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**Dr. A Selvaraju**
Assistant Professor,
PG Department of Botany
Sri Vidya Mandir Arts and Science College, Katteri,
Uthangarai, Tamil Nadu, India

**S Kaviya**
PG Scholar, PG Department of Botany, Sri Vidya Mandir Arts and Science College, Katteri,
Uthangarai, Tamil Nadu, India

**Dr. K. Manoj Dhanraj**
Research Fellow, Department of Wildlife Science, Madras Veterinary College, Vepery,
Chennai, Tamil Nadu, India

**Correspondence**
**Dr. A Selvaraju**
Assistant Professor,
PG Department of Botany
Sri Vidya Mandir Arts and Science College, Katteri,
Uthangarai, Tamil Nadu, India

# Phytochemical analysis and anthelmintic potential of *Nigella sativa* against the trematode, *Cotylophoron cotylophorum*

**Dr. A Selvaraju, S Kaviya and Dr. K Manoj Dhanraj**

**Abstract**
Paramphistomosis caused by amphistomes constitutes a major group of diseases, causing considerable economic loss to livestock industry in India. Chemical control of helminths coupled with improved management has important parasitic control strategy throughout the world. However increasing problems of development of resistance in helminths against anthelmintics led to the proposal to screening medicinal plants for their anthelmintic activity. Hence the present study was undertaken to elucidate the phytochemical analysis and anthelmintic potential of *Nigella sativa* against the trematode, *Cotylophoron cotylophorum*. The flukes were exposed to five different sub-lethal concentration of ethanol extract of *Nigella sativa* (*Ns*EE) for 2, 4 and 8h. The motility response of the drug- treated parasites was recorded with the aid of Electronic micromotility meter (EMM). The maximum inhibition of motility was observed at 0.5 mg/ml concentration after 8 h of exposure. Phytochemical and GC – MS analysis of ethanol extract of *Nigella sativa* revealed various compounds possessed remarkable anthelmintic activity. The present study enlightened the anthelmintic effect of ethanol extracts of *Nigella sativa* suggest that could be used as phytotherapeutic agents to combat the paramphisotmes infection in livestock.

**Keywords:** *Nigella sativa, Cotylophoron cotylophorum,* Motility, GC - MS

## Introduction
*Cotylophoron cotylophorum* is a gastrointestinal digenetic trematode, lives in the rumen of livestock. It has a complex life cycle which requires an intermediate host, planorbid snail. It parasitizes a wide range of hosts. Heavy infection of *C. cotylophorum* causes paramphistomosis in cattle, sheep and goats. Paramphistomosis has been a neglected trematode infectious disease; recently, it emerged as an important cause of productivity loss (Sharma and Katoch, 2007 and Ozdal *et al*., 2010) [33, 27]. Synthetic anthelmintics are used to combat helminth infection in livestock. However, synthetic drugs are usually associated with many limitations, such as nonavailability of desired type, cost, drug resistance, environment pollution and presence of residues in milk, meat, wool and on pasture. Therefore, there is a need for developing cheaper, less toxic and eco-friendly novel anthelmintic drug. The use of medicinal plants for the prevention and treatment of gastro-intestinal parasitism has its origin in ethnoveterinary medicine (Athanasiadou *et al*., 2007) [4]. Plants are known to provide a rich source of potent botanical anthelmintics. The use of medicinal plants for the prevention and treatment of gastro intestinal parasitism has its origin in ethno veterinary medicine (Satyavati *et al*., 1976; Lewis, and Lewis, 1977) [32, 25].

As, application of plant based anthelmintic drugs appears promising, an attempt has been made in the present study to elucidate the anthelmintic potential of seeds of *Nigella sativa* against the paramphistome *C. cotylophorum. Nigella sativa* commonly called black seed belongs to the Family Ranunculaceae is emerging as a miracle herb with a rich historical and religious background since many researches revealed its wide spectrum of pharmacological potential. Seeds of *Nigella sativa* possessed biological activities such as diuretic, antihypertensive, antidiabetic, anticancer, immunomodulatory, analgesic, antimicrobial, anthelmintics, analgesics, anti-inflammatory, spasmolytic, bronchodilator, gastroprotective, hepatoprotective, renal protective and antioxidant properties. The seeds of *N. sativa* are used to treat bronchitis, asthma, diarrhea, rheumatism, skin disorders, liver tonic, digestive, anti-diarrheal, appetite stimulant, parasitic infections (Abel-Salam, 2012; Khaled, 2009; Assayed, 2010; Abdel-Zaher *et al*., 2011; Boskabady *et al*., 2010 and Goreja, 2003) [2, 22, 3, 1, 6, 14]. *Nigella sativa* has many different chemical ingredients including thymoquinone (TQ) (30- 48%), flavonoids, anthocyanins, alkaloids and essential fatty acids, particularly linoleic and oleic acid (Desai *et al*., 2015) [11].

