incubation**		
	2h	4h	8h
AsEE			
0.005	24.50 \pm 0.47	59.80 \pm 0.31	70.30 \pm 0.57
0.01	31.00 \pm 0.31	65.20 \pm 0.47	74.90 \pm 0.39
0.05	35.20 \pm 0.40	71.00 \pm 0.40	79.40 \pm 0.39
0.1	47.70 \pm 0.57	74.00 \pm 0.47	83.20 \pm 0.30
0.5	52.20 \pm 0.41	78.00 \pm 0.38	88.50 \pm 0.50
* Inhibitory effects of the extracts among the different concentrations of the respective plants are duration of incubation (P < 0.05) using Duncan principle comparisons.			
** Inhibitory effects of the extracts among the different hours of incubation is significantly different for each concentration of the respective plants (P < 0.01) Duncan principle comparisons.			

Table 4: Effect of ethanol extract of *A. sativum* on the AChE of *H. contortus*

Conc. mg/ml*	inhibition (mean \pm SD of n=5) at various periods of incubation**		
	2h	4h	8h
AsEE			
0.005	25.00 \pm 0.29	60.07 \pm 0.41	75.03 \pm 0.13
0.01	33.33 \pm 0.37	67.37 \pm 0.67	80.31 \pm 0.21
0.05	37.50 \pm 0.16	70.48 \pm 0.68	83.75 \pm 0.14
0.1	41.66 \pm 0.2	73.64 \pm 0.20	88.35 \pm 0.14
0.5	50.00 \pm 0.1	82.43 \pm 0.12	90.25 \pm 0.17
* Inhibitory effects of the extracts among the different concentrations of the respective plants are duration of incubation (P < 0.05) using Duncan principle comparisons.			
** Inhibitory effects of the extracts among the different hours of incubation is significantly different for each concentration of the respective plants (P < 0.01) Duncan principle comparisons.			

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