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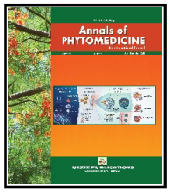

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In vitro nematicidal evaluation of *Terminalia bellirica* (Gaertn.) Roxb. fruit against *Meloidogyne incognita*

Rajni Kant Sharma*, Anil Kumar, Ekta*◆, Komal*, Lochan Sharma**, Savita Rani***and Man Mohan Baghel******

** Department of Chemistry, CCS Haryana Agricultural University, Hisar-125004, Haryana, India*
*** Department of Nematology, CCS Haryana Agricultural University, Hisar-125004, Haryana, India*
**** Department of Horticulture, CCS Haryana Agricultural University, Hisar-125004, Haryana, India*
***** Department of Plant Pathology, CCS Haryana Agricultural University, Hisar-125004, Haryana, India*

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Abstract

Terminalia bellirica (Gaertn.) Roxb. is a medicinally important plant widely used traditionally in the health system of Ayurveda. The present study consists of an *in vitro* nematicidal evaluation of ethanolic and aqueous fruit extracts of *T. bellirica* fruit against the root-knot nematode *Meloidogyne incognita*, a major phytoparasitic pest affecting avariety of agricultural crops. Ethanolic and aqueous fruit extracts were tried for their efficacy in egg hatching inhibition and second-stage juveniles (J2) per cent mortality of *M. incognita*. The ethanolic extract exhibited 76.4% egg hatching inhibition and 91.6% juvenile mortality at 20% concentration, compared to 47.3% and 51% for the aqueous extract at 200 ppm, after 72 h of exposure time, respectively. The cytotoxicity evaluation of ethanolic extract estimated CC_{50} value as 0.57 µg/ml. These findings suggest that *T. bellirica* fruit can be possessed as eco-friendly botanical nematicides for sustainable management of *M. incognita*.

1. Introduction

Root-knot nematodes (*Meloidogyne* sp.) rank among the most destructive plant-parasitic nematodes, causing substantial yield losses across numerous economically important crops. These nematodes induce the formation of characteristic root galls, disrupt water and nutrient uptake, and make crops more vulnerable to secondary infections by fungi and bacteria. Globally, plant-parasitic nematodes are responsible for an average annual loss of 12.3% across 40 major crops, with developing countries experiencing greater losses (14.6%) compared to developed countries (8.8%)(Kumar *et al.*, 2020). According to an extensive worldwide assessment, plant-parasitic nematodes inflict an estimated USD 173 billion in annual economic losses across major crops (Elling, 2013). Conventional management tactics have deeply relied on man-made nematicides. However, their use is gradually restricted due to high mammalian toxicity, environmental tenacity, and harmful effects on non-target organisms.

Many plants produce various secondary metabolites such as alkaloids,flavonoids, tannins, terpenoids and phenolic compounds, many of them exhibit potent nematicidal properties. Among these, species of the genus *Terminalia* (Combretaceae) have gained considerable attention for their broad-spectrum biological activities, including anti-inflammatory, antimicrobial, pesticidal and antioxidant properties (Ndo *et al.*, 2024; Fabiyi, 2021; Bag *et al.*, 2013; Basu *et al.*, 2017; Cheesman *et al.*, 2019; Debnath *et al.*, 2013). Some *Terminalia* species are widely utilized medicinal plants in global ethnopharmacology as traditional medical practices including Chinese, Tibetan, and Ayurvedic medicine (Zhang *et al.*, 2019).

T. bellirica, widely referred to as 'Bahera' is a deciduous tree inherent to the Indian subcontinent and a key component of the traditional Ayurvedic formulation "Triphala". Literature studies revealed that *T. bellirica* showed antioxidant, antiobesity, anticancer and antimicrobial properties (Jayesh *et al.*, 2017; Makihara *et al.*, 2012; Dharmaratne *et al.*, 2018). The previous studies have reported the presence of numerous bioactive secondary metabolites in this species such as phenolic compounds, flavones, alkaloids, coumarins, lignans, terpenoids, tannins, saponins and glycosides (Abraham *et al.*, 2014). While previous studies have established the antimicrobial potential of *T. bellirica* fruit, its efficacy as a nematicidal agent against the root-k not nematode *M. incognita* has not yet been reported.

