



# Efficacy of Garlic Extract on the Reproductive Parameters of Root Knot Nematode, *Meloidogyne incognita* (Kofoid and White) Chitwood

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## ABSTRACT

The present study aimed to assess the efficacy of garlic aqueous extract (GAE) on the reproduction of the root knot nematode (RKN), *Meloidogyne incognita*, in tomato plants. Four experiments were conducted during this investigation using different concentrations of GAE (50%, 75%, and 100%). The effects of GAE were evaluated on the mortality and egg hatching of *M. incognita* under *in vitro* conditions. In field conditions, GAE was applied using both root dipping and soil drenching methods. In the soil drench method, 20ml of GAE was applied around the root zone, and 1000 freshly hatched juveniles of *M. incognita* were inoculated. In the root dipping method, tomato roots were immersed in GAE for 20 min before transplantation, and then the plants were inoculated with 1000 freshly hatched juveniles. Each treatment was replicated five times, and various parameters including the number of galls, egg masses, the number of juveniles per 100g of soil, shoot and root length, weight, number of branches, leaves, and stem girth were recorded. The results showed significant juvenile mortality under *in vitro* conditions, with the highest mortality and the lowest egg hatching rates observed in the 100% solution of GAE. Furthermore, a significant reduction in RKN reproduction parameters was observed when using the soil drench application method compared to the root dipping method. The active substance in garlic extract, diallyl polysulfide, acts on cellular targets. Overall, soil drench treatment with garlic extract resulted in reduced root galling in tomato plants compared to root dipping treatment. Therefore, garlic extract could be successfully used for the management of the root knot nematode, *M. incognita* in field conditions.

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## Authors' Contribution

AJ, HA and SAK planned, designed and executed experimental work. MB and AA assisted in processing of samples. MK and AH reviewed, edited and analysed the data. AI and ME helped in writing the manuscript. All authors have read and approved the final version of the manuscript.

## Key words

Root knot, Nematode, *Meloidogyne incognita*, Diallyl polysulfide

## INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is a significant crop, yielding approximately 182.3 million tons of fruit on 4.85 million hectares annually, making it the second most important fruit and vegetable crop after potato (*Solanum tuberosum* L.). These are a valuable source of nutrients, including vitamin A, carotenoids,  $\beta$ -carotene, and lycopene, and are beneficial for reducing cardiovascular risks associated with type-2 diabetes (Shifdar *et al.*, 2011).

However, tomatoes are susceptible to a wide range of diseases, with approximately 200 diseases identified, each exhibiting unique symptoms and economic impacts. Among these, root knot nematodes (RKN) are common plant-parasites, found in soil. In England, the *Meloidogyne* species of RKN was initially observed in the roots of cucumber plants by Berkeley in 1855. RKNs disrupt the plant's ability to absorb nutrients and water, leading to stunted growth, yellowing foliage, and reduced yields.

The life cycle of RKN begins with the deposition of eggs into a gelatinous matrix that protects and holds them together (Maggenti and Fortuner, 1987). The first-stage RKN larvae (J<sub>1</sub>) shed their skins before hatching into eggs, often independently of plant root stimuli. The freshly hatched J<sub>2</sub>s move over the soil in search of roots to feed on, heading straight to the root tissue tips and entering the roots (Jones *et al.*, 2013). Once a J<sub>2</sub> locates a root, it secretes enzymes and uses its stylet to penetrate the plant's roots. The larvae's bodies undergo two additional moults, with the third and fourth juvenile stages possessing a

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functional stylet but not feeding. In the final moult, mature females form, comprising a functional stylet, median bulb, uterus, and vagina (Perry *et al.*, 2009; Jones *et al.*, 2013). Under ideal conditions, RKNs can complete their life cycle in three to four weeks, with five to eight generations reproducing in a single growing season (Noling, 2014).

In Pakistan, estimates of crop loss due to nematodes range from 5 to 20% (Maqbool *et al.*, 1988), with disease occurrence in Punjab reaching between 75 and 100% (Shahid *et al.*, 2007; Khan, 2009). In some areas, such as Okara, yield losses due to RKNs can be as high as 85.2%, with an incidence frequency of 38.89% (Hussain *et al.*, 2012). Globally, the economic impact of nematodes is substantial, with assessments reaching up to US \$78 billion (Mokrini, 2016).

