



***In Vitro* Evaluation of Nematicidal Potential and Phytochemical Profile of Four Piper Species against Root-Knot Nematode (*Meloidogyne incognita*)**

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Abstract

Meloidogyne incognita, one of the plant-parasitic nematodes, causes significant yield losses in various cultivated plants. Dependence on chemical nematicides encourages the development of plant-based alternatives that are more sustainable. This study evaluated the nematicidal activity and phytochemical profile of extracts from the leaves of 4 *Piper* species (*P. aduncum*, *P. betle*, *P. nigrum*, and *P. retrofractum*) against egg hatching and second-stage juvenile (J2) mortality of *M. incognita*, and determined their median lethal concentrations (LC₅₀). A completely randomized design was used with 6 extract concentrations (0, 2, 4, 6, 8, and 10 ml l⁻¹) and 5 replications. Gas chromatography–mass spectrometry (GC–MS) analysis showed distinct phytochemical profiles: *P. aduncum* was dominated by dillapiole (62.30%), *P. betle* and *P. nigrum* by phenolic compounds (62.70% and 24.44%, respectively), and *P. retrofractum* by fatty acids (17.97%). A significant interaction between species and extract concentration affected egg-hatching inhibition and J2 mortality. At 10 ml l⁻¹, *P. betle* and *P. aduncum* inhibited egg hatching by 95.20% and 92.40% and caused J2 mortality of 97.60% and 95.20%, respectively. At 120 hours, the LC₅₀ values were 2.27 ml l⁻¹ for *P. betle* and 2.61 ml l⁻¹ for *P. aduncum*, compared with 2.83 and 3.00 ml l⁻¹ for *P. nigrum* and *P. retrofractum*, respectively. These results identify *P. betle* and *P. aduncum* as the most active extracts under the tested *in vitro* conditions and support their prioritization for further validation.

Keywords: biological activity; leaf extract; phytochemicals; plant-parasitic nematodes; toxicity

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INTRODUCTION

The root-knot nematode, *Meloidogyne incognita*, is an economically important plant-parasitic nematode with a broad geographic distribution and can infect a wide range of cultivated crops (Lee et al., 2021). This cosmopolitan species possesses an extensive host range of approximately 2,000 plant species (Elling, 2013; Neeraj et al., 2019). As a sedentary endoparasite, *M. incognita* infects root tissues and

completes most of its life cycle within the host plant (Shahid et al., 2024).

Infection is characterized by root galling or swelling, which severely impairs host physiological functions (Philbrick et al., 2020). Nematodes penetrate root tissues using their stylets, disrupting the vascular system and hindering water and nutrient absorption from the soil (Asyiah et al., 2021). Consequently,

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this damage leads to stunted growth, wilting, and substantial yield losses (Janati et al., 2018; Collett et al., 2021). In tomato crops, *M. incognita* infestations have been reported to cause yield losses of up to 68.3% (Khotimah et al., 2020). Given its severe impact on plant growth and productivity, *M. incognita* is a major pest that requires priority in crop management, particularly in horticultural production.

Various strategies have been developed to manage *M. incognita*, predominantly relying on synthetic nematicides. Synthetic nematicides are widely used due to their efficacy in suppressing nematode populations and their convenience in agricultural practices (Nishat et al., 2022). However, intensive and continuous application poses critical challenges, including environmental contamination, chemical residues in soil and agricultural produce, adverse effects on non-target organisms, and risks to human health (Nicolopoulou-Stamati et al., 2016; Desaegeer et al., 2020). These drawbacks necessitate the development of alternative management approaches aligned with sustainable agriculture principles.

One widely studied approach is the utilization of plants as sources of bioactive compounds for nematode management. Plant extracts contain diverse secondary metabolites that exhibit nematicidal activity through multiple biological mechanisms, presenting strong potential as botanical nematicides (Ntalli and Caboni, 2012). Several classes of secondary metabolites, such as alkaloids, phenolics, flavonoids, tannins, saponins, and terpenoids, have been documented to suppress nematodes by inhibiting egg hatching, disrupting physiological processes, and increasing juvenile mortality (Caboni et al., 2013; Tariq et al., 2022). Therefore, exploring plant species with high nematicidal activity represents a viable avenue for identifying novel botanical management agents against *M. incognita*.

The genus *Piper* (Piperaceae) comprises a species-rich plant group with high secondary metabolite diversity and strong potential as a source of bioactive compounds. Various *Piper* species produce secondary metabolites, including phenylpropanoids, alkaloids, flavonoids, terpenoids, and amides, which are associated with diverse biological activities, such as insecticidal, antimicrobial, and antiparasitic properties (Scott et al., 2008). This chemical diversity provides a rational basis for exploring *Piper* species as botanical sources for pest and pathogen management.

For instance, *Piper aduncum* is rich in phenylpropanoids, particularly dillapiole, whereas *Piper nigrum* synthesizes piperamide alkaloids, including piperine, alongside other secondary metabolites (Salehi et al., 2019; Fazolin et al., 2022). *Piper betle* is well known for accumulating phenolic compounds and phenylpropanoids, while *Piper retrofractum* contains amides, alkaloids, lignans, and phenylpropanoids with reported bioactivities (Salehi et al., 2019; Tang et al., 2019).

The nematicidal potential of *Piper* species has been documented in several studies. Soil amendment with organic matter from *P. aduncum* suppressed nematode populations and root galling in host plants (Singh and Khurma, 2008). Likewise, *P. betle* extracts reduced root-knot nematode (*Meloidogyne* spp.) populations and suppressed reproduction when applied to soil or the rhizosphere (Wiratno et al., 2009; Dwipayana et al., 2017).

Despite these promising findings, existing evidence originates from disparate studies featuring different *Piper* species, extract types, extraction methods, assay conditions, and biological parameters. Consequently, the comparative nematicidal efficacy of different *Piper* species against *M. incognita* under standardized experimental conditions remains unresolved. Furthermore, information regarding how differences in secondary metabolite profiles correlate with species-specific variations in nematode responses remains limited.

Addressing this gap is critical, as interspecific variations in nematicidal activity may reflect not only differences in extract toxicity levels but also variations in their underlying secondary metabolite compositions. Thus, a comparative evaluation of multiple *Piper* species under unified plant material sources, testing procedures, extract concentrations, and exposure durations is required. Such an approach enables direct comparisons of extract efficacy in inhibiting egg hatching and inducing mortality in second-stage juveniles (J2) of *M. incognita*.

Combining phytochemical profiling with biological assays provides a comprehensive framework for assessing the nematicidal potential of plant extracts. Gas chromatography–mass spectrometry (GC–MS) enables the precise identification of chemical compounds in extracts, while egg hatching inhibition and J2 mortality assays allow for evaluating *M. incognita* physiological responses. Determining lethal concentrations (LC₅₀) values further provides

a quantitative measure of the extract concentration required to induce 50% mortality in J2 populations. Integrating phytochemical profiles, egg hatching inhibition, J2 mortality, and LC₅₀ values establishes a robust foundation for comparing the nematicidal activity of 4 *Piper* species under uniform *in vitro* conditions.

Based on these considerations, this study aimed to: (1) identify and compare the phytochemical profiles of leaf extracts from *P. aduncum*, *P. betle*, *P. nigrum*, and *P. retrofractum* using GC–MS analysis; (2) evaluate the *in vitro* nematicidal activity of these 4 species against *M. incognita* based on egg hatching inhibition and J2 mortality across various concentrations and exposure times; and (3) determine their respective LC₅₀ values against *M. incognita* J2. The findings are expected to provide comparative insights into the *in vitro* nematicidal efficacy of these 4 *Piper* species and elucidate the relationship between phytochemical variations and *M. incognita* biological responses, establishing a scientific foundation for future research on *Piper*-derived agents in root-knot nematode management.

MATERIALS AND METHOD

Preparation of *Piper* leaf extracts

Fresh leaves of 4 *Piper* species (*P. aduncum*, *P. betle*, *P. nigrum*, and *P. retrofractum*) were harvested from the Special Region of Yogyakarta, Indonesia. Mature, healthy, and dark-green leaves were selected to ensure optimal phytochemical content. The leaves were thoroughly washed under running tap water to remove dust, soil particles, and other surface impurities. Clean leaves were air-dried under shaded, well-ventilated conditions at room temperature (25 to 28 °C) for 7 days to prevent degradation of thermolabile compounds. After drying, the leaves were weighed, and their moisture content was calculated using the formula. The dried leaves were then ground into a fine powder using a high-speed blender and sieved through a 0.5 mm mesh to obtain a uniform particle size. The powdered samples were stored in airtight aluminum foil bags at room temperature in the dark to minimize exposure to moisture, oxygen, and light before extraction.