Therefore, this study was designed to assess the *in vitro* nematicidal activity of different percentages of aqueous and ethanolic extracts of *T. bellirica* fruit against *M. incognita*, with particular emphasis on egg hatching inhibition and second-stage juveniles (J_2) mortality. Moreover, cytotoxic evaluation of ethanolic extract of *T. bellirica* fruit was also conducted.

2. Materials and Methods

2.1 Nematode inoculation

The second-stage infective juveniles (J_2) of *M. incognita* used in this study were obtained from a pure culture maintained on tomato plants grown in micro-plots within a screen house at the NematologicalField, CCSHAU, Hisar, Haryana, India. Galled tomato roots bearing mature egg masses were gently washed to remove adhering soil, after which the egg masses were isolated under a stereo-microscope and rinsed

Corresponding author: Ms. Ekta
Department of Chemistry, CCS Haryana Agricultural University, Hisar-125004, Haryana, India
E-mail: ektarewari@gmail.com
Tel.: +91-7015913910

thoroughly with sterile distilled water (SDW). The egg masses were then transferred to a 0.5% sodium hypochlorite (NaOCl) solution and agitated for 4 min to dissolve the gelatinous matrix. The released eggs were subsequently washed three times with SDW on a 26 µm mesh sieve and used within 1 h of extraction. J2s were obtained by incubating the cleaned eggs on modified Baermann funnels at ambient temperature (30°C ± 2°C).

2.2 Collection and extraction of plant material

T. bellirica fruits were collected in December and identified by taxonomist of Dravyaguna Department of Shri Krishna AYUSH University, Kurukshetra. Fruit Specimen (Herbarium) has been in record (CHEM/0010) for future reference. The *T. bellirica* fruits were shade-dried and pulverized into a fine powder. The fruit powder (70 g) was extracted with absolute ethanol using Buchi universal extractor. After 6 h extraction, the ethanolic filtrate was concentrated under vacuum using a rotary evaporator at 40°C to produce the semisolid ethanolic crude extract (11.8 g). Similarly, the fruit powder (50 g) was extracted with distilled water using Buchi universal extractor to get aqueous filtrate which was lyophilized to form hygroscopic off-white extract (4.6 g). Both ethanolic and aqueous extracts were kept at 4°C until further use.

2.3 Chemicals

All chemicals used in this study were purchased from Hi Media and Sigma-Aldrich and were of AR grade.

2.4 Toxicity studies

2.4.1 Sample preparation

The ethanolic and aqueous extracts of *T. bellirica* fruits were dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA) to prepare stock solution at a concentration of 10 mg/ml. The stock solutions were sonicated and stored at 4°C until used in biological studies.

2.4.2 Cell line

African green monkey kidney (Vero) cells were procured from the National Centre for Veterinary Type Cultures (NCVTC), ICAR-National Research Centre on Equines (ICAR-NRCE), Hisar. The cells were cultured in Minimum Essential Medium (MEM) (Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, USA) and antibiotics (Penicillin and Streptomycin 1X, Sigma, St. Louis, USA).