To combat nematodes, various plant extracts have been used as effective methods for control. These extracts in combination with fertilizers and biopesticides are cost-effective, easy to use, environmentally friendly, and capable of improving soil health (Sultana *et al.*, 2010; Abbas *et al.*, 2023). Garlic (*Allium sativum* L.), a member of the Allium family, has been found to contain diallyl polysulfide as its active substance, which targets cellular mechanisms. This action, observed particularly in the roots, can lead to the development of resistance against RKNs. GAE has been found to have nematocidal and nematode-hatching inhibitory effects, reducing nematode infestations such as gall formation, egg masses, and hatched juveniles on tomato roots, as well as the number of juveniles in the soil. In view of above the current investigation was designed to evaluate the effectiveness of GAE in reducing juvenile mortality and egg hatching of *M. incognita*, and to assess its efficacy through soil drenching and root dipping treatments against *M. incognita* in tomato plants.

## MATERIALS AND METHODS

### *Collection and culturing of nematodes*

Diseased samples of eggplant roots and soil infested with RKNs were collected and processed for mass culturing of nematodes. Nursery raising of the tomato cultivar “Money Maker” was carried out, and after three weeks of transplantation into pots, approximately 1000 freshly hatched juveniles were inoculated around the plant rhizosphere by creating holes in each pot. For each experiment, five replications were conducted.

### *Preparation of garlic aqueous extract (GAE)*

GAE was prepared by soaking 25g of crushed garlic cloves in 1000ml of distilled water for 72 h and then filtered through Whatman no.1 filter paper. The prepared GAE was kept in sterilized vessel at 4°C temperature till

needed. The crude extract and diluted extract were used for experiment purposes at different concentrations of 50 % (v/v), 75 % (v/v), 100 % (v/v).

### *Effect of GAE on egg hatching of RKN*

Five ml of GAE was poured into Petri dishes. In separate plates having different concentration of (50%, 75%, 100%) treatments were inoculated with 50 *M. incognita* eggs. Control treatment was kept with distilled water. Data were recorded after the 3<sup>rd</sup>, 5<sup>th</sup>, and 7<sup>th</sup> days of interval after treatments applications and observed the no. of hatched eggs and calculated.

$$\text{Egg inhibition\%} = \frac{t - c}{100 - c} \times 100$$

### *Effect of GAE on juvenile mortality of RKN*

Five ml of GAE was poured into each Petri plate. In separate plates having different concentration of treatments (50%, 75%, 100%) were inoculated with 100 freshly hatched juveniles of RKN. Control treatment was kept with distilled water. The total juveniles mortality was detected 24, 48 and 72 h after the treatment using the formula:

$$\% \text{ juvenile's mortality} = \frac{\text{No. of juveniles killed}}{\text{total no. of juveniles}} \times 100$$

### *Root dipping of tomatoes seedling in GAE*

The test plant roots of tomato seedlings were dipped into 20ml of solutions of different concentrations of (50%, 75%, 100%) in GAE for 15-20 min before transplanting them into pots, filled with sterilized soil. The control plants were dipped in equal volume of water for the same period. After three weeks of transplantation, tomato seedlings were inoculated with 1000 J<sub>2</sub> of *M. incognita*. Around the plant root rhizosphere three holes was made at a distance of 2cm away from tomato plant. Inoculation was done by adding water suspension containing juveniles into these holes and covering it with soil to avoid desiccation.

### *Soil drenching of tomato seedlings by GAE*

The soil around the root zone was drenched with 20 ml of the extract at various concentrations. After one week, inoculation of 1000 freshly hatched juveniles into the tomato plants was conducted by creating three holes around the plant stem. The roots were then covered with topsoil. The same process was performed for the control plants using water. Plant growth parameters and nematode reproduction parameters were recorded.

After 15 days of treatment application, a second application of garlic extract was administered to the soil-drenched plants. Experiments were repeated to confirm the results.

*Data recorded*

After 60 days, harvesting was done. Following parameters were measured: shoot and root length, stem girth, fresh shoot and root weight, number of leaves per plant, number of branches per plant, number of flowers per plant, number of fruits per plant, egg mass of *M. incognita*, number of root galls, number of juveniles, and number of females.