All effective parasitic drugs affect the motility of the parasites. Motility of helminths is a good indicator of fluke's viability and can serve as a valuable parameter for evaluation of the spectrum of sensitivity of a specific helminth species to a variety of anthelmintics. Hence, motility is considered as an important parameter in evaluating the efficacy of drugs. Visual assessment of motility involved observation for presence of free spontaneous movement in medium (Bowen and Vitayavirasak, 2005) [7]. However, interpretation of anthelmintic property by visual observation is very time consuming, demanding close microscopical examinations and the results are also highly variable. Electronic micromotility meter provides a more dependable quantitative measure of the motor activity of drug-treated parasites and could ascertain the effects of the drug on the parasites (Veerakumari, 2003) [36].

Plants are known to have beneficial therapeutic effects documented in Traditional Indian System of Medicine. Much work has been done on ethnomedicinal plants in India. Interest in a large number of traditional natural products has increased. It has been suggested that phytochemical extracts from plants holds promise to be used in allopathic medicine as they have potential biological activity (Nair *et al*., 2005; Ramya *et al*., 2008) [26, 28]. If natural plant products are to be exploited as medicines for human use or for the treatment of livestock, then isolation and characterization of their active principles becomes an essential requirement to further progress (Behnke *et al*., 2008) [5]. Natural products, as a pure compound or as a standardized plant extracts provide unlimited opportunities for new drug lead because of the unmatched availability of chemical diversity (Jaiganesh and Arunachalam, 2013) [18]. The pharmacologically active ingredients of many ayurvedic medicines today are being identified and their usefulness in drug therapy was determined (Samy *et al*., 2008) [30]. Gas Chromatography-Mass Spectrometry (GC-MS) with the use of internal standards, provides a multidimensional drug identification and quantization procedure that is the leading confirmation method for forensic drug testing. Hence the present was undertaken to elucidate the phytochemical analysis and anthelmintic effect of *Nigella sativa* on *Cotylophoron cotylophorum*.

## Materials and methods

In the present investigations, phytochemical analysis and anthelmintic potential of *Nigella sativa* against the trematode, *Cotylophoron cotylophorum* was studied *in vitro*

### *In vitro* maintenance of *Cotylophoron cotylophorum*

*Cotylophoron cotylophorum* (Fig.1) (Fischoeder) were collected from the rumen of infected sheep, slaughtered at Perambur abbatoir, Chennai. Adult live flukes were washed thoroughly in physiological saline and maintained in Hedon-Fleig solution (pH-7.0) which is the best medium for the *in vitro* maintenance of *C. cotylophorum* (Veerakumari, 1996) [35].

### Collection and preparation of plant Extract

Seeds of *Nigella sativa* (Fig.2) were collected and powdered and stored in closed bottles at room temperature in the dark until needed. Powdered Seeds of *Nigella sativa* (200 gm) was soaked in ethanol. The extract was then filtered using Whatman No. 1 filter paper and concentrated by distillation using Rotary evaporator. The concentrated extract was completely dried to remove the last traces of the solvent using Freeze Dryer. Different concentrations of ethanol extract of bark of *N. sativa* (*Na*EE) were prepared using Hedon-Fleig solution. Twenty five millilitre of Hedon-Fleig solution containing various concentrations (0.1, 0.2, 0.3, 0.4 and 0.5%) of the extracts were individually distributed to air tight containers. In each container five live flukes were introduced. The activities of the flukes were checked at various time intervals (5 min, 15 min, 30 min, 1, 2, 4, 6, 8, 12 and 24 h). Control was also maintained simultaneously in Hedon-Fleig solution without *Na*EE. Based on the motility of the flukes, the observations were categorized as very active (++++), active (+++), moderately active (++), sluggish (+) and dead (-). The flukes with no movement were regarded as dead. Based on the motility of the parasites for 24 h exposure to the plant extract, five different sub-lethal concentrations were selected for further studies.

### Quantitative measurement of motility of flukes using Electronic Micromotility Meter (EMM)

EMM has been found to be highly sensitive, and suitable for *in vitro* quantitative assay of the motility of helminths. In the present study, the motility of the control and treated flukes was measured. The motility of the flukes of different concentrations of *Na*EE was quantitatively assayed using Electronic micromotility meter (EMM).