2.4.3 Cytotoxic assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity assay was performed with minor modifications (Mosmann, 1983). The cytotoxic activity of *T. bellirica* fruit ethanolic extract was assessed against African green monkey kidney (Vero) cells. Vero cells, in triplicate, were cultured in 96-well tissue culture plates and treated with 3-fold serial dilutions of *T. bellirica* fruit than olic or vehicle control (100% ethanol) in a total volume of 100 µl of growth medium for 96 h. To each well, 20 µl of freshly prepared 5 mg/ml MTT solution was added, and the plates were incubated at 37°C for 5 h. Following incubation, the medium was removed, and 200 µl of DMSO was added to each well to dissolve the purple formazan product. The plates were then incubated at 37°C for an additional 5 min to allow bubbles to escape. MTT absorbance was measured photometrically at 570 nm. Doxorubicin was used as a positive control for cytotoxicity, while untreated cells and vehicle controls (0.1% DMSO) served as negative controls. Cell viability (%) was calculated relative to the untreated control. The percentage of cell viability was calculated using the following formula:

$$\text{Cell viability (\%)} = \frac{\text{Mean absorbance of the sample}}{\text{Mean absorbance of the control}} \times 100$$

2.5 Nematicidal studies

2.5.1 Sample preparation

The TBFEE stock solution (6 g/50 ml) was prepared in distilled water using ultrasonicator. 18 ml of each concentrated solution, *i.e.,* 1%, 5%, 10%, 15% and 20% were prepared for the experiment under laboratory conditions. Similarly, the stock solution of TBFAE was prepared in distilled water using ultrasonicator.

2.5.2 Egg hatching inhibition assay

For egg hatching assay, two egg masses were added in each cavity block containing 3 ml of the different concentration of TBFEE and TBFAE. They were exposed to 3 ml of the test extract at five concentrations, *i.e*., 1%, 5%, 10%, 15% and 20% of TBFEE and TBFAE in 4 cm diameter plastic cavity block. Each treatment was performed in triplicate. All treatments were kept at 28 ± 2°C. Egg hatching inhibition was recorded after 24, 48 and 72 h. The per cent egg hatching inhibition was calculated by the formula (Kaur *et al*., 2021):

$$\text{Per cent egg hatching inhibition} = \frac{\text{Egg hatching in control} - \text{Egg hatching in treatment}}{\text{Egg hatching in control}} \times 100$$

2.5.3 Percent mortality assay

In this assay, 20 freshly hatched second stage juveniles (J_2) were taken in each cavity block containing 3 ml of the different concentration of TBFEE and TBFAE. Carbofuran was taken as standard and water was considered as control for the comparison of results. Stereoscopic microscope was used after 24, 48 and 72 h of time intervals at magnification 4x to study the mortality percentage. All the experiments were done in triplicates and samples were stored at 25°C. Juveniles were considered dead when no movement was observed after mechanical nudge, their irreversible mobility was confirmed by transferring them to distilled water. The per cent mortality was calculated by the formula (Kaur *et al*., 2021):

$$\text{Per cent mortality} = \frac{\text{Number of dead juvenile nematodes}}{\text{Total number of juvenile nematodes}} \times 100$$

3. Results

3.1 Cytotoxic study

The cytotoxic evaluation of ethanolic extract of *T. bellirica* fruit was assessed against African green monkey kidney (Vero) cells to determine the per cent cell viability using the MTT assay. The cell viability results at different concentrations (100, 20, 4, 0.8, 0.16 and 0.32 µg/ml) of *T. bellirica* fruit ethanolic extractare summarize in Table 1. It demonstrated a good effect on Vero cells within the concentration range of 0.16 µg/ml to 0.032 µg/ml (Figure 1). The highest viability

of the ethanolic extract against Vero cells was found at a concentration of 0.032 µg/ml, with 64.5% cell viability. The median cytotoxic concentration (CC_{50}) was estimated as 0.57 µg/ml.

3.2 *In vitro* nematicidal assessment of *T. bellirica* fruit ethanolic and aqueous extracts on J_2s mortality and egg hatching

The effect of different concentration of *T. bellirica* fruit aqueous and ethanolic extracts (TBFAE and TBFEE) was studied on the egg hatching and mortality of J_2s juveniles of *M. incognita* for 24, 48 and 72 h interval under laboratory conditions. The viability of J_2s of *M. incognita* was decreased significantly over time by all concentration of ethanolic extract of *T. bellirica* fruit with respect to control. Different concentrations of TBEE extract, *i.e.*,1,5,10,15 and 20% had good effect on J_2s mortality with 20% being the most effective. The EC_{50}value of percent juvenile mortality was observed as 5% concentration after 72 h exposure. Results presented in Table 2 indicated that TBFEE showed 91.6% juvenile mortality and 76.4% egg hatching inhibition at 20% concentration (Figures 2-3). Mortality increased with exposure period. The EC_{50} value for the J_2 mortality was estimated to be 5% in case of TBFEE.