**RESULTS***Effect of GAE on egg hatching and juvenile mortality*

The efficacy of GAE was observed on egg hatching 3<sup>rd</sup>, 5<sup>th</sup>, and 7<sup>th</sup> days under *in vitro* conditions at different concentrations (Table I). The highest percentage of egg hatching was observed in the control plate with water, while the lowest percentage of egg hatching was observed in the 100% crude extract of garlic after 3 days.

**Table I. Effect of garlic extract (GAE) on egg hatching and juveniles mortality of *M. incognita* under *in vitro* condition.**

GAE treatment	Egg hatching			Juveniles mortality after		
	3 <sup>rd</sup> day	5 <sup>th</sup> day	7 <sup>th</sup> day	24 h	48 h	72 h
Control	26.24 <sup>d</sup>	35.14 <sup>b</sup>	48.03 <sup>a</sup>	0 <sup>j</sup>	0 <sup>j</sup>	0 <sup>j</sup>
50% GAE	16.50 <sup>f</sup>	25.88 <sup>d</sup>	31.67 <sup>e</sup>	7 <sup>i</sup>	19 <sup>g</sup>	46 <sup>d</sup>
75% GAE	11.03 <sup>g</sup>	20.33 <sup>e</sup>	28.73 <sup>cd</sup>	12 <sup>h</sup>	22 <sup>f</sup>	64 <sup>b</sup>
100% GAE	5.09 <sup>h</sup>	12.11 <sup>g</sup>	19.03 <sup>cd</sup>	24 <sup>e</sup>	48 <sup>c</sup>	84 <sup>a</sup>

\*Mean values sharing similar letters do not differ significantly  $\alpha = 0.05$

The efficacy of GAE was examined regarding juvenile mortality under *in vitro* conditions at different concentrations (Table I). Juvenile mortality was recorded after 24 h, 48 h, and 72 h. An increase in juvenile mortality was observed with 100% crude garlic extract. At lower concentrations of GAE, specifically 50%, the minimum number of juvenile mortalities were observed. No mortality was observed in the control plate containing only water.

*Effect of GAE on RKN reproduction and plant growth parameters by soil drench method**First application*

Table II shows the effect of GAE on RKN reproduction parameters and plant growth parameters by the soil drench method. A significant reduction in the number of galls, the number of juveniles ( $J_2$ ), the number of egg masses, and the number of females was observed due to the application of GAE at different concentrations. The maximum number of galls was observed in diseased control plants where no treatment was applied. In healthy control plants, no galling

was observed. A significant reduction in the number of juveniles ( $J_2$ ) was observed due to the application of GAE at different concentrations. At higher concentrations of GAE, the number of juveniles per 100g of soil was reduced compared to diseased control plants where the maximum number of juveniles per 100g of soil was present. At a higher concentration, the minimum number of egg masses were obtained compared to diseased plants where the maximum number of egg masses were observed. A higher concentration of GAE minimized the number of nematode females compared to diseased plants where a higher number of females were observed. Therefore, a higher concentration of GAE was effective in controlling nematode reproduction parameters.

**Table II. Effect of first and second application of aqueous garlic extract (GAE) on root knot nematode reproduction parameters by soil drench method.**

GAE treatment	No. of galls	No. of $J_2$ / 100g of soil	No. of egg masses	No. of females
<b>First application</b>				
Control (healthy)	0 <sup>e</sup>	0 <sup>e</sup>	0 <sup>e</sup>	0 <sup>e</sup>
Control (diseased)	180.39 <sup>a</sup>	1047.43 <sup>a</sup>	167.49 <sup>a</sup>	186.31 <sup>a</sup>
50% GAE	73.3 <sup>b</sup>	487.18 <sup>b</sup>	68.11 <sup>b</sup>	77.37 <sup>b</sup>
75% GAE	54.51 <sup>c</sup>	321.45 <sup>c</sup>	42.18 <sup>c</sup>	58.43 <sup>c</sup>
100% GAE	43.56 <sup>d</sup>	202.33 <sup>d</sup>	32.35 <sup>d</sup>	47.38 <sup>d</sup>
<b>Second application</b>				
Control (healthy)	0 <sup>e</sup>	0 <sup>e</sup>	0 <sup>e</sup>	0 <sup>e</sup>
Control (diseased)	175.33 <sup>a</sup>	1040.2 <sup>a</sup>	159.27 <sup>a</sup>	177.25 <sup>a</sup>
50% GAE	69.12 <sup>b</sup>	481.15 <sup>b</sup>	61.01 <sup>b</sup>	70.46 <sup>b</sup>
75% GAE	46.39 <sup>c</sup>	318.37 <sup>c</sup>	38.24 <sup>c</sup>	52.31 <sup>c</sup>
100% GAE	37.13 <sup>d</sup>	198.52 <sup>d</sup>	27.41 <sup>d</sup>	40.35 <sup>c</sup>