The percent inhibition of motility of drug-treated flukes was calculated using the formula,

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

where,

C - deviation of voltage signal in the control flukes

T - deviation of voltage signal in the fluke treated with plant extracts

### Statistical analysis

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 of the flukes was assessed using analysis of variance (ANOVA) for different concentrations of ethanol extract of *Nigella sativa* (*Na*EE). 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.

## Results and discussion

The present study elucidated the anthelmintic potential of *Nigella sativa* against the trematode, *Cotylophoron cotylophorum* and phyto pharmacological of ethanol extract of *Nigella sativa* were studied.

Preliminary investigations on the efficacy of the ethanol extracts of *Nigella sativa* (Table 1) on the flux of *Cotylophoron cotylophorum* was studied. The control flukes were highly motile and active throughout the experimental period, whereas the movement of the drug–treated flukes was severely curbed. It is evident from Table 2 that ethanol extract of *Nigella sativa* were effective in decelerating the motility of *Cotylophoron cotylophorum*. The inhibition of the motility recorded with the aid of EMM showed that the percentage inhibition of motility is directly proportional to the concentration and period of incubation; Maximum inhibition (81.02 %) of the motility was observed at 5% concentration after 8h of exposure (Fig. 3). The inhibitory effects *Na*EE, of on the motility at different concentrations, for each period of

incubation are significant (P<0.005). The survival status of any living organism depends on the motility. Several synthetic anthelmintic drugs were assessed for their efficacy based on their inhibitory effect on the motility of the parasites against helminth parasites (Fairweather *et al*., 1984; Holmes and Fairweather, 1984; Kumar and Tripathi, 1998; Veerakumari and Priya, 2006) [13, 15, 23, 34]. Challam *et al*. (2010) [10] reported that a similar kind of dose dependent motility was also recorded among trematode and cestode parasites, treated *in vitro* with the crude extract of several plants like *Flemingia vestita*, *Alpinia nigra*, *Millettia pachycarpa*, *Acacia oxyphylla* and *Lysimachia ramose*. Several investigators reported the inhibitory effect of plant extracts on the motility of *Haemonchus contortus*, *Fasciola gigantica*, *Gigantocotyle explanatum*, *Gastrothylax crumenifer*, *Fasciolopsis buski*, *Ascaris suum* and *Raillietina echinobothrida* (Iqbal *et al*., 2010; Kushwaha *et al*., 2004; Jeyathilakan *et al.,* 2010; 2012) [16, 24, 19, 20].

**Phytochemical and GC -MS analysis of ethanol extract of *Nigella sativa***

Preliminary screening of ethanol extract of *Nigella sativa* was found to contain various phytochemical constituents' viz., Steroid, Triterpenoid, Flavonoid, tannin, phenol and alkaloid (Table 3). Visweswari *et al*. (2013) [38] observed the phytochemical screening *Withania somnifera* root extracts exhibits phenols, flavonoids, tannins, saponins, alkaloids, steroids, terpenoids, glycosides and reducing sugars which could account for its varied medicinal properties like anti-inflammatory, anti-spasmodic, anti-analgesic, neuroprotective and diurectic effects. Desai *et al*. (2015) [12] observed the phytochemical analysis of *Nigella sativa* possessed phenols, flavonoids, tannins, saponins, alkaloids, steroids, terpenoids. Similar observations were made by several investigators (Ishtiaq *et al*., 2013; Kazemi, 2014; Yessuf, 2015; Saleh *et al*., 2018) [17, 21, 39, 30].