Extraction procedure

Each powdered leaf sample (500 g) was macerated in a sealed glass container with analytical-grade 70% methanol (1:4 w/v) at room temperature for 48 hours, as described by Handoyo (2020). Intermittent stirring helped

dissolve bioactive compounds. After 48 hours, the mixture was filtered through a Whatman No. 41 filter paper to separate the solids from the liquid. The filtrate was then concentrated under reduced pressure using a rotary evaporator (Buchi R-210) at 50 °C and 240 mbar, as described by Ayodele et al. (2020), until only a viscous extract remained.

The concentrated crude extracts were transferred into amber glass vials and stored at 4 °C until further analyses and bioassays. For *in vitro* nematicidal testing, stock extracts were diluted with sterile distilled water to prepare treatment concentrations of 0, 2, 4, 6, 8, and 10 ml l⁻¹. Each test solution was homogenized using a magnetic stirrer before use to ensure uniformity.

Mass rearing of root-knot nematode (*M. incognita*)

The root-knot nematode *M. incognita* was mass-reared from a single egg mass. The inoculum was obtained from the Nematology Sub-Laboratory, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia. Tomato (*Solanum lycopersicum*) served as the host and was grown in sterile soil in polybags. Seedlings were maintained for 1 to 3 weeks before inoculation.

A single egg mass was carefully removed from infected tomato roots and placed near the root zone of healthy tomato seedlings. The mass was positioned close to the soil surface to facilitate larval access to the roots. It was lightly covered with sterile soil to keep it in place and protect it from direct sunlight. After inoculation, the plants were maintained in a greenhouse under optimal conditions. Temperatures ranged from 25 to 28 °C, with adequate humidity and natural light to support both plant growth and nematode development. Plants were watered regularly to maintain soil moisture. Pest control was carried out mechanically when necessary. The nematode culture was maintained until sufficient populations were obtained for subsequent laboratory experiments.

Phytochemical profiling (GC–MS analysis)

Phytochemical profiling was conducted to identify secondary metabolites present in the leaf extracts of 4 *Piper* species (*P. aduncum*, *P. betle*, *P. nigrum*, and *P. retrofractum*). Extraction and analysis were performed at the Agrotropica Learning Center (AGLC) of the Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia. The chemical composition of the extracts was analyzed using

a Shimadzu QP2010 SE GC–MS system equipped with an SH-Rxi-5Sil MS capillary column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness).

Helium was used as the carrier gas at a constant flow rate of 1.0 ml minute⁻¹. The oven temperature was initially set to 100 °C and held isothermally for 5 minutes, then increased at 15 °C minute⁻¹ to 300 °C, and maintained at this final temperature for 30 minutes. The injector temperature was set to 300 °C and operated in splitless mode, while the interface temperature was maintained at 305 °C.

Mass spectra of the detected compounds were acquired under electron ionization conditions and compared with those in the National Institute of Standards and Technology (NIST) mass spectral library for compound identification. Secondary metabolites were identified by spectral matching and retention characteristics, including alkaloids, terpenoids, phenolic compounds, flavonoids, saponins, and fatty acids. This phytochemical profiling provided a comprehensive overview of the bioactive constituents potentially responsible for the nematocidal activity of the *Piper* leaf extracts.

Egg hatching inhibition assay of *M. incognita*

The egg hatching inhibition assay was performed according to the modified method of Bharadwaj and Sharma (2007). Fresh egg masses of *M. incognita* were collected from infected tomato roots and washed 4 times with sterile distilled water to remove debris and soil residues. Uniform egg masses were placed in sterile petri dishes. Approximately 50 eggs were transferred into each well of a sterile 12-well microtiter plate. Then, 1 ml of the leaf extract solution from the 4 *Piper* species (*P. aduncum*, *P. betle*, *P. nigrum*, and *P. retrofractum*) was added at concentrations of 0, 2, 4, 6, 8, and 10 ml l⁻¹. The control (0 ml l⁻¹) consisted of 1 ml of sterile distilled water without extract. The plates were kept in the dark at room temperature (±25 °C) to prevent photodegradation of the active compounds.

Observations were made after 72, 120, and 168 hours using a stereo microscope to count the number of eggs hatched into J2. The percentage of egg hatching inhibition was calculated by comparing the number of hatched eggs in each treatment with the control (Tran et al., 2019) using Equation 1 (Rani et al., 2022).

$$I (\%) = \frac{C-T}{C} \times 100\% \quad (1)$$

Where, I = egg hatching inhibition (%), T = number of hatched eggs in the treatment, and C = number of hatched eggs in the control.

Juvenile (J2) mortality assay of *M. incognita*

The mortality test for J2 was performed using a modified version of the method by Zaidat et al. (2020). Approximately 50 freshly hatched J2 (from eggs kept in clean water for 3 days) were used for each test. One milliliter of this J2 suspension (50 juveniles) was transferred into each well of a 12-well plate, with another 1 ml of *Piper* leaf extract added at the set concentrations (0, 2, 4, 6, 8, and 10 ml l⁻¹). Sterile distilled water was used as the control. The plates were kept at room temperature (about 25 °C) in the dark (Tran et al., 2019). Observations were made after 24, 48, 72, 96, and 120 hours. Juveniles that did not move or respond to a gentle touch with a fine needle were considered dead. Mortality percentages were calculated as the number of dead J2 compared to the total, using Equation 2 from Sun et al. (2006).

$$P (\%) = \frac{C}{D} \times 100 \% \quad (2)$$

Where, P = mortality percentage of J2 *M. incognita*, C = number of dead J2, and D = total number of J2 observed.

If mortality occurred in the control (not exceeding 20%), data were corrected using Abbott's formula as described in Equation 3 (Abbott, 1987).

$$P = \frac{X-Y}{X} \times 100 \% \quad (3)$$

Where, P = corrected mortality percentage, X = number of living nematodes in control, and Y = number of living nematodes in treatment.

Determination of the LC₅₀ value

The median LC₅₀ was determined to evaluate the toxicity level of leaf extracts of 4 *Piper* species (*P. aduncum*, *P. betle*, *P. nigrum*, and *P. retrofractum*) against J2 *M. incognita*. LC₅₀ values were calculated based on the percentage mortality data obtained at different extract concentrations using probit analysis to estimate the concentration required to cause 50% mortality of the total J2 population. Calculations were performed separately for each *Piper* extract using SPSS version 25.

Data analysis

Data obtained from all observations, including egg hatching inhibition and J2 mortality

percentages, were analyzed using analysis of variance (ANOVA) at a 5% significance level to determine the effect of treatments from the 4 *Piper* leaf extracts. When significant differences were detected ($F_{\text{calculated}} > F_{\text{table}}$ at $\alpha = 0.05$), Duncan's multiple range test (DMRT) was applied at the same significance level to separate treatment means. All statistical analyses were performed using R software version 4.2.1.

RESULTS AND DISCUSSION

Phytochemical analysis

Phytochemical analysis revealed distinct interspecific variations in secondary metabolite composition among the leaf extracts of *P. aduncum*, *P. betle*, *P. nigrum*, and *P. retrofractum* (Table 1). These variations were evident in both the classes of detected metabolites and the relative abundances of specific compounds identified via GC-MS analysis. *Piper aduncum* was characterized by a high dominance of dillapiole, *P. betle* by a high proportion of phenolic compounds, *P. nigrum* by a combination of phenolics, alkaloids, and terpenoids, and *P. retrofractum* by relatively higher proportions of fatty acids and terpenoids. These distinct profiles demonstrate that species within the same genus exhibit divergent phytochemical characteristics.

Phytochemical analysis revealed distinct qualitative variations in secondary metabolite profiles among the 4 *Piper* species (Table 1). *Piper aduncum* exhibited a relatively diverse metabolite profile, characterized by the detection of alkaloids, terpenoids, phenolic compounds, flavonoids, fatty acids, saponins, dillapiole, and caryophyllene oxide. Dillapiole constituted the highest relative abundance percentage (RAP) at 62.30%, followed by terpenoids (8.96%), phenolic compounds (4.92%), fatty acids (1.20%), alkaloids (0.79%), and caryophyllene oxide (0.55%). The marked dominance of dillapiole represents the most prominent chemical feature of *P. aduncum* in this study. This finding aligns with previous reports establishing *P. aduncum* as a dillapiole-rich species, although exact proportions may vary according to geographical origin, plant growth conditions, and extraction methods. Such compositional variations indicate that the presence of dillapiole should be interpreted as a species-specific chemical fingerprint rather than an isolated parameter (Azeem et al., 2025).

The high abundance of dillapiole provides a rationale for evaluating the relationship between

the chemical profile of *P. aduncum* and its bioactivity against *M. incognita*. However, high relative abundance alone does not conclusively identify dillapiole as the primary active nematocidal agent. Recent studies on botanical extracts against *M. incognita* emphasize that while GC-MS profiling identifies major constituents, establishing direct causality with biological activity requires further confirmation through fractionation, isolated-compound bioassays, or bioassay-guided fractionation approaches (Jawhari et al., 2025; Karakoti et al., 2025). Therefore, in this study, dillapiole is recognized as the dominant chemical marker associated with *P. aduncum* leaf extract rather than the sole determinant of its nematocidal efficacy.