Table 1: *In vitro* cytotoxicity assessment of *T. bellirica* fruit ethanolic extract

S. No.	*T. bellirica* fruit ethanolic extract (µg/ml)	% Cell viability
1	100	12.3
2	20	12.8
3	4	63.1
4	0.8	56.5
5	0.16	54.5
6	0.032	64.5

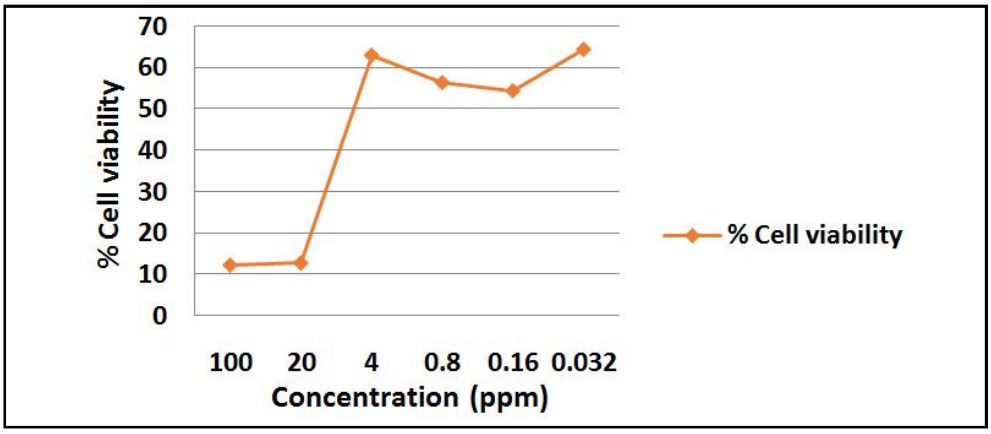

Figure 1: Effect of different concentration of TBFEE on cell viability.

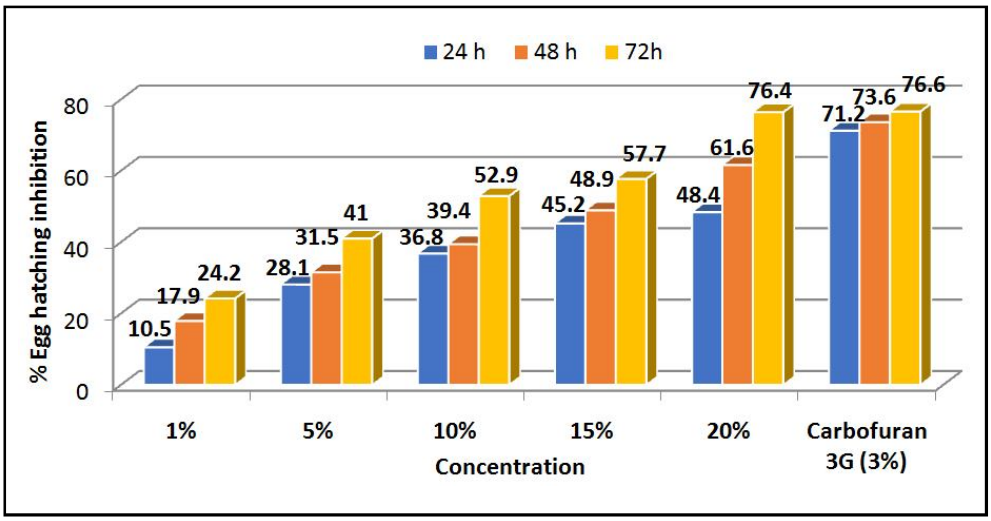

Figure 2: Per cent egg hatching inhibition of different concentration of TBFEE.