GC–MS analysis of ethanol extract of *Nigella sativa* revealed the presence of 8 compounds namely Thymine, Vinylidene diacetate, Methyl salicylate, D-Mycoceranic Acid, Palmitic acid, 1,10-Decanediol, Dioxolan and 1-Heptatriacontanol and their molecular formula, molecular weight (MW), and RT value were presented in (Table 4; Fig.4). Saleh *et al*. (2018) [30] who studied the GC–MS analysis of *Nigella sativa* oil possessed eight peaks and the major compound was thymol. Reddy *et al*. (2018) [29] also reported the GC–MS analysis of *Nigella sativa* exhibits various phytoconstituents. Secondary metabolites present in the plants are predominantly used to treat various diseases. Secondary metabolites are also called as plant constituents or natural compounds which exert significant pharmacological and toxicological effects in humankind. The chemical compounds present in the plant sources are categorized as primary and secondary metabolites based on the chemical structure and biosynthetic derivation. Secondary metabolites exhibit different series of pharmacological activity which can be further classified based on their chemical structure and functional groups present in it. The most important secondary metabolites include terpenoids, phenolics, flavonoids, alkaloids and glycosides which act as an important source for single bioactive ingredients in nutraceuticals and modern medicines. Secondary metabolites have a very good antioxidant property which can be used as an effective natural antioxidants source in nutraceuticals. Most of the secondary metabolites have a broad range of their therapeutic activity and they directly interact with the receptors, cell membranes, and nucleic acids (Velu *et al*., 2018). GC–MS analysis of ethanol extract of *Nigella sativa* revealed the 8 compounds and the major compounds are thymol and palmitic acid. Thymol and palmitic acid possessed anthelmintic activity were reported by Camurça-Vasconcelos *et al*. (2005) [9] and Camurça-Vasconcelos *et al*. (2007) [8].

*Ns*EE exhibits a potential anthelmintic activity against *C. cotylophorum*. It may serve as an alternative for synthetic anthelmintics to avoid theirtoxic side effects and development of resistance in a safe and eco friendly manner. In depth field trials of plant based anthelmintics along with best farm management practices can play a great role in parasite control strategies and in enhancing productivity of livestock farming.

**Table 1:** Visual observations on the motility of *C. cotylophorum* treated with *Ns*EE

| Extract | Conc. mg/ml | 5min | 15min | 30min | 1h | 2h | 4h | 6h | 8h | 12h | 24h |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| *Ns*EE | 0.1 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++ | ++ |
| | 0.2 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++ |
| | 0.3 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | +++ | ++ | + |
| | 0.4 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | +++ | ++ | - | - |
| | 0.5 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++ | + | - | - |

very active (++++); moderately active (+++); slightly active (++); sluggish (+); dead (-).

**Table 2:** Quantitative measure on the motility of *C. cotylophorum* treated with *Ns*EE

| Conc. mg/ml* | Percentage of inhibition at various periods of incubation | | |
|---|---|---|---|
| | 2h | 4h | 8h |
| 0.1 | 9.12 ± 0.12 | 20.61 ± 0.12 | 48.31 ± 0.42 |
| 0.2 | 12.32 ± 0.14 | 31.20 ± 0.18 | 51.42 ± 0.92 |
| 0.3 | 18.17 ± 0.21 | 39.40 ± 0.21 | 62.81 ± 0.51 |
| 0.4 | 23.15 ± 0.16 | 42.16 ± 0.30 | 69.21 ± 0.11 |
| 0.5 | 31.12 ± 0.20 | 55.02 ± 0.12 | 81.02 ± 0.72 |

**Table 3:** Phytochemical screening of ethanol extract of seeds of *Nigella sativa* (*Ns*EE)

| S.no | Phytochemical tests | *Ns*EE |
|---|---|---|
| 1 | Liebermann– Burchad test(Steroid) | + |
| 2 | Noller's test (Triterpenoid) | + |
| 3 | Shinoda test (Flavonoid) | + |
| 4 | Furan test (Furanoid) | – |
| 5 | Coumarin test | – |
| 6 | Sugar test | – |
| 7 | Quinone test | – |
| 8 | Saponin test | – |
| 9 | Acid test | – |
| 10 | Tannin test | + |
| 11 | Phenol test | + |
| 12 | Alkaloid test | + |

**Table 4:** Phytochemicals present in ethanol extract of seeds of *Nigella sativa*

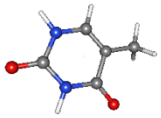
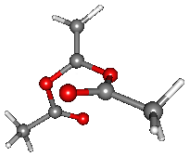
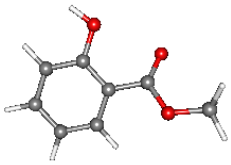
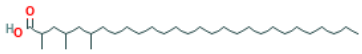
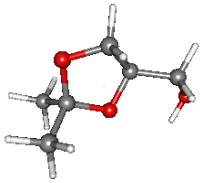
| S. No | Compound Name | Retention Time | Molecular Formula | Compound structure |
|---|---|---|---|---|
| 1 | Thymine | 126 | $C_5H_6O_2N_2$ |  |
| 2 | Vinylidene diacetate | 144 | $C_6H_8O_4$ |  |
| 3 | Methyl salicylate | 152 | $C_8H_8O_3$ |  |
| 4 | D-Mycoceranic Acid; | 466 | $C_{31}H_{62}O_2$ |  |
| 5 | Palmitic acid | 256 | $C_{16}H_{32}O_2$ |  |
| 6 | 1,10-Decanediol | 174 | $C_{10}H_{22}O_2$ |  |
| 7 | Dioxolan | 132 | $C_6H_{12}O_3$ |  |
| 8 | 1-Heptatriacontanol | 536 | $C_{37}H_{76}O$ |  |