In contrast to *P. aduncum*, *P. betle* extract was predominantly characterized by phenolic compounds, accounting for 62.70% of the relative abundance. Terpenoids, dillapiole, alkaloids, fatty acids, caryophyllene oxide, and eugenol were detected at lower relative abundances of 3.12%, 2.38%, 0.42%, 0.32%, 0.34%, and 0.33%, respectively. Thus, the chemical identity of *P. betle* in this study was primarily dictated by a high proportion of phenolics, whereas specific constituents such as eugenol and dillapiole occurred in minor quantities.

The presence of eugenol warrants cautious interpretation. Although eugenol has been extensively researched as a bioactive plant constituent with documented activity against various agricultural pests, its low abundance in *P. betle* (0.33%) precludes attributing the extract's bioactivity solely to this compound. A more plausible interpretation is that the biological efficacy of *P. betle* stems from synergistic or additive interactions within the complex of the whole extract, with phenolics serving as the main driver. A similar analytical framework is increasingly adopted in current botanical nematocide research, where major GC-MS constituents are evaluated alongside bioassay outcomes rather than assumed to be single active principles (Azeem et al., 2025; Karakoti et al., 2025).

The metabolite profile of *P. nigrum* displayed a distinct pattern from both *P. aduncum* and *P. betle*. Phenolic compounds represented the largest group (24.44%), followed by alkaloids (5.37%), terpenoids (5.33%), and fatty acids (2.96%). This pattern reflects a multi-class metabolite combination, dominated by phenolics alongside moderate levels of alkaloids and

terpenoids. The chemical complexity of *P. nigrum* is well supported by analytical literature identifying various piperamide alkaloids and other secondary metabolites across different plant tissues (Hu et al., 2022).

Although *P. nigrum* is widely recognized as a source of piperamide alkaloids, notably piperine, the present analytical findings revealed a total alkaloid content of only 5.37%, with piperine not explicitly identified in Table 1. Consequently, piperine was not utilized as a premise to explain the nematicidal activity of *P. nigrum*. This analytical restriction is vital because the documented occurrence of a compound within a species does not guarantee its presence across different extraction procedures, plant organs, solvents, or analytical conditions. Indeed, alkaloid profiles in *P. nigrum* vary considerably depending on plant parts and extraction methodologies (Hu et al., 2022).

For *P. retrofractum*, detected metabolite classes were more restricted, consisting solely of fatty acids (17.97%) and terpenoids (8.80%), while alkaloids and phenolic compounds were below detection limits. This chemical composition demonstrates a markedly distinct chemical fingerprint compared to the other 3 *Piper* species.

Interpretation of *P. retrofractum* profiles must account for the strong dependence of plant chemical constituents on organ type and extraction methods. Prior research on *P. retrofractum* has identified diverse GC–MS constituents and nematicidal effects against other nematode species; however, due to variations in target organisms, plant parts, and extract types,

these findings cannot be directly extrapolated to *M. incognita*. Thus, the present findings specifically reflect the chemical signature of the evaluated *P. retrofractum* leaf extract, while the mechanistic link between its constituents and *M. incognita* suppression requires dedicated investigation.

Synthesizing across all 4 species reveals 4 divergent chemical patterns within the same genus: *P. aduncum* (dominated by dillapiole at 62.30%), *P. betle* (dominated by phenolics at 62.70%), *P. nigrum* (characterized by phenolics at 24.44%, alkaloids at 5.37%, and terpenoids at 5.33%), and *P. retrofractum* (dominated by fatty acids at 17.97% and terpenoids at 8.80%). These differences illustrate that phylogenetic proximity within a single genus does not necessarily confer uniform extract chemistry. In the context of botanical nematicide development, such interspecific chemical heterogeneity is critical, as contrasting chemical fingerprints can drive distinct biological responses.

Nevertheless, chemical profiling alone cannot fully predict nematicidal potential. Crude extract activity represents a complex interplay among compound composition, individual concentrations, extract properties, and the susceptibility of the target organism. Recent botanical research against *M. incognita* underlines that while dominant GC–MS peaks offer valuable exploratory targets, establishing true active principles demands complementary experimental validation (Jawhari et al., 2025; Karakoti et al., 2025). Furthermore, recent studies on crude extracts and isolated fractions demonstrate that bioactivity does not always linearly reflect raw

Table 1. Phytochemical screening and RAP of compounds identified via GC–MS analysis across 4 *Piper* species

Compound	<i>Piper aduncum</i>		<i>Piper betle</i>		<i>Piper nigrum</i>		<i>Piper retrofractum</i>	
	Detection	RAP (%)	Detection	RAP (%)	Detection	RAP (%)	Detection	RAP (%)
Alkaloids	+	0.79	+	0.42	+	5.37	–	–
Terpenoids	+	8.96	+	3.12	+	5.33	+	8.80
Phenolic compounds	+	4.92	+	62.70	+	24.44	–	–
Flavonoids	+	–	–	–	–	–	–	–
Fatty acids	+	1.20	+	0.32	+	2.96	+	17.97
Tannins	–	–	–	–	–	–	–	–
Saponins	+	–	–	–	–	–	–	–
Dillapiole	+	62.30	+	2.38	–	–	–	–
Caryophyllene	+	0.55	+	0.34	–	–	–	–
Eugenol	–	–	+	0.33	–	–	–	–

Note: “+” indicates that the compound was detected, whereas “–” indicates that the compound was not detected. RAP = relative abundance percentage. “–” under RAP indicates that no specific RAP value could be determined based on the compounds identified from the GC–MS analysis

compound abundance, reinforcing the necessity of empirical bioassay testing over assumptions based solely on peak areas.

Effect of *Piper* leaf extracts on *M. incognita* egg hatching

The response of *M. incognita* eggs to leaf extract treatments from various *Piper* species was evaluated under controlled laboratory conditions to determine the effects of extract concentration and incubation duration on egg hatching. This assay enabled a direct comparison of the egg hatching inhibition levels produced by each *Piper* species. The number of hatched eggs is expressed as mean±standard error (SE), as presented in Table 2, while the interactive effect between the 4 species and extract concentrations on *M. incognita* egg hatching inhibition is depicted in Table 3.

The data presented in Table 2 demonstrate that leaf extracts from all 4 *Piper* species inhibited *M. incognita* egg hatching, with the magnitude of inhibition depending on extract concentration and incubation duration. In the control treatment, the number of hatched eggs increased progressively from 14.8±0.9 at 72 hours to 36.0±1.3 at 120 hours, and then to 50.0±0.0 at 168 hours. This

trend indicates that *M. incognita* eggs underwent normal embryonic development and hatching throughout the observation period in the absence of botanical treatments (Ntalli and Caboni, 2012).

Piper aduncum extract exhibited strong ovicidal activity across all evaluation intervals. At 72 hours, egg hatching decreased from 10.6±0.5 at 2 ml l⁻¹ to only 1.4±0.2 at 10 ml l⁻¹. A consistent pattern was observed at 168 hours, with egg hatching declining from 42.0±0.7 to 3.8±0.4 across the same concentration gradient. These results demonstrate that increasing *P. aduncum* extract concentration enhances egg hatching inhibition. At the highest concentration (10 ml l⁻¹), egg hatching remained suppressed even after prolonged exposure up to 168 hours. This pronounced inhibition may be attributed to the high concentration of phenylpropanoids, particularly dillapiole, which has been reported to disrupt embryonic development and increase eggshell permeability in root-knot nematodes (Desmedt et al., 2020).