\*Mean values sharing similar letters do not differ significantly  $\alpha = 0.05$

There was a significant effect on the plant growth parameters of tomato plants due to the application of GAE as a soil drench method at various concentrations (Table III). A higher shoot length of tomato plants was observed in healthy control plants compared to diseased control plants where the minimum shoot length was recorded. Significantly, the shoot weight of tomato plants was recorded as the maximum in healthy control plants compared to disease control plants where the minimum shoot weight was observed. The root length of tomato plants was higher in healthy control plants compared to diseased control plants where the minimum root length was observed. As the concentrations of GAE were increased, the root weight of tomato plants decreased because the number of galls was reducing due to the nematicidal activity of GAE. The maximum number of

**Table III. Effect of first and second application of GAE on plant growth parameters by soil drench method**

GAE treatment	Shoot length	Shoot weight	Root length	Root weight	Number of leaves	Number of branches	Stem girth
<b>First application</b>							
50% GAE	31.13 <sup>c</sup>	9.63 <sup>cd</sup>	9.75 <sup>b</sup>	6.17 <sup>ab</sup>	7.58 <sup>c</sup>	5.56 <sup>bc</sup>	1.46 <sup>c</sup>
75% GAE	33.13 <sup>bc</sup>	11.40 <sup>bc</sup>	10.39 <sup>b</sup>	5.15 <sup>bc</sup>	8.04 <sup>bc</sup>	6.27 <sup>abc</sup>	1.55 <sup>c</sup>
100% GAE	34.32 <sup>ab</sup>	12.62 <sup>ab</sup>	11.62 <sup>ab</sup>	4.48 <sup>c</sup>	9.51 <sup>b</sup>	7.47 <sup>ab</sup>	1.82 <sup>b</sup>
Control (healthy)	36.5 <sup>a</sup>	13.82 <sup>a</sup>	12.79 <sup>a</sup>	4.92 <sup>bc</sup>	11.63 <sup>a</sup>	8.12 <sup>a</sup>	2.16 <sup>a</sup>
Control (diseased)	19.52 <sup>d</sup>	8.16 <sup>d</sup>	6.67 <sup>c</sup>	7.0 <sup>a</sup>	4.34 <sup>d</sup>	5.34 <sup>c</sup>	0.92 <sup>d</sup>
<b>Second application</b>							
50% GAE	32.03 <sup>b</sup>	10.14 <sup>c</sup>	10.53 <sup>c</sup>	7.17 <sup>ab</sup>	8.55 <sup>b</sup>	4.47 <sup>b</sup>	1.17 <sup>bc</sup>
75% GAE	34.02 <sup>ab</sup>	12.02 <sup>b</sup>	11.21 <sup>bc</sup>	6.31 <sup>bc</sup>	9.34 <sup>b</sup>	5.76 <sup>ab</sup>	1.36 <sup>b</sup>
100% GAE	35.04 <sup>ab</sup>	13.41 <sup>ab</sup>	12.39 <sup>ab</sup>	5.39 <sup>c</sup>	10.47 <sup>ab</sup>	6.49 <sup>ab</sup>	1.54 <sup>b</sup>
Control (healthy)	37.03 <sup>a</sup>	14.55 <sup>a</sup>	13.52 <sup>a</sup>	5.61 <sup>c</sup>	12.46 <sup>a</sup>	7.46 <sup>a</sup>	2.18 <sup>a</sup>
Control (diseased)	19.09 <sup>c</sup>	8.10 <sup>d</sup>	7.30 <sup>d</sup>	8.29 <sup>a</sup>	5.69 <sup>c</sup>	4.38 <sup>b</sup>	0.96 <sup>c</sup>

\*Mean values sharing similar letters do not differ significantly  $\alpha = 0.05$

leaves on tomato plants was observed in healthy control plants compared to diseased control plants where the minimum number of branches were recorded. The stem girth of tomato plants was maximum in healthy control plants compared to diseased control plants where the minimum stem girth was observed. As the concentrations of GAE were increased, the plant growth parameters, including shoot length and weight, root length and weight, stem girth, number of leaves, and branches, also increased due to the plant growth-promoting factors present in garlic extract.