Table 2: ***In vitro*** **nematicidal evaluation of TBFEE on J_{2s} per cent juvenile mortality and per cent egg hatching inhibition of *M. incognita***

Concentration (%)	24 h		48 h		72 h	
	Per cent juvenile mortality	Per cent egg hatching inhibition	Per cent juvenile mortality	Per cent egg hatching inhibition	Per cent juvenile mortality	Per cent egg hatching inhibition
1	6.6	10.5	20	17.9	38.3	24.2
5	15	28.1	25	31.5	50	41
10	15	36.8	30	39.4	58.3	52.9
15	21.6	45.2	36.6	48.9	75	57.7
20	33.3	48.4	46.6	61.6	91.6	76.4
Carbofuran 3G (3%)	71.2	71.2	73.6	73.6	76.6	76.6

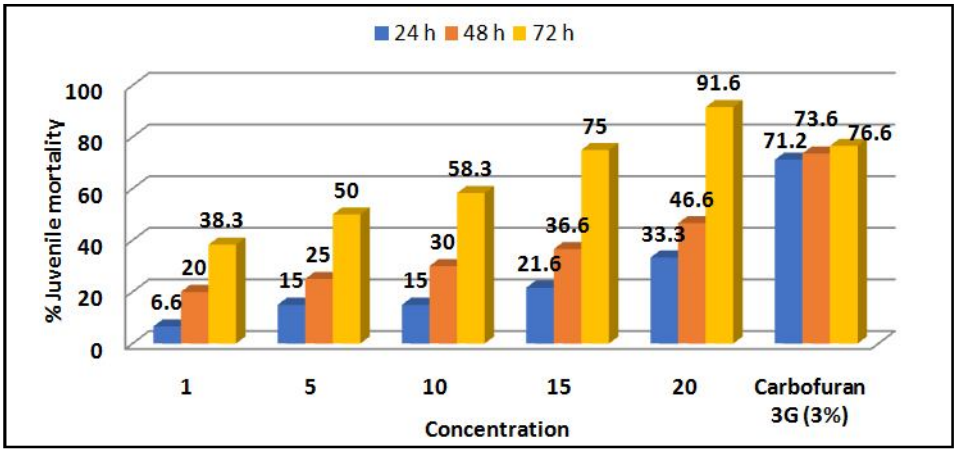

Figure 3: **Per cent J_2s juvenile mortality at different concentration of TBFEE.**

Table 3: ***In vitro*** **nematicidal evaluation of TBFAE on J_2s per cent juvenile mortality and per cent egg hatching inhibition of *M. incognita***

Nematicidal activity	24 h			48 h			72 h		
	100 ppm	200 ppm	400 ppm	100 ppm	200 ppm	400 ppm	100 ppm	200 ppm	400 ppm
Percent juvenile mortality	5.3	13.7	15.0	11.3	15.0	29.3	25.7	30.3	51.0
Percent egg hatching inhibition	3.3	11.0	11.3	9.3	12.3	25.3	22.7	27.4	47.3

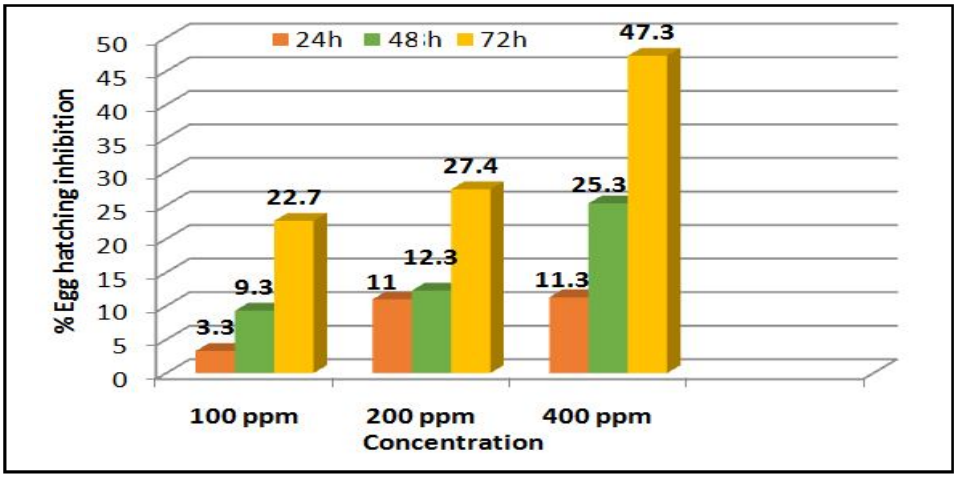

Figure 4: **Per cent egg hatching inhibition of TBFAE at different concentration.**

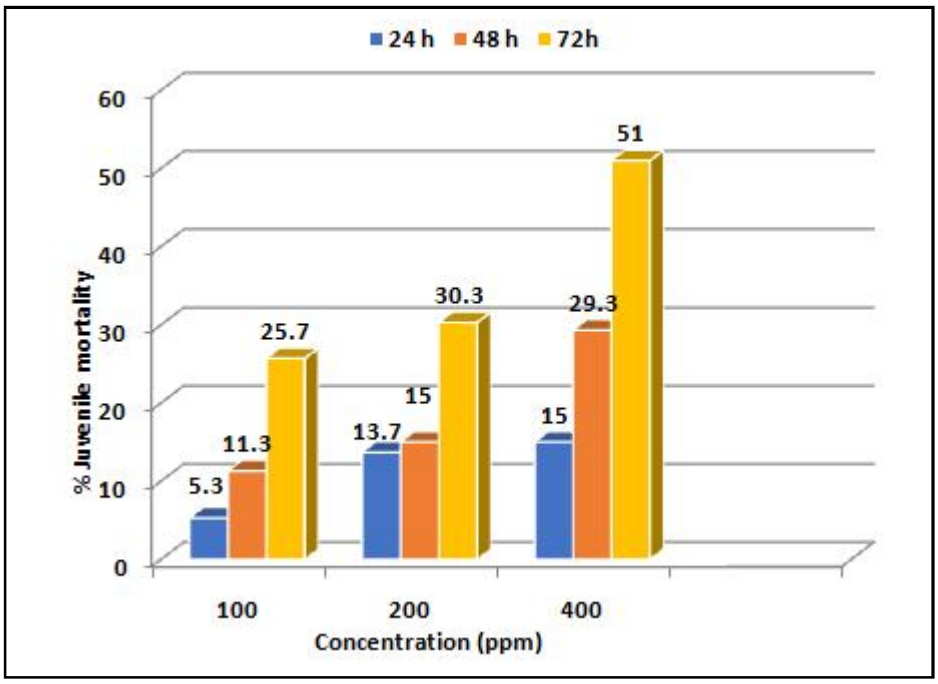

Figure 5: Per cent juvenile mortality of TBFAE at different concentration.

Similarly,the viability of *M. incognita* J_2s decreased significantly over time at all tested concentrations of aqueous extract of *T. bellirica* fruit. Hatching inhibition was significantly increased in eggs incubated at 100, 200 and 400 ppm. Data presented in Table 3 showed that TBFAE exhibited 51% juvenile mortality and 47.3% egg hatching inhibition at 400 ppm concentration as compared to blank control, in a dose-dependent manner (Figures 3-4). The dead juveniles appeared immobile with a characteristic straightened morphology.

4. Discussion

The objective of the present study was to evaluate the efficacy of *T. bellirica* fruit against the globally prevalent root-knot nematode *M. incognita*. The results of *in vitro* experiment demonