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Experimental Investigation of the Anti-Root-Knot Nematode Activity of Vidhara Leaf Extract (Argyreia Nervosa) in Pomegranate from Maharashtra State

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Abstract

Pomegranate (*Punica granatum* L.) is an economical and commercial crop of tropical and subtropical India. Among the plant-parasitic nematodes, root-knot nematode (*Meloidogyne* spp.) is considered to be an economically important pest of pomegranate cultivation in Maharashtra. Almost all pome orchards are noticed to be infested with this nematode and incur a huge monetary loss to the growers. Furthermore, nematode parasitism has observed to increase the tree's susceptibility to soil-borne pathogens and induce wilt, subsequently tree succumb to death. Nematicides have been recommended so far but have limited use as these nematicides are costlier to the growers, toxic to the environment and polluting bio flora and fauna. In the present study, the root samples of nematode infested pome plants were collected washed and females were isolated from galls. From the perineal pattern, the nematode species is identified as *M. javanica*. An aqueous leaf extract of Vidhara plant has assessed for its anti-nematode property against root-knot nematode. The leaves of Vidhara were harvested and dried into the oven at 70℃ for 3 days and powder prepared. A sample (5g) of powder was crushed into distilled water and filtered, centrifuged and the clear aqueous suspension was taken for study. The bio efficacy study showed that 46.65%, 58.3%, 69.33%, 83.45% and 90% mortality of juveniles (J2s) were noticed at 10%, 20%, 30%, 40% and 50% concentration of Vidhara leaf extract at room temperature for 48 hours of exposure. Utilization of locally available plants for nematode management would be the best strategy as eco-friendly, non-contaminating and cheap to the

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farmer. Therefore, an aqueous leaf extract of Vidhara plant holds a potential botanical for root-knot nematode management in pomegranate.

Key words: *Root-knot Nematode, Pomegranate, Bio-efficacy, Vidhara.*

INTRODUCTION

Pomegranate (*Punica granatum* L.) is a native to the Republic of Iran and belongs to the family of *Lythraceae*. It is mostly cultivated in the tropical and subtropical regions of the world *viz*., Afghanistan, Iran, Pakistan, Chine, UAE and India (National Horticulture Board of India, 2018). Globally, pome fruits are consumed in various cuisine dishes on the table and in the form of the processed products (juice, syrup, jam and wine) which have widespread delicious acceptability among the consumers. Nutritionally, the pomegranate arils are rich in Vitamins (C, E, K and A), iron, calcium and various antioxidants which helps medicinally to cure against several diseases like diabetes, cancer, stomach disorders, blood pressure, etc. India ranked the first position in pomegranate production all over the world, cultivated over an area of 2.62 lakh ha. and producing 30.36 lakh metric tons in 2018-19 (ICAR- National Research Centre on Pomegranate, Solapur 2018). The state of Maharashtra is at the top position in pomegranate production, grown in an area about 90,000 ha with the production of 9.45 lakh metric tons (National Horticultural Board of India, 2018**)**. The popular varieties of pomegranate cultivated in Maharashtra are Ganesh, Bhagwa, Arakta and Mridula. These varieties are released by Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri and ICAR-NRCP, Solapur.

The productivity has remained constant low and hindered due to uncertain meteorological situations and attack of insect pests and diseases at the farm level. Major insect pests *viz*., anar butterfly, bark eating caterpillar, whitefly, fruit borer and thrips etc. while most important diseases are bacterial blight, oily spot and Fusarium wilt etc. are observed in pome orchards.

Root-knot nematode (RKN) (*Meloidogyne* spp.) is considered to be an economically important pest of pomegranate cultivation worldwide. RKN is responsible for causing about 30 % to 40 % yield losses. The species of RKN (*M. incognita*, *M. javanica* and *M. arenaria*) are observed to be associated with pomegranate orchards in Maharashtra. The second juvenile (J2s) stages of RKN are microscopic, cylindrical and searching for the roots of plants. With the help of stylet

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(cuticular pointed tube-like structure in the head), J2 punctures the root primordial cells and takes entry into the root. Inside the roots, J2s travel intracellularly by dissolving the cell walls with help of enzymes emanated from the J2s oesophageal glands secretions during feeding. J2 selects one or few cells adjacent to the xylem and phloem (pericycle) cells of root and become motionless, sedentary and moults into subsequent generations (J3 and J4 stage) and finally into adults. The oesophageal gland secretions emitted by nematodes contain hydrolytic enzymes (tryptophan) which is a precursor of indole acetic acid (IAA) which disrupts the cell cycle mechanism and induces cell enlargement. RKN induced cells have undergoes a repetitive mitotic phage i. e. endoreduplication without cytokinesis, which establishes a large cell encompassing several nuclei (multinucleate), dense cytoplasm, full of cell organelle called as 'giant cell' that supply the food and nutrition to the feeding nematode continuously. As a result, adjacent cells of giant cells undergo rapid cell division, successively producing lump or root gall. These root galls thereby hinder the process of translocation of water and nutrient flow in the plants that consequently affect the plant vigour and withering or wilting of plants. RKN affected plants later susceptible to other soil-borne pathogens (phytopathogenic bacteria and fungi) which ultimately succumbed to the death. RKN completes its life cycle within 28 to 30 days at optimum temperature. If plenty of food is available, most of J2s moults into females which reproduces parthenogenetically and lays the eggs in a gelatinous matrix secreted by females herself as egg mass. One female can lay 300-500 eggs in one egg mass. Eggs hatch at optimum temperature and soil moisture and J2 hatch out in the search for new roots. Above-ground symptoms of RKN affected pomegranate plants seen as yellowing of leaves, wilting from top to bottom and stunted growth.

Vidhara (*Argyreia nervosa* [Burm.f.] Bojer) is a woody climber belonging to the Family of *Convolvulaceae*, commonly called samundrasokh, vidhara. The vidhara grows up to 10 M in height. The leaves pattern is an alternate, simple and has a long petiole. The leaf is 20-30 cm in length and 20-25 cm broad. The flower is large and funnels shaped and purple. The vidhara fruit is yellow-brown with smooth glow indehiscent. The vidhara plant contains various alkaloids, glycosides, flavonoids, and steroids. The leaves mostly contain kaempferol, quercetin, kaempferol 3-O-rhamnopyranoside, 7,8,3′,4′,5′-penta hydroxy flavone and 5-O-β-D-

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glucopyranoside (Jaiswal et. al. 2018).The vidhara plant shows various medicinal properties such as antioxidant, anti-inflammatory, anti-rheumatic, immunomodulatory, adaptogenic and hepatoprotective (Joseph *et. al.* 2011).

MATERIALS AND METHODS

3.1 Materials:

The present study was undertaken at the Department of Botany, Padmashri Vikhe Patil College of Arts, Science and Commerce located at Pravaranagar, Tehsil: Rahata, Dist. Ahmednagar (MS), India. Various instruments *viz*., autoclave, biological incubator (BOD), hot air oven, set of sieves and compound microscope (Olympus CX23 Upright microscope) were available in the laboratory and used whenever necessary. The chemicals *viz*., alcohol, double distilled water, acetic acid, acid fuchsin powder, sodium hypochlorite, acetone, tap water, hydrochloric acid, glycerine etc. have been used for the present experiment. Plastic wares such as tubs, beakers, micropipettes, Petri dishes etc. while glassware's such as Petri dishes, beakers, watch glass, test tubes, cavity blocks, glass bottles, conical flasks, pipettes, measuring cylinders, microscope slides, coverslips etc. were used for the present study. The photographs of the nematode were taken with a compound microscope (Olympus CX23 Upright microscope).

3.2 Methodology:

3.2.1 Soil and Root Sampling:

A random field survey was carried out in the search of root-knot nematode infested pomegranate orchards in the Nimgaonjali village (19˚34'18" N 74˚24'07" E) near Kolhar-Ghoti highway (Tehsil: Sangamner, Dist. Ahmednagar). The popular pomegranate variety (Bhagwa) cultivated over an area of 1 acre and the orchard was almost 8-9 years old. Withered and drooped pome plants were spotted in the orchard and soil sampling was done under such diseased plants. Approximately, 1 kg of soil was collected at three different directions of each tree and a depth of 15 cm, 30 cm and 40 cm with 1 to 1.5-meter-wide distance from the plant. Soil samples were gathered into polyethene bags, labelled properly and brought to the laboratory. The nematode infected roots along with galls were also collected and put into test tubes. In the laboratory, the

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soil samples were stored in the refrigerator at 4℃ while root samples were washed under tap water to remove adhered soil debris and stored in the refrigerator at 4℃.

3.2.2 Isolation of nematodes from soil samples:

The soil samples were taken out and mixed well with each other. Plant debris, sandstones, gravels and weed roots were removed from the soil samples. A composite of 250 gm soil was taken for further soil processing. In the plastic tub, soil samples were mixed with tap water where large soil lumps were crushed, mixed and stirred well, so that soil was uniformly mixed with water. The soil suspension was passed through a 20-mesh size sieve and collected into another plastic tub. The collected soil suspension was then passed into a 60-mesh sieve size and collected into another plastic tub. Finally, collected soil suspension was poured on to 325-mesh size sieve and the residue was collected into plastic beakers. Thereafter, a soft tissue paper was placed on the wire gauge supported by a Petri dish and fill with water. Soil suspension collected in the beaker was poured into a wire gauge and kept for 24 hours at room temperature for nematode isolation. The next day, nematodes were seen under the stereomicroscope (Cobb 1918).

3.2.3 Identification and staining of the nematodes:

3.2.3.1 Staining of nematode induced root galls:

The nematode infected roots along with galls were washed thoroughly under the tap to dislodge soil particles attached to the roots. The galls were immersed into 4% sodium hypochlorite solution for 5 minutes for clearing the root and washed repetitively under tap water to remove the chemical residue. A stock solution of the acid fuchsin stain was prepared with 3.5 g of acid fuchsin powder into 250 ml of glacial acetic acid and 750 ml of distilled water and stored in a glass bottle. Approximately, 20 ml of freshly prepared stain stock solution was taken into a glass beaker and boiled. Washed roots along with galls were wrapped into a muslin cloth and immersed into boiled acid fuchsin stain solution and kept for 5-6 minutes. The roots are then put on blotting paper to remove an extra stain and placed in a glycerine dish acidified with 1% HCl. Next day, the stained nematodes and females were seen under a stereomicroscope (Byrd *et. al*. 1983).

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3.2.3.2 Identification of root-knot nematode species by the perineal pattern:

The females were separated from the root galls and placed in glycerine for observation. A sharp cut was made on the posterior portion of the female with a blade and cleared for the perineal area. A temporary mount of the perineal part of the female was prepared and observed under a compound microscope for species identification. (Eisenback *et. al.* 1980)

3.2.4 Preparation of aqueous leaf extract:

On average, 10 leaves of vidhara (*Argyreia nervosa* (Burm.f.) Bojer) plants were harvested and cleaned under tap water (3 to 4 times). The leaves were then oven-dried at a temperature of 70^0C in a hot air oven for successive 3 days to remove moisture within them. The completely dried leaves were ground into sterilized mortar and pestle to prepare to a fine powder. Thereafter, 5 g of powder was added to 80 ml of distilled water and homogeneously stirred for uniform mixing. The suspension was filtered through a double-layered muslin cloth and the filtrate was collected and centrifuged at a rate of 5000 rpm for 20 minutes. The supernatant was carefully transferred to a glass bottle and stored as stock solution as aqueous leaf extract and used for further experiments (Plant extract stored at 4°C temperature in the refrigerator.) (Vinodhini *et. al.* 2019).

3.2.5 Bio efficacy studies of aqueous leaf extract of Vidhara against root-knot nematode:

The bioefficacy experiment was conducted into two parts: i.e. the effect of aqueous leaf extract of vidhara against egg hatching and juveniles (J2s) longevity. Various concentration of vidhara aqueous leaf extract (10%, 20%, 30%, 40% and 50%) were prepared with distilled water separately and experiments were designed in such way that each petri dish has 9 ml of a leaf extract from each concentration and also have 20 juveniles of root-knot nematode per Petri dish and kept for 24, 48, 72, 96 and 120 hours for incubation at room temperature. Each set of experiments have three replications and the mortality of J2s was recorded at each interval. In another experiment, egg masses were collected from galls and exposed them to the different concentrations of leaf extracts as mentioned above and observed the effect of leaf extracts on egg hatching and juvenile longevity after each interval mentioned above. Each set of experiments has three replications and data was subjected for statistical analysis.

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RESULT AND DISCUSSION

4.1 Identification and characterization of root-knot nematode species:

The females were isolated from galls and perineal pattern were observed under the microscope. We observed the ridges in perineum run the entire width of the pattern, but gradually disappear near the tail terminus. The dorsal arch is low and rounded to high and squarish and often contains a whorl in the tail terminal area. The lateral lines were distinct and the striae are smooth to slightly wavy, and some striae may bend toward the vulval edge that confirms the nematode species is *Meloidogyne javanica* (Fig. 1) (Eisenback et. al. 1980).

4.2 Bio efficacy studies of aqueous leaf extract of vidhara against J2s of *Meloidogyne javanica*:

To assess the nematocidal effect of aqueous vidhara leaf extract, various concentrations (10%, 20%, 30%, 40% and 50%) were prepared with dilution factor while untreated control was kept as check with only distilled water and observations were recorded on juvenile mortality after 24, 48, 72, 96 and 120 hours after nematode inoculation. We noticed that the nematocidal effect of vidhara leaf extract was found to be effective to cause juvenile mortality, however, the increasing concentration of leaf extract and exposure period was observed to have higher numbers of juvenile's mortality.

Table No. 1 Per cent juvenile mortality (%) after exposure to various concentrations of aqueous leaf extract of vidhara at different exposure periods

Concentrations / Exposure Time (h)	Untreated control	10%	20%	30%	40%	50%
24 h	0	23.44	33.33	44.33	53.65	59.33
48 h	0	46.65	58.33	69.33	83.45	90
72 h	0	61.65	71.65	90.33	96.65	100
96 h	0	83.33	95.62	100	100	100
120 h	2.21	96.15	100	100	100	100

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(Values are in percentage % and mean of three replications of each set of the experiment)

4.3 Bio efficacy studies of aqueous leaf extract of vidhara against egg hatching of *M.javanica*:

Table No. 2: Per cent egg hatching (%) of *M. javanica* at a various concentration of aqueous leaf extract of vidhara at different exposure periods

Concentrations / Exposure Time (h)	Untreated control	10%	20%	30%	40%	50%
24 h	21.71	17.96	15.59	14.11	10.36	8.19
48 h	38.20	29.41	26.06	21.81	19.14	10.66
72 h	42.56	39.58	31.09	27.54	21.81	16.48
96 h	50.34	48.96	47.58	32.47	28.82	22.30
120 h	63.77	61.99	59.52	42.05	33.36	30.01

(Values are in percentage % and the mean of three replications of each set of the experiment)