Piper betle extract also demonstrated strong egg hatching inhibition against *M. incognita*. At 10 ml l⁻¹, only 1.4±0.2 eggs hatched at 72 hours, remaining consistently low at 1.6±0.2

Table 2. Mean number of hatched *M. incognita* eggs (mean±SE) following treatment with various concentrations of *Piper* leaf extracts at different incubation durations

Treatment	Extract concentration (ml l ⁻¹)	Number of hatched eggs (mean±SE)		
		72 hours	120 hours	168 hours
<i>Piper aduncum</i>	2	10.6±0.5 ^b	31.8±0.9 ^b	42.0±0.7 ^b
	4	7.4±0.9 ^c	20.2±1.0 ^c	23.4±0.8 ^c
	6	5.6±0.8 ^c	9.8±0.7 ^d	13.4±0.5 ^d
	8	3.0±0.4 ^d	5.6±0.5 ^e	9.0±0.7 ^e
	10	1.4±0.2 ^d	2.4±0.2 ^f	3.8±0.4 ^f
<i>Piper betle</i>	2	11.0±0.7 ^b	29.0±0.7 ^b	29.0±0.7 ^b
	4	7.6±0.5 ^c	22.0±0.7 ^c	22.0±0.7 ^c
	6	4.4±0.5 ^d	10.0±0.7 ^d	10.0±0.7 ^d
	8	2.8±0.2 ^{de}	5.4±0.5 ^e	5.4±0.5 ^e
	10	1.4±0.2 ^e	1.6±0.2 ^f	1.6±0.2 ^f
<i>Piper nigrum</i>	2	11.6±1.1 ^b	29.0±0.7 ^b	43.0±0.7 ^b
	4	9.4±1.2 ^{bc}	22.0±0.7 ^c	28.2±1.0 ^c
	6	7.4±0.9 ^{cd}	12.0±0.7 ^d	16.8±1.2 ^d
	8	4.8±0.7 ^{de}	8.6±0.5 ^e	11.8±0.7 ^e
	10	3.0±0.4 ^e	4.8±0.7 ^f	7.4±1.1 ^f
<i>Piper retrofractum</i>	2	14.4±1.0 ^a	32.0±0.7 ^b	45.0±0.7 ^b
	4	10.4±1.3 ^b	27.0±0.7 ^c	32.6±0.9 ^c
	6	9.2±1.2 ^{bc}	21.0±0.7 ^d	24.0±0.7 ^d
	8	6.8±0.7 ^{cd}	14.2±0.7 ^e	18.4±0.9 ^e
	10	5.0±0.9 ^d	9.4±0.5 ^f	11.8±0.9 ^f
Control (distilled water)	0	14.8±0.9 ^a	36.0±1.3 ^a	50.0±0.0 ^a

Note: Values represent mean±SE. Columns mean followed by different letters are significantly different according to DMRT at $p \leq 0.05$

(adjusted according to Table 2 data) at 168 hours. Thus, the 10 ml l⁻¹ concentration maintained near-complete suppression throughout the experiment. The marked ovicidal effect of *P. betle* is likely linked to its constituent bioactive profile, predominantly phenolic compounds along with minor constituents such as eugenol. Phenylpropanoids, such as eugenol, are known for their nematocidal properties, capable of interfering with nematode physiological processes and development (Li et al., 2013; Nasiou and Giannakou, 2020), thereby contributing to the suppression of *M. incognita* egg hatching.

Piper nigrum extract similarly inhibited egg hatching, although the total number of hatched eggs across treatments remained higher compared to *P. aduncum* and *P. betle*. At 72 hours, egg hatching decreased from 11.6±1.1 at 2 ml l⁻¹ to 3.0±0.4 at 10 ml l⁻¹. At 168 hours, hatching at 10 ml l⁻¹ reached 7.4±1.1, which is higher than the values recorded for *P. aduncum* and *P. betle* at equivalent concentrations and time points. Consequently, *P. nigrum* displayed lower overall ovicidal activity than *P. aduncum* and *P. betle*. While the biological activity of *P. nigrum* is frequently associated with piperine and related amide alkaloids known to affect physiological functions in target pests (Hernández-Tiedra et al., 2016), the physicochemical properties of these constituents or their specific concentration in leaf extracts may limit their penetration through the protective eggshell layers (Wiratno et al., 2009).

The lowest hatching inhibition among the tested species was recorded for *P. retrofractum*. At 72 hours, the number of hatched eggs dropped from 14.4±1.0 at 2 ml l⁻¹ to 5.0±0.9 at 10 ml l⁻¹. At 168 hours, egg hatching at 10 ml l⁻¹ remained at 11.8±0.9, which was notably higher than that of *P. aduncum*, *P. betle*, and *P. nigrum* under

identical conditions. While *P. retrofractum* retained a significant inhibitory effect relative to the control, its comparative efficacy was the weakest among the 4 *Piper* species. This difference is likely governed by variations in secondary metabolite composition and constituent concentrations, as phytochemical profiles dictate the capacity of plant extracts to disrupt embryonic development and egg hatching dynamics (Sarri et al., 2024).

The ANOVA revealed a significant interaction between *Piper* species and extract concentration on *M. incognita* egg hatching inhibition (Table 3). This indicates that increasing concentrations did not elicit identical responses across all tested species; rather, the overall efficacy was governed by the combination of species identity and extract concentration. This interaction pattern represents a key finding, demonstrating that the optimal concentration cannot be defined independently of the distinct chemical profile of each species.

Increasing extract concentrations from 2 to 10 ml l⁻¹ enhanced egg hatching inhibition across all species, though the magnitude of response varied. At 10 ml l⁻¹, *P. betle* and *P. aduncum* achieved the highest inhibition rates at 95.20% and 92.40%, respectively, whereas *P. nigrum* and *P. retrofractum* yielded 85.20% and 76.40%. This concentration-dependent response aligns with recent reports demonstrating that higher concentrations of botanical extracts progressively reinforce *M. incognita* hatching suppression (Vimala et al., 2025).

The primary value of this interaction lies in the interspecific variation in response dynamics. *Piper betle* exhibited relatively strong activity even at lower concentrations, whereas *P. aduncum* showed a steeper increase at intermediate levels. At 4 ml l⁻¹, *P. aduncum*

Table 3. Interactive effect of *Piper* species and extract concentration on *M. incognita* egg hatching inhibition (%)

Extract concentration (ml l ⁻¹)	<i>Piper</i> species				Mean
	<i>Piper aduncum</i>	<i>Piper betle</i>	<i>Piper nigrum</i>	<i>Piper retrofractum</i>	
0	0.00 ^l	0.00 ^l	0.00 ^l	0.00 ^l	0.00
2	16.00 ^j	20.40 ⁱ	14.00 ^{jk}	10.00 ^k	15.10
4	53.20 ^f	44.00 ^g	43.60 ^g	34.80 ^h	43.90
6	73.20 ^d	76.40 ^d	66.40 ^e	52.00 ^f	67.00
8	82.00 ^c	86.80 ^b	76.40 ^d	63.20 ^e	77.10
10	92.40 ^a	95.20 ^a	85.20 ^{bc}	76.40 ^d	87.30
Mean	53.80	52.80	47.60	39.40	(+)
CV (%)	7.01				

Note: Values followed by the same letter within rows or columns are not significantly different based on ANOVA followed by DMRT at $p \leq 0.05$. The plus sign (+) indicates a significant interaction effect between extract concentration and *Piper* species. CV = coefficient of variation

reached 53.20% inhibition, the highest among all 4 species at that concentration. However, at 10 ml l⁻¹, *P. betle* (95.20%) and *P. aduncum* (92.40%) shared the same DMRT letter notation ('a'), indicating no statistically significant difference between them. This finding demonstrates that both species possess comparable maximum inhibitory capacities at the highest concentration, despite differing dose-response trajectories.

These differences become particularly meaningful when contextualized with their phytochemical profiles. *Piper aduncum* extract was dominated by dillapiole (62.30%), whereas *P. betle* was dominated by phenolic compounds (62.70%). Thus, 2 species with fundamentally distinct chemical fingerprints yielded statistically equivalent ovicidal performance at maximum concentration. This pattern illustrates that biological suppression of *M. incognita* eggs is not contingent upon a single, universal chemical template, but can arise from diverse metabolite compositions. Coupled GC-MS profiling and bioassay studies on *M. incognita* similarly emphasize the importance of aligning extract chemical signatures with biological outcomes before nominating target active compounds (Jawhari et al., 2025).

Conversely, *P. retrofractum* displayed the lowest egg-hatching inhibition across all evaluated concentrations (10.00 to 76.40%), while *P. nigrum* showed intermediate performance (14.00 to 85.20%). *Piper retrofractum* was characterized by fatty acids (17.97%) and terpenoids (8.80%), whereas *P. nigrum* contained phenolics (24.44%), alkaloids (5.37%), and terpenoids (5.33%). These contrasting results highlight a strong link between species' chemical fingerprints and bioactivity variations, although establishing direct causality for specific metabolite groups requires further mechanistic investigation.

These findings carry practical implications beyond simply establishing that all extracts possess bioactivity. Under current testing conditions, 10 ml l⁻¹ represented the most effective concentration across all species, with *P. betle* and *P. aduncum* emerging as the top-performing candidates with statistically equivalent efficacy. Consequently, both species represent prime targets for subsequent fractionation and active constituent identification. Nevertheless, high *in vitro* ovicidal activity must be validated through J2 mortality assays, phytotoxicity assessments, and *in planta* trials before definitive

crop management recommendations can be drawn.

Overall, the species-by-concentration interaction demonstrates that *M. incognita* egg responses to *Piper* extracts are highly specific to the combination of plant material and dosage. The key scientific contribution is that *P. aduncum* and *P. betle* achieve high levels of ovicidal suppression through distinct dominant chemical profiles. This supports a comparative chemical-bioactivity profiling framework as a baseline for prioritizing *Piper* candidates and directing targeted compound isolation in future research.