#### *Second application*

A significant reduction in the number of galls, the number of juveniles ( $J_2$ ), the number of egg masses, and the number of females was observed due to the 2<sup>nd</sup> application of GAE at different concentrations (Table II). The maximum number of galls was observed in diseased control plants. The number of egg masses was significantly reduced by the 2<sup>nd</sup> application of GAE, as a higher concentration of GAE resulted in a minimum number of egg masses compared to diseased plants where the maximum number of egg masses was observed. A significant reduction in the number of females was also observed after the 2<sup>nd</sup> application of GAE, especially at a higher concentration.

There was a significant effect on the plant growth parameters of tomato plants due to the 2<sup>nd</sup> application of GAE as a soil drench method at various concentrations (Table III). The maximum shoot length was observed in healthy control plants compared to diseased control plants, where the minimum shoot length was recorded. The

maximum number of leaves on tomato plants was observed in healthy control plants compared to diseased control plants, where the minimum number of branches was recorded. The stem girth of tomato plants was maximum in healthy control plants compared to diseased control plants, where the minimum stem girth was observed. As the concentrations of the 2<sup>nd</sup> application of GAE were increased, the plant growth parameters also increased.

**Table IV. Effect of GAE on root RKNs reproduction parameters by root dipping method.**

GAE treatment	No. of galls	No. of $J_2$ /100g of soil	No. of egg masses	No. of females
Control (healthy)	0e	0e	0e	0e
Control (diseased)	191.08 <sup>a</sup>	1124.41 <sup>a</sup>	183.19 <sup>a</sup>	193.08 <sup>a</sup>
50% GAE	93.94 <sup>b</sup>	494.13 <sup>b</sup>	89.25 <sup>b</sup>	98.02 <sup>b</sup>
75% GAE	57.16 <sup>c</sup>	375.13 <sup>c</sup>	51.44 <sup>c</sup>	64.49 <sup>c</sup>
100% GAE	44.25 <sup>d</sup>	221.55 <sup>d</sup>	40.22 <sup>d</sup>	51.12 <sup>d</sup>

\*Mean values sharing similar letters do not differ significantly  $\alpha = 0.05$

#### *Effect of GAE on RKN reproduction and plant growth parameters by root dipping method*

Table IV shows the effect of GAE on RKN reproduction parameters by the root dipping method. A significant reduction in the number of galls was observed due to the application of GAE at different concentrations. The maximum number of galls was observed in diseased control plants. There was a significant reduction in the number of juveniles ( $J_2$ ) due to the application of GAE

**Table V. Effect of GAE on plant growth parameters by root dipping method.**

GAE treatment	Shoot length	Shoot weight	Root length	Root weight	No. of leaves	Number of branches	Stem girth
Control (healthy)	38.35 <sup>a</sup>	14.0 <sup>a</sup>	13.17 <sup>a</sup>	4.43 <sup>c</sup>	11.99 <sup>a</sup>	9.78 <sup>a</sup>	2.12 <sup>a</sup>
Control (disease)	21.39 <sup>c</sup>	10.56 <sup>b</sup>	8.01 <sup>d</sup>	7.77 <sup>a</sup>	5.38 <sup>d</sup>	6.23 <sup>c</sup>	0.89 <sup>c</sup>
50% GAE	25.08 <sup>d</sup>	7.11 <sup>c</sup>	9.26 <sup>cd</sup>	6.64 <sup>ab</sup>	8.1 <sup>c</sup>	6.65 <sup>bc</sup>	1.41 <sup>b</sup>
75% GAE	30.35 <sup>c</sup>	8.26 <sup>bc</sup>	11.07 <sup>bc</sup>	5.21 <sup>bc</sup>	9.05 <sup>bc</sup>	7.52 <sup>bc</sup>	1.66 <sup>ab</sup>
100% GAE	35.0 <sup>b</sup>	9.15 <sup>bc</sup>	12.13 <sup>ab</sup>	3.47 <sup>c</sup>	10.74 <sup>ab</sup>	8.35 <sup>ab</sup>	1.83 <sup>ab</sup>

\*Mean values sharing similar letters do not differ significantly  $\alpha = 0.05$

at higher concentrations. The number of egg masses was significantly reduced by the application of aqueous garlic extract at higher concentrations, with the minimum number of egg masses obtained compared to diseased plants where the maximum number of egg masses was observed. Higher concentrations of GAE also reduced the number of nematode females compared to diseased plants, where a higher number of females were observed.

There was a significant effect on plant growth parameters in tomato plants due to the application of aqueous garlic extract by the root dipping method at different concentrations (Table V). The shoot weight of tomato plants was significantly higher in healthy control plants compared to diseased control plants, where the minimum shoot weight was observed. The root length of tomato plants was greater in healthy control plants compared to diseased control plants, where the minimum root length was observed. The maximum number of leaves on tomato plants was observed in healthy control plants compared to diseased control plants, where the minimum number of branches was recorded. The stem girth of tomato plants was maximum in healthy control plants compared to diseased control plants, where the minimum stem girth was observed. As the concentrations of aqueous garlic extract increased, the stem girth also increased.

## DISCUSSION

The present study was conducted to assess the efficacy of GAE on the plant growth parameters and reproduction parameters of RKNs, specifically *Meloidogyne incognita*, in tomato plants. Severe infections resulting from nematode attacks lead to a significant decrease in plant height, crop losses, and reduced product quality. Due to their broad host range, RKN are challenging to manage, causing worldwide losses estimated at up to US \$78 billion (Mokrini, 2016). RKNs are known to cause damage ranging from 50% to 85% in tomato production.

In this study GAE with laboratory test at different concentrations (50%, 75%, and 100%) inhibited the egg

hatching of *M. incognita* compared to the control treatment. These findings are in line with those of Adomako and Kwoseh (2013), who observed increased egg hatching in control treatments compared to 100% crude garlic extract, where less egg hatching occurred. Lower concentrations of the treatments were less effective compared to higher concentrations in all treatments examined. These results align with those of Adegbite and Adesiyun (2005) suggesting that the inhibitory impact of botanicals may be attributed to the presence of compounds in the extract with larvicidal and ovicidal characteristics. The results revealed that the maximum juvenile mortality was observed at a 100% concentration of GAE, while the minimum egg hatching was observed at the same concentration.

Under field conditions, GAE showed a significant impact on nematode reproduction, and plant parameters indicated increased growth soil drenching and root dipping methods. GAE has been reported to contain complex growth-promoting substances such as vitamins, saponins, carbohydrates, proteins, alkaloids, and sugars like fructose (Martins *et al.*, 2016). A well-maintained dose of these compounds actively participates in promoting the growth of recipient plants.

The shoot length of tomato plants was maximized in the 100% GAE, which proved to be effective. Maximum shoot weight was observed at higher concentrations of GAE. Increased root length was observed at higher concentrations of GAE, while minimum root weight was detected at the 100% concentration. These nematodes deprive plants of nutrition by forming galls on roots and colonizing root tissue (Bird, 1974). The minimum number of galls was observed in the 100% GAE treatment, along with the minimum number of egg masses. Additionally, the minimum number of juveniles per 100g of soil was observed in the 100% GAE treatment. In contrast to nematode-infested plants, soil treatment with GAE reduced the nematode root gall index and increased the activity of catalase and B-1,3 glucanase enzymes in tomato plants, as stated by Abd-Elgawad *et al.* (2009). The active substance in garlic extract is diallyl polysulfide, and its mode of

action targets cells. This pattern was consistent in the roots, indicating the development of resistance to RKN. GAE caused no phytotoxicity in plants (Sukul *et al.*, 1974).

## CONCLUSION

The most beneficial impact of GAE was a considerable reduction in the galling index and other reproductive factors, such as egg mass count, number of females, and number of juveniles. The results of this investigation demonstrate that GAE has great potential for nematode control and significantly enhances the growth of tomato plants.

The soil drenching application method was more effective compared to the root dipping method. Therefore, the crude aqueous garlic extract has proven to be effective for the management of root-knot nematodes under both *in vitro* and field conditions.

## DECLARATIONS

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### Statement of conflict of interest

The authors have declared no conflict of interest.

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