**Fig 1:** *Cotylophoron cotylophorum*

**Fig 2:** Seeds of *Nigella sativa*

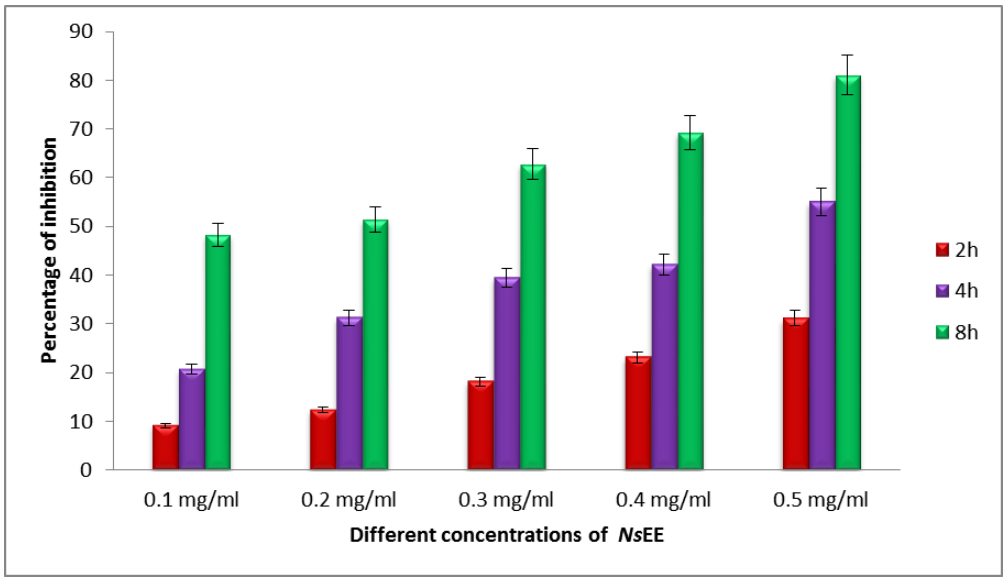



**Fig 3:** Effect of *Ns*EE on motility of *C. cotylophorum*

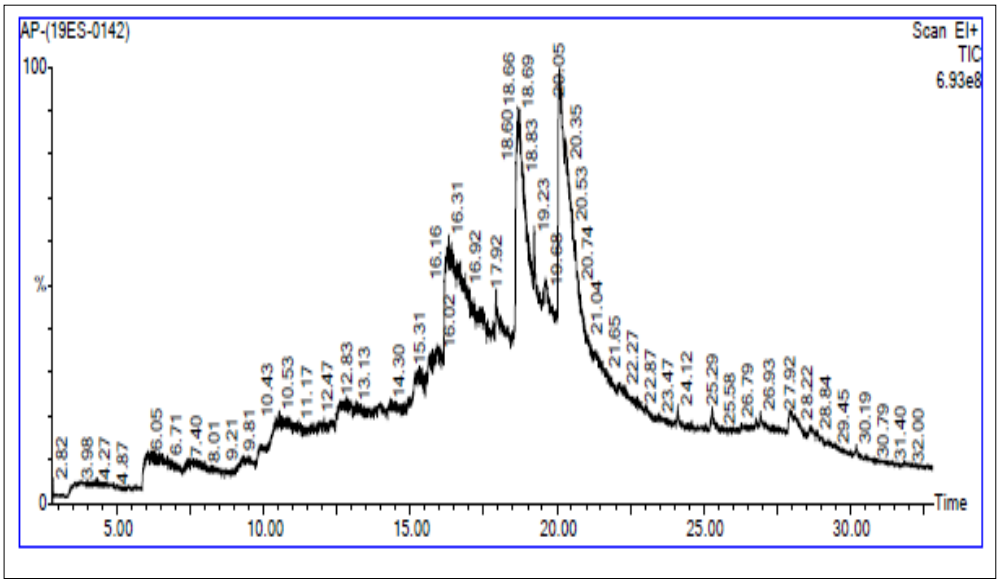



**Fig 4:** GC-MS chromatogram of ethanol extract of seeds of *Nigella sativa*

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