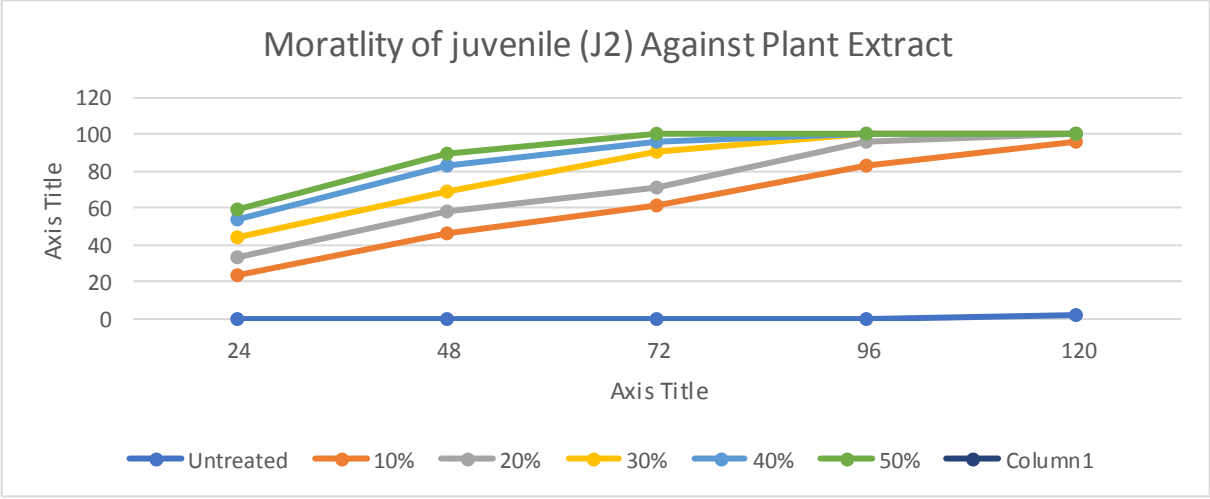

Figure:1 Graphical representation on juvenile mortality of *M. javanica* due to nematocidal effect of aqueous leaf extract of Vidhara plant

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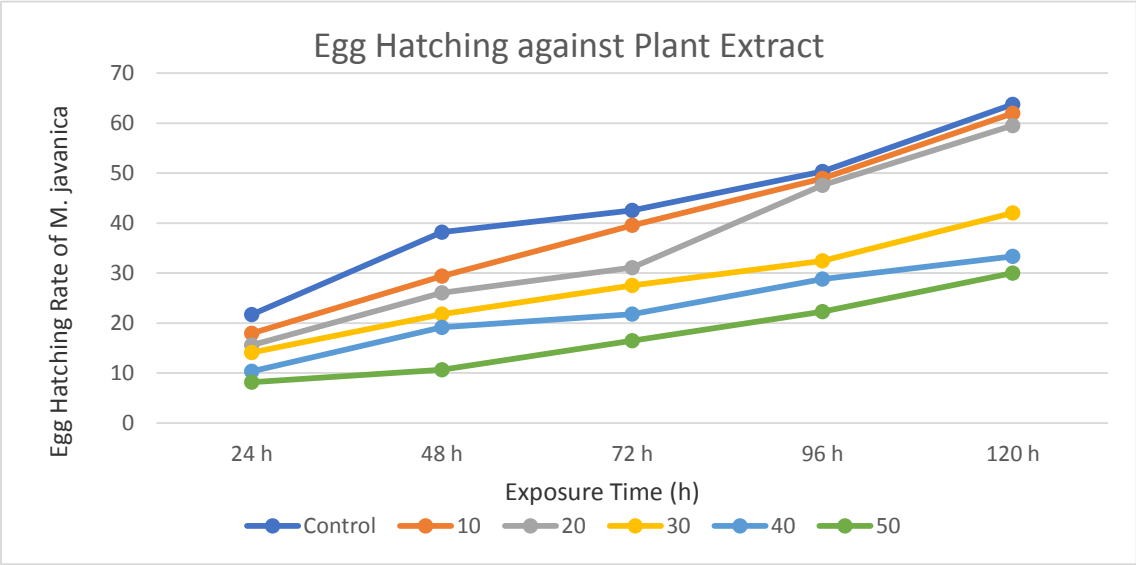

Figure: 2 Graphical representation on Egg Hatching of *M. javanica* due to nematocidal effect of aqueous leaf extract of Vidhara plant

DISCUSSION

Root-knot nematodes (RKNs) (*Meloidogyne* spp.) are the most notorious pests of fruits and vegetable crops in India causing ginormous monetary loss to the farmers. RKNs attacks a wide range of host plants from monocot to dicotyledons groups, thus rotation with crops as an agronomic strategy for nematode management found to be ineffective. Use of chemical nematicides has suggested so far controlling the nematode population, but due to their hazardous effects on the biotic environment, the extensive application of nematicides have restricted or banned. Furthermore, nematode parasitism has found to aggravate the infestations of soil-borne pathogens, thereby concomitantly intensify the wilting. As an alternative, there are a lot of scopes to use botanicals (extracts of locally available plants) for nematode management as these botanicals and their products are eco-friendly, non-contaminating and easily available to the growers. In this connection, the present study was carried out to investigate the nematocidal properties of vidhara plants against RKNs infesting pomegranate under laboratory conditions.

In the first objective, the exploratory survey was conducted in diseased and nematode infested pomegranate orchard at specified locations and sampling (root and soil) was done. The females

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were isolated from nematode induced galls and stored in glycerine for characterization. The perineal pattern confirms *M. javanica* species. At the same time, egg masses were detached from root galls and put for incubation at 28^0C for 2 days. The hatched juveniles were collected and made aliquot for bioassay studies.

We have used the various concentrations of vidhara leaf extracts (10%, 20%, 30%, 40%and 50%) to assess their anti-nematode properties under laboratory conditions. By a standard protocol mentioned in materials and methods chapter, a water-soluble or aqueous suspension of vidhara leaf extract was prepared and maintained as a stock solution. We tested all concentrations against juveniles and eggs of *M. javanica* at various exposure periods (from 24 h- to 120 h) and compared with untreated control. We found that almost all treatments were effective to cause juveniles mortality and inhibited the egg hatching, however, 50% concentration of the extract was recorded to be best which recorded 100% juvenile mortality within 72 h exposure period and at the same concentration, the rate of egg hatching was 30% after 120 hours of exposure.

Our findings are concomitant with the results of Vinodhini *et. al*. (2019) who showed 100% concentration of asparagus, hibiscus, jasmine and ixora leaf extract observed to cause 90.2%, 74.9%, 50.4% and 35.4% juvenile mortality of M. incognita in 48 hours of exposure. Azhagumurugan and Rajan (2014) showed the leaf extract of nilkumil (*G. asiatica*) having concentrations of 5 ppm to 25 ppm to cause 27% juvenile mortality of *M. incognita* in 24 hours of exposure. Rehman *et. al.* (2012) showed the S/2 concentration of *Ageratum conyzoides* to induce 59.5% nematode mortality and S/2 concentration of *Coccinia grandis* to cause 42.1% nematode mortality. As well as, the same S/2 concentrations of *Ageratum conyzoides* and *Coccinia grandis* extract inhibited 88.82% and 81.76% egg hatching of *Meloidogyne* spp. Nimbalkar and Rajurakar (2009) observed the anti-nematode and hatching inhibition properties of siam weed, castor bean, lemongrass and neem against *M. incognita*. The 20% concentration of siam weed extract to cause 98.8% juvenile mortality and 92.5% inhibited the egg hatching in 24 hours of exposure, 20% neem extract kills 89% of juveniles and inhibited 88.7% egg hatching, 10% concentration of castor bean extract induced 23.6% juvenile mortality and inhibited 58.5%

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of egg hatching whereas 20% of lemongrass extract kills 26.8% juveniles and inhibited 76.1% of egg hatching.

By looking the results and various researchers and our findings, we could opine that the leaf extract of vidhara plant have anti-nematode and hatching inhibitory properties which have a drastic influence on nematode biology and life cycle, thereby affecting nematode parasitism. However, the variation in causing juvenile mortality and inhibition of egg hatching might be due to the dose/concentration and exposure period. Higher the dose with a longer exposure period has found to be effective in influencing nematode parasitism. Our findings also paved the way for identification of nematocidal compounds present in vidhara plant as their leaves are rich in 1-triacontanol, epifriedelinol acetate, epifriedelinol, β-sitosterol, quercetin, flavonoids, quercetin and kaempferol together with the glycoside kaempferol-3-o-lrhamnopyranoside. These compounds or their combination might act as nematocidal or egg hatching inhibitor which needs to study in detail.

SUMMARY AND CONCLUSION

Our findings showed that with 10%, 20%, 30%, 40% and 50% concentrations, the juvenile mortality were recorded from 25%, 33.3%, 48.3%, 56.65% and 58.33, respectively after 24 hours of exposure. With the same concentrations, 46.6%, 55%, 98.3%, 80% and 91.6% juvenile mortality was monitored after 120 hours of exposure. In other experiments, with the same concentrations, the egg hatching inhibition was studied. At 50% concentration significant (30%) egg hatching inhibited after 120 hours of the exposure period. Therefore, present study under investigation could deduce that leaf extract of vidhara plant have anti-nematode and egg hatching inhibition properties which might affect nematode biology, life cycle and thereby influence nematode parasitism. In future, the role of individual or in the combination of chemical constituents of vidhara plants needs to be explored in detail for nematode management.

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