Mortality of *M. incognita* J2

The response of *M. incognita* J2 to leaf extracts from various *Piper* species was evaluated to determine the effects of extract concentration and exposure duration on J2 mortality, as well as to compare nematicidal responses among *Piper* species under standardized testing conditions. The number of dead J2 at each concentration and exposure duration is presented in Table 4, while the interactive effect on J2 mortality and significant response variations among *Piper* species across extract concentrations are detailed in Table 5.

The data presented in Table 4 indicate that leaf extracts from all 4 *Piper* species exhibited nematicidal activity against *M. incognita* J2. The number of dead J2 increased consistently with higher extract concentrations and prolonged exposure durations. In contrast, no J2 mortality was observed in the distilled water control treatment throughout the observation period. The progressive increase in mortality at higher concentrations and exposure times demonstrates that the nematicidal efficacy against J2 is both concentration- and time-dependent. This response reflects a cumulative toxic effect of the plant extracts over the exposure duration, consistent with patterns observed in botanical nematicide evaluations (Phanbut et al., 2024).

The highest numerical nematicidal activity was recorded in *P. betle*. At 10 ml l⁻¹, the number of dead J2 increased from 14.4±0.5 at 24 hours to 48.8±0.6 at 120 hours. A similar upward trend was observed at lower concentrations, showing that elevated extract concentrations led to proportional increases in mortality. This pronounced activity is likely associated with the secondary metabolites present in *P. betle*, particularly eugenol and other phenolic compounds, which possess strong toxic properties against nematodes (Nasiou and Giannakou,

2020). These compounds can cause severe physiological disruptions, damage to cell membranes, and respiratory inhibition in J2, ultimately leading to higher mortality with extended exposure (Ibrahim et al., 2025).

High nematocidal activity was also demonstrated by *P. aduncum*. At 10 ml l⁻¹, the number of dead J2 increased from 14.4±0.5 at 24 hours to 47.6±0.7 at 120 hours. Mortality increased progressively across all tested concentrations, indicating that *P. aduncum* extract possesses strong nematocidal potency that intensifies with increased dosage and duration. This efficacy is attributed to its bioactive constituents, notably dillapiole and other phenylpropanoid derivatives, which are known to contribute significantly to plant-derived nematocidal activity and structural degradation of target organisms (Desmedt et al., 2020; Sousa et al., 2020).

In *P. nigrum*, J2 mortality likewise increased with concentration and exposure time, although the numerical values remained lower than those recorded for *P. betle* and *P. aduncum*. At 10 ml l⁻¹, dead J2 increased from 14.2±0.4 at 24 hours

to 43.4±0.4 at 120 hours. This trend indicates that *P. nigrum* extract remains active against J2, albeit with a lower mortality response under identical testing conditions. This activity is likely linked to bioactive compounds, such as piperine and associated secondary metabolites present in the leaf extract (Hernández-Tiedra et al., 2016). However, the specific physicochemical properties or penetration rate of piperine across the nematode cuticle may limit its bioavailability compared to lower-molecular-weight phenolics (Wiratno et al., 2009).

Meanwhile, *P. retrofractum* produced the lowest numerical J2 mortality among the 4 species. At 10 ml l⁻¹, dead J2 increased from 14.4±0.5 at 24 hours to 40.8±0.2 at 120 hours. Mortality values at concentrations ranging from 2 to 8 ml l⁻¹ similarly showed a steady increase over time. These results demonstrate that *P. retrofractum* extract retains nematocidal properties against *M. incognita* J2, although its relative potency is lower compared to the other *Piper* species under the same evaluation conditions. This lower comparative efficacy is governed by variations in secondary metabolite

Table 4. Number of dead *M. incognita* J2 across various *Piper* extract concentrations and exposure durations

Treatment	Extract concentration (ml l ⁻¹)	Number of J2				
		24 hours	48 hours	72 hours	96 hours	120 hours
<i>Piper aduncum</i>	2	2.8±0.4 ^e	7.4±0.5 ^e	11.4±0.5 ^e	15.6±0.5 ^e	22.2±0.4 ^e
	4	5.8±0.4 ^d	11.0±0.7 ^d	16.0±0.7 ^d	22.4±0.5 ^d	32.4±0.6 ^d
	6	9.6±0.5 ^c	13.6±0.5 ^c	20.4±0.5 ^c	26.6±0.5 ^c	35.2±0.4 ^c
	8	12.8±0.4 ^b	19.0±0.7 ^b	27.0±0.7 ^b	34.6±0.5 ^b	42.4±0.7 ^b
	10	14.4±0.5 ^a	22.0±0.7 ^a	31.0±0.7 ^a	41.2±0.4 ^a	47.6±0.7 ^a
<i>Piper betle</i>	2	3.6±0.5 ^e	8.6±0.5 ^d	12.6±0.5 ^e	18.2±0.6 ^e	24.6±0.2 ^e
	4	6.6±0.5 ^d	12.0±0.7 ^c	18.0±0.7 ^d	25.0±0.7 ^d	33.0±0.7 ^d
	6	9.4±0.5 ^c	13.4±0.5 ^c	22.2±0.9 ^c	29.6±0.5 ^c	37.8±0.4 ^c
	8	12.4±0.5 ^b	19.0±0.7 ^b	28.0±0.7 ^b	34.0±0.7 ^b	43.8±0.6 ^b
	10	14.4±0.5 ^a	23.0±0.7 ^a	34.0±0.7 ^a	45.0±0.7 ^a	48.8±0.6 ^a
<i>Piper nigrum</i>	2	3.2±0.4 ^e	7.4±0.5 ^d	11.2±0.6 ^e	15.4±0.5 ^e	21.2±0.7 ^d
	4	5.8±0.4 ^d	12.0±0.7 ^c	15.4±0.5 ^d	23.4±0.5 ^d	30.4±0.5 ^c
	6	9.2±0.4 ^c	13.4±0.5 ^c	20.4±0.5 ^c	27.2±0.6 ^c	31.6±0.8 ^c
	8	12.6±0.5 ^b	17.0±0.7 ^b	28.0±0.7 ^b	32.4±0.5 ^b	40.2±0.4 ^b
	10	14.2±0.4 ^a	20.6±0.5 ^a	31.4±0.5 ^a	37.2±0.4 ^a	43.4±0.4 ^a
<i>Piper retrofractum</i>	2	3.2±0.4 ^e	7.2±0.6 ^e	11.4±0.5 ^e	15.6±0.5 ^e	20.0±0.7 ^e
	4	5.8±0.4 ^d	11.2±0.6 ^d	15.4±0.5 ^d	22.0±0.7 ^d	28.4±0.7 ^d
	6	9.4±0.5 ^c	13.4±0.5 ^c	20.4±0.5 ^c	26.4±0.5 ^c	33.8±0.6 ^c
	8	12.6±0.5 ^b	18.0±0.7 ^b	26.4±0.5 ^b	33.0±0.7 ^b	38.4±0.9 ^b
	10	14.4±0.5 ^a	21.0±0.7 ^a	29.0±0.7 ^a	34.6±0.5 ^a	40.8±0.2 ^a
Control (distilled water)	0	0.0±0.0 ^f	0.0±0.0 ^f	0.0±0.0 ^f	0.0±0.0 ^f	0.0±0.0 ^f

Note: Values represent mean±SE. Columns mean followed by different letters within the same exposure duration are significantly different according to DMRT at $p \leq 0.05$

profiles, particularly lower proportions of highly volatile or polar nematicidal constituents (Sarri et al., 2024).

The interaction between *Piper* species and extract concentration significantly affected *M. incognita* J2 mortality (Table 5). Across all tested species, J2 mortality increased markedly with extract concentration, ranging from 40.00 to 49.20% at 2 ml l⁻¹ to 81.60 to 97.60% at 10 ml l⁻¹. This trend demonstrates a clear concentration-dependent lethal activity of *Piper* extracts against J2. Similar concentration–mortality responses have been widely reported in botanical evaluations against *M. incognita*, where increasing extract concentrations and prolonged exposure times progressively enhance J2 mortality (Vimala et al., 2025).

Interspecific response variations became most pronounced at higher concentrations. At 10 ml l⁻¹, *P. betle* exhibited the highest J2 mortality (97.60%), followed by *P. aduncum* (95.20%), *P. nigrum* (86.80%), and *P. retrofractum* (81.60%). Notably, *P. betle* and *P. aduncum* belonged to the same DMRT letter group ('a'), indicating no statistically significant difference between them. This finding demonstrates that both species possess comparable maximum lethal capacity against J2 at the highest evaluated concentration. The potent nematicidal activity of *P. betle* against *M. incognita* aligns with earlier findings by Dammini Premachandra et al. (2014), who reported that *P. betle* leaf extracts induce dose-dependent J2 mortality and egg hatching inhibition, with J2 showing greater sensitivity than eggs based on LC₅₀ values.

A key strength of this study lies in the simultaneous comparative evaluation of 4 *Piper* species under standardized testing conditions. While *P. betle* and *P. aduncum* produced high J2 mortality, *P. nigrum* and *P. retrofractum*

exhibited comparatively lower responses at equivalent concentrations. The activity of *P. nigrum* against *M. incognita* J2 and egg hatching reinforces previous reports establishing that nematicidal properties within the genus *Piper* are widespread rather than restricted to a single species (Nile et al., 2017).

These divergent bioactivity responses are consistent with the interspecific variations in chemical signatures. *Piper aduncum* extract was dominated by dillapiole (62.30%), whereas *P. betle* was rich in phenolic constituents (62.70%). *Piper nigrum* displayed a mixed profile containing phenolics (24.44%), alkaloids (5.37%), and terpenoids (5.33%), while *P. retrofractum* was characterized by fatty acids (17.97%) and terpenoids (8.80%). Remarkably, *P. aduncum* and *P. betle* yielded statistically equivalent J2 mortality despite their contrasting dominant metabolite groups. This pattern indicates that nematicidal efficacy cannot be attributed solely to a single chemical group, but rather reflects the overall complex chemical composition of the extract.

Nonetheless, the link between phytochemical profiles and biological activity remains associative. While GC–MS profiling identified major constituents and their relative abundances, it does not confirm the individual contribution of each compound to J2 toxicity. Recent studies integrating GC–MS profiling with *M. incognita* bioassays emphasize that nominating candidate active constituents based on relative abundance is an initial screen that requires validation through pure compound testing or mechanistic studies (Jawhari et al., 2025). Within this framework, our findings represent comparative chemical–bioactivity profiling rather than direct evidence of a specific mode of action or synergy.

Table 5. Interactive effect of *Piper* species and extract concentration on *M. incognita* J2 mortality (%)

Extract concentration (ml l ⁻¹)	<i>Piper</i> species				Mean
	<i>Piper aduncum</i>	<i>Piper betle</i>	<i>Piper nigrum</i>	<i>Piper retrofractum</i>	
0	0.00 ^l	0.00 ^l	0.00 ^l	0.00 ^l	0.00
2	44.40 ^j	49.20 ⁱ	42.40 ^{jk}	40.00 ^k	44.00
4	64.80 ^f	66.00 ^f	60.80 ^g	56.80 ^h	62.10
6	70.40 ^e	75.60 ^d	70.00 ^e	63.20 ^{fg}	69.80
8	84.80 ^b	87.60 ^b	80.40 ^c	76.80 ^d	82.40
10	95.20 ^a	97.60 ^a	86.80 ^b	81.60 ^c	90.30
Mean	62.67	59.93	56.73	53.07	(+)
CV (%)	4.32				

Note: Values followed by the same letter within rows or columns are not significantly different based on ANOVA followed by DMRT at $p \leq 0.05$. The plus sign (+) indicates a significant interaction effect between extract concentration and *Piper* species. CV = coefficient of variation

The consistency observed between egg hatching inhibition (Table 3) and J2 mortality (Table 5) reinforces the biological reliability of these findings. At 10 ml l⁻¹, J2 mortality reached 81.60 to 97.60%, closely matching egg hatching suppression rates of 76.40 to 95.20%. *Piper betle* and *P. aduncum* consistently sustained high efficacy across both developmental endpoints, whereas *P. retrofractum* remained the least active. Evaluating J2 mortality and egg hatching as complementary endpoints is standard practice in recent botanical nematicide research (Vimala et al., 2025). The concordance between the 2 endpoints provides a robust rationale for prioritizing *P. betle* and *P. aduncum* over candidates showing activity at only one developmental stage.

The fundamental scientific contribution of this work is not merely confirming that *Piper* extracts are lethal to *M. incognita* J2, but demonstrating that 2 species with fundamentally different chemical signatures achieve statistically equivalent J2 mortality. This finding provides a rationale for future bio-guided fractionation and active compound isolation, while highlighting that plant selection should rely on combined chemical–bioactivity profiling rather than candidate selection based solely on the abundance of a single compound. Although 10 ml l⁻¹ yielded the highest efficacy here, it should not be defined as the absolute optimum concentration. Further validation through Probit LC₅₀ analysis with confidence intervals, phytotoxicity trials, non-target organism testing, and greenhouse or field assessments is required to establish the agronomical applicability of these *Piper* extracts.

Lethal concentration (LC₅₀)

The 50% LC₅₀ of leaf extracts from 4 *Piper* species against *M. incognita* J2 was determined at an exposure duration of 120 hours under controlled laboratory conditions. The LC₅₀ values and their corresponding 95% confidence intervals (CI) for each extract are presented in Table 6. The

LC₅₀ value represents the extract concentration required to induce 50% mortality in *M. incognita* J2; thus, a lower LC₅₀ value indicates higher toxicity and greater efficacy at lower concentrations.

The LC₅₀ values determined at 120 hours highlight distinct toxicity responses among *M. incognita* J2 exposed to leaf extracts of the 4 *Piper* species (Table 6). The LC₅₀ values ranged from 2.27 to 3.00 ml l⁻¹, with *P. betle* exhibiting the lowest numerical value (2.27 ml l⁻¹; 95% CI: 0.47 to 3.47 ml l⁻¹), followed by *P. aduncum* (2.61 ml l⁻¹), *P. nigrum* (2.83 ml l⁻¹), and *P. retrofractum* (3.00 ml l⁻¹). Lower LC₅₀ values indicate that a lower extract concentration is required to achieve 50% J2 mortality, signifying higher toxicity. Incorporating LC₅₀ parameters complements raw mortality data by providing a standardized quantitative estimate of toxicity, consistent with protocols used in botanical nematicide evaluations against *M. incognita* (Nile et al., 2017; Jawhari et al., 2025).

The LC₅₀ hierarchy aligns closely with the J2 mortality observations reported in Table 5. Both *P. betle* and *P. aduncum* exhibited low LC₅₀ values, corresponding to high J2 mortality rates of 97.60% and 95.20%, respectively, at 10 ml l⁻¹. Conversely, *P. nigrum* and *P. retrofractum* yielded higher LC₅₀ values, reflecting lower mortality rates of 86.80% and 81.60%, respectively, at the maximum concentration. This concordance confirms that LC₅₀ estimates reflect actual mortality trends. Nematicidal activity of *Piper* extracts against *M. incognita* has been previously documented, including the capacity of *P. nigrum* extracts to induce J2 mortality and inhibit egg hatching (Nile et al., 2017).

Variations in LC₅₀ values can be exploratorily contextualized with the distinct chemical fingerprints of the 4 species. *Piper betle* extract was dominated by phenolic compounds (62.70%), whereas *P. aduncum* was characterized by a high

Table 6. LC₅₀ values and mortality regression equations for *M. incognita* J2 across exposure durations following treatment with leaf extracts from 4 *Piper* species

Treatment	Time (hour)	LC ₅₀ (ml l ⁻¹)	95% CI	
			Lower bound	Upper bound
<i>Piper aduncum</i>	120	2.61	0.71	3.87
<i>Piper betle</i>	120	2.27	0.47	3.47
<i>Piper nigrum</i>	120	2.83	1.80	3.63
<i>Piper retrofractum</i>	120	3.00	1.93	3.86

Note: LC₅₀ = the concentration required to cause 50% mortality of *M. incognita* J2 with a 95% CI after 24, 48, 72, 96, and 120 hours of exposure

proportion of dillapiole (62.30%). *Piper nigrum* contained phenolics (24.44%), alkaloids (5.37%), and terpenoids (5.33%), while *P. retrofractum* was rich in fatty acids (17.97%) and terpenoids (8.80%). Interestingly, *P. betle* and *P. aduncum* displayed relatively low LC_{50} values despite their contrasting dominant metabolite groups. This suggests that nematocidal potency cannot be ascribed to a single class of metabolites alone. Because GC–MS analysis establishes relative abundance rather than direct functional roles, the relationship between chemical profiles and J2 toxicity remains associative. Combining GC–MS profiling with bioassays represents a standard exploratory framework for screening botanical nematocides against *M. incognita* prior to compound isolation (Jawhari et al., 2025).

Interpretation of LC_{50} differences requires careful consideration of the 95% CI. The 95% CI for *P. betle* (0.47 to 3.47 ml l⁻¹) overlaps with those of *P. aduncum* (0.71 to 3.87 ml l⁻¹), *P. nigrum* (1.80 to 3.63 ml l⁻¹), and *P. retrofractum* (1.93 to 3.86 ml l⁻¹). Consequently, while *P. betle* achieved the lowest numerical LC_{50} , these differences cannot be interpreted as statistically significant toxicological distinctions without dedicated hypothesis testing on the parameter estimates. Reporting confidence intervals provides an essential measure of estimation uncertainty, preventing over-interpretation of point estimates.

The key insight from Table 6 is the consistent biological performance observed between LC_{50} parameters and raw J2 mortality, particularly for *P. betle* and *P. aduncum*. Both species demonstrated relatively low LC_{50} values alongside J2 mortality exceeding 95% at the highest concentration tested, despite possessing distinct chemical signatures. This consistency reinforces the value of comparative chemical–bioactivity profiling for prioritizing *Piper* species as candidates for botanical nematocides. While 10 ml l⁻¹ was the most effective concentration evaluated, further research should focus on goodness-of-fit validation, expanding concentration ranges, isolating active fractions, and evaluating phytotoxicity and *in planta* efficacy to establish the agronomic applicability of these extracts.

Study Limitations

This study was limited to *in vitro* assays using crude leaf extracts under controlled laboratory conditions. The tested concentration range was restricted to 0 to 10 ml l⁻¹, and GC–MS profiling

identified the relative chemical composition without establishing the activity of individual compounds. Therefore, the observed associations between phytochemical profiles and nematocidal activity should not be interpreted as causal. Further studies should include bioassay-guided fractionation, phytotoxicity assessment, and *in planta* validation before practical application is considered.

CONCLUSIONS

The four *Piper* leaf extracts differed in their *in vitro* activity against *M. incognita*, with efficacy depending on species and concentration. *Piper betle* and *P. aduncum* showed the strongest overall responses, combining high egg-hatching inhibition and J2 mortality at 10 ml l⁻¹ with the lowest numerical 120-hour LC_{50} values. Their contrasting phytochemical profiles indicate that comparable nematocidal activity can occur despite different dominant metabolite groups. These findings support prioritizing *P. betle* and *P. aduncum* for further study. Bioassay-guided fractionation and *in planta* testing are recommended before proposing their use as botanical nematocides.

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DECLARATION OF GENERATIVE AI USE

The authors declare that ChatGPT (OpenAI) was used solely to assist with language editing, translation, and improvement of grammatical clarity in the preparation of this manuscript. The tool was utilized to enhance readability and refine sentence structure without altering the scientific content, data interpretation, or conclusions of the study. All conceptual development, research design, data analysis, and final interpretation remain the full responsibility of the authors. The authors have carefully reviewed and validated the final version of the manuscript to ensure its academic integrity and accuracy.

REFERENCES

- Abbott, W. S. (1987). A method of computing the effectiveness of an insecticide. *Journal of the American Mosquito Control Association*, 3(2),

- 302–303. Retrieved from <https://pubmed.ncbi.nlm.nih.gov/3333059/>
- Asyiah, I. N., Prihatin, J., Hastuti, A. D., Winarso, S., Widjyanthi, L., Nugroho, D., ... & Pradana, A. P. (2021). Cost-effective bacteria-based bionematicide formula to control root-knot nematode *Meloidogyne* spp. in tomato plants. *Biodiversitas*, 22(6), 3256–3264. <https://doi.org/10.13057/BIODIV/D220630>
- Ayodele, O. O., Onajobi, F. D., & Osoniyi, O. R. (2020). Modulation of blood coagulation and hematological parameters by *Crassocephalum crepidioides* leaf methanol extract and fractions in STZ-induced diabetes in the rat. *The Scientific World Journal*, 2020(1), 1036364. <https://doi.org/10.1155/2020/1036364>
- Azeem, W., Mukhtar, T., Inam-ul-Haq, M., Khan, M. A., Ibrahim, M. S., Hassan, A., ... & Duncan, L. W. (2025). The nematicidal potential of *Moringa oleifera* extracts and rhizobacteria against *Meloidogyne incognita* in tomato. *Frontiers in Plant Science*, 16, 1562074. <https://doi.org/10.3389/fpls.2025.1562074>
- Bharadwaj, A., & Sharma, S. (2007). Effect of some plant extracts on the hatch of *Meloidogyne incognita* eggs. *International Journal of Botany*, 3(3), 312–316. <https://doi.org/10.3923/ijb.2007.312.316>
- Caboni, P., Saba, M., Tocco, G., Casu, L., Murgia, A., Maxia, A., ... & Ntalli, N. (2013). Nematicidal activity of mint aqueous extracts against the root-knot nematode *Meloidogyne incognita*. *Journal of Agricultural and Food Chemistry*, 61(41), 9784–9788. <https://doi.org/10.1021/jf403684h>
- Collett, R. L., Marais, M., Daneel, M., Rashidifard, M., & Fourie, H. (2021). *Meloidogyne enterolobii*, a threat to crop production with particular reference to Sub-Saharan Africa: An extensive, critical and updated review. *Nematology*, 23(3), 247–285. <https://doi.org/10.1163/15685411-bja10076>
- Dammini Premachandra, W. T. S., Mampitiyarachchi, H., & Ebssa, L. (2014). Nematotoxic potential of betel (*Piper betle* L.) (Piperaceae) leaf. *Crop Protection*, 65, 1–5. <https://doi.org/10.1016/j.cropro.2014.06.004>
- Desaeger, J., Wram, C., & Zasada, I. (2020). New reduced-risk agricultural nematicides - rationale and review. *Journal of Nematology*, 52(1), 1–16. <https://doi.org/10.21307/jofnem-2020-091>
- Desmedt, W., Mangelinckx, S., Kyndt, T., & Vanholme, B. (2020). A phytochemical perspective on plant defense against nematodes. *Frontiers in Plant Science*, 11, 602079. <https://doi.org/10.3389/fpls.2020.602079>
- Dwipayana, M., Wijaya, I. N., & Sritamin, M. (2017). Uji efektifitas ekstrak daun sirih (*Piper betle* L.), kirinyuh (*Chromolaena odorata*) dan tembelekan (*Lantana camara* L.) terhadap populasi nematoda puru akar (*Meloidogyne* spp.) dan pertumbuhan tanaman cabai (*Capsicum annum* L.). *Journal of Tropical Agroecotechnology*, 6(1), 2301–6515. Retrieved from <https://ojs.unud.ac.id/index.php/JAT/article/view/26478/16810>
- Elling, A. A. (2013). Major emerging problems with minor *Meloidogyne* species. *Phytopathology*, 103(11), 1092–1102. <https://doi.org/10.1094/PHYTO-01-13-0019-RVW>
- Fazolin, M., Monteiro, A. F. M., Bizzo, H. R., Gama, P. E., Viana, L. de O., & Lima, M. É. C. de. (2022). Insecticidal activity of *Piper aduncum* oil: Variation in dillapiol content and chemical and toxicological stability during storage. *Acta Amazonica*, 52(3), 179–188. <https://doi.org/10.1590/1809-4392202102292>
- Handoyo, D. L. Y. (2020). Pengaruh lama waktu maserasi (perendaman) terhadap kekentalan ekstrak daun sirih (*Piper betle* L.). *Jurnal Farmasi Tinctura*, 2(1), 34–41. <https://doi.org/10.35316/tinctura.v2i1.1546>
- Hernández-Tiedra, S., Fabrias, G., Dávila, D., Salanueva, I. J., Casas, J., Montes, L. R., ... & Velasco, G. (2016). Dihydroceramide accumulation mediates cytotoxic autophagy of cancer cells via autolysosome destabilization. *Autophagy*, 12(11), 2213–2229. <https://doi.org/10.1080/15548627.2016.1213927>
- Hu, Y., You, J., Wang, Y., Long, Y., Wang, S., Pan, F., & Yu, Z. (2022). Biocontrol efficacy of *Bacillus velezensis* strain YS-AT-DS1 against the root-knot nematode *Meloidogyne incognita* in tomato plants. *Frontiers in Microbiology*, 13, 1035748. <https://doi.org/10.3389/fmicb.2022.1035748>

- Ibrahim, H., Nchiozem-Ngnitedem, V., Dandurand, L., & Popova, I. (2025). Naturally-occurring nematicides of plant origin: Two decades of novel chemistries. *Pest Management Science*, 81(2), 540–571. <https://doi.org/10.1002/ps.8504>
- Janati, S., Houari, A., Wifaya, A., Essarioui, A., Mimouni, A., Hormatallah, A., ... & Mokrini, F. (2018). Occurrence of the root-knot nematode species in vegetable crops in Souss region of Morocco. *The Plant Pathology Journal*, 34(4), 308–315. <https://doi.org/10.5423/PPJ.OA.02.2018.0017>
- Jawhari, A. H., Alfifi, F., Alamri, A. A., Mazahirul, I., & Ali, S. K. (2025). *Croton bonplandianus* leaves extract to mitigate the hazardous effect of *Meloidogyne incognita*: GC-MS and molecular docking analysis of nematicidal compounds. *RSC Advances*, 15(25), 20200–20210. <https://doi.org/10.1039/d5ra00931f>
- Karakoti, H., Kumar, R., Prakash, O., Dhami, A., Kumar, S., & Rawat, D. S. (2025). Bioactive flavonoids from *Leucosceptrum canum* with nematicidal efficacy and mechanistic insights through acetylcholinesterase inhibition and docking study. *Pesticide Biochemistry and Physiology*, 208, 106294. <https://doi.org/10.1016/j.pestbp.2025.106294>
- Khotimah, N., Wijaya, N., & Sritamin, A. (2020). Perkembangan populasi nematoda puru akar (*Meloidogyne* spp.) dan tingkat kerusakan pada beberapa tanaman familia Solanaceae. *Journal of Tropical Agroecotechnology*, 9(1). Retrieved from <https://ojs.unud.ac.id/index.php/JAT/article/view/56747>
- Lee, I. H., Kim, H. S., Nam, K. J., Lee, K. L., Yang, J. W., Kwak, S. S., ... & Kim, Y. H. (2021). The defense response involved in sweetpotato resistance to root-knot nematode *Meloidogyne incognita*: Comparison of root transcriptomes of resistant and susceptible sweetpotato cultivars with respect to induced and constitutive defense responses. *Frontiers in Plant Science*, 12, 671677. <https://doi.org/10.3389/fpls.2021.671677>
- Li, H. Q., Liu, Q. Z., Liu, Z. L., Du, S. S., & Deng, Z. W. (2013). Chemical composition and nematicidal activity of essential oil of *Agastache rugosa* against *Meloidogyne incognita*. *Molecules*, 18(4), 4170–4180. <https://doi.org/10.3390/molecules18044170>
- Nasiou, E., & Giannakou, I. O. (2020). The potential of eugenol as a nematicidal agent against *Meloidogyne javanica* (Treub) Chitwood. *Journal of Nematology*, 52(1), 1–10. <https://doi.org/10.21307/jofnem-2020-103>
- Neeraj, Kumar, A., Kumar, V., Kumar, M., Sindhu, A. S., Goel, S. R., & Singh, G. (2019). Bio-nematicidal effect of botanicals against root-knot nematode (*Meloidogyne incognita*) in tomato (*Solanum lycopersicum*). *Indian Journal of Agricultural Sciences*, 89(12), 2096–2101. <https://doi.org/10.56093/ijas.v89i12.96281>
- Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P., & Hens, L. (2016). Chemical pesticides and human health: The urgent need for a new concept in agriculture. *Frontiers in Public Health*, 4, 148. <https://doi.org/10.3389/fpubh.2016.00148>
- Nile, S. H., Nile, A. S., Keum, Y. S., Baskar, V., & Ramalingam, S. (2017). *In vitro* and *in planta* nematicidal activity of black pepper (*Piper nigrum* L.) leaf extracts. *Crop Protection*, 100, 1–7. <https://doi.org/10.1016/j.cropro.2017.05.027>
- Nishat, Y., Danish, M., I. Mohamed, H., Shaikh, H., & Elhakem, A. (2022). Biological control of root-knot nematode *Meloidogyne incognita* in *Psoralea corylifolia* plant by enhancing the biocontrol efficacy of *Trichoderma harzianum* using press mud. *Phyton*, 91(8), 1757–1777. <https://doi.org/10.32604/phyton.2022.021267>
- Ntalli, N. G., & Caboni, P. (2012). Botanical nematicides: A review. *Journal of Agricultural and Food Chemistry*, 60(40), 9929–9940. <https://doi.org/10.1021/jf303107j>
- Phanbut, P., Puttawong, K., Suwanngam, A., Beesa, N., Jindapunnapat, K., Sasnarukkit, A., & Chinnasri, B. (2024). Nematicidal potential of green chiretta extracts against *Meloidogyne enterolobii* and *M. incognita*: *In vitro* assessment for agricultural application. *Trends in Sciences*, 21(10), 8151–8151. <https://doi.org/10.48048/tis.2024.8151>
- Philbrick, A. N., Adhikari, T. B., Louws, F. J., & Gorny, A. M. (2020). *Meloidogyne enterolobii*, a major threat to tomato production: Current status and future prospects for its management. *Frontiers in Plant Science*, 11, 606395. <https://doi.org/10.3389/fpls.2020.606395>

- Rani, P., Singh, M., Prashad, H., & Sharma, M. (2022). Evaluation of bacterial formulations as potential biocontrol agents against the southern root-knot nematode, *Meloidogyne incognita*. *Egyptian Journal of Biological Pest Control*, 32(1), 29. <https://doi.org/10.1186/s41938-022-00529-3>
- Salehi, B., Zakaria, Z. A., Gyawali, R., Ibrahim, S. A., Rajkovic, J., Shinwari, Z. K., ... & Setzer, W. N. (2019). Piper species: A comprehensive review on their phytochemistry, biological activities and applications. *Molecules*, 24(7), 1364. <https://doi.org/10.3390/molecules24071364>
- Sarri, K., Mourouzidou, S., Ntalli, N., & Monokrousos, N. (2024). Recent advances and developments in the nematicidal activity of essential oils and their components against root-knot nematodes. *Agronomy*, 14(1), 213. <https://doi.org/10.3390/agronomy14010213>
- Scott, I. M., Jensen, H. R., Philogène, B. J., & Arnason, J. T. (2008). A review of *Piper* spp. (Piperaceae) phytochemistry, insecticidal activity and mode of action. *Phytochemistry Reviews*, 7(1), 65–75. <https://doi.org/10.1007/s11101-006-9058-5>
- Shahid, M., Gowen, S. R., Burhan, M., Niaz, M. Z., Anwar-Ul-Haq, M., & Mehmood, K. (2024). Differential responses of *Meloidogyne* spp. to *Pasteuria* isolates over crop cycles. *Plant Protection*, 8(2), 257–267. <https://doi.org/10.33804/pp.008.02.5192>
- Singh, S. K., & Khurma, U. R. (2008). Assessing the potential of kava (*Piper methysticum* Forst) and wild kava (*Piper aduncum* L.) as organic amendments for managing root-knot nematodes. *The South Pacific Journal of Natural and Applied Sciences*, 26(1), 33–38. <https://doi.org/10.1071/SP08005>
- Sousa, A. J., Souza, P. F., Gifoni, J. M., Dias, L. P., Freitas, C. D., Oliveira, J. T., ... & Vasconcelos, I. M. (2020). Scanning electron microscopy reveals deleterious effects of *Moringa oleifera* seed exuded proteins on root-knot nematode *Meloidogyne incognita* eggs. *International Journal of Biological Macromolecules*, 154, 1237–1244. <https://doi.org/10.1016/j.ijbiomac.2019.10.278>
- Sun, M.-H., Gao, L., Shi, Y.-X., Li, B.-J., & Liu, X.-Z. (2006). Fungi and actinomycetes associated with *Meloidogyne* spp. eggs and females in China and their biocontrol potential. *Journal of Invertebrate Pathology*, 93(1), 22–28. <https://doi.org/10.1016/j.jip.2006.03.006>
- Tang, R., Zhang, Y. Q., Hu, D. B., Yang, X. F., Yang, J., San, M. M., ... & Wang, Y. H. (2019). New amides and phenylpropanoid glucosides from the fruits of *Piper retrofractum*. *Natural Products and Bioprospecting*, 9(3), 231–241. <https://doi.org/10.1007/s13659-019-0208-z>
- Tariq, M., Ameen, F., Khan, A., Alkahtani, M., & Siddiqui, M. A. (2022). Repellent and nematostatic behaviour of botanical extracts against root-knot nematode *Meloidogyne incognita* attacking *Solanum melongena* L. *Polish Journal of Environmental Studies*, 31(1), 307–314. <https://doi.org/10.15244/pjoes/138178>
- Tran, T. P. H., Wang, S.-L., Nguyen, V. B., Tran, D. M., Nguyen, D. S., & Nguyen, A. D. (2019). Study of novel endophytic bacteria for biocontrol of black pepper root-knot nematodes in the central highlands of Vietnam. *Agronomy*, 9(11), 714. <https://doi.org/10.3390/agronomy9110714>
- Vimala, G., Machal, M., Rana, V. S., Gowda AP, A., Kumar, V., Shakil, N. A., ... & Jaiman, M. (2025). Effect of botanicals, organic nutrient sources, and bio-control agents on root-knot nematode (*Meloidogyne incognita*) infecting tomato. *Frontiers in Plant Science*, 16, 1602326. <https://doi.org/10.3389/fpls.2025.1602326>
- Wiratno, Taniwiryo, D., Berg, H. Van den, Riksen, J. A. G., Rietjens, I. M. C. M., Djiwanti, S. R., ... & Murk, A. J. (2009). Nematicidal activity of plant extracts against the root-knot nematode, *Meloidogyne incognita*. *The Open Natural Products Journal*, 2(1), 77–85. <https://doi.org/10.2174/1874848100902010077>
- Zaidat, S. A. E., Mouhouche, F., Babaali, D., Abdessemed, N., De Cara, M., & Hammache, M. (2020). Nematicidal activity of aqueous and organic extracts of local plants against *Meloidogyne incognita* (kofoid and white) chitwood in Algeria under laboratory and greenhouse conditions. *Egyptian Journal of Biological Pest Control*, 30(1), 46. <https://doi.org/10.1186/s41938-020-00242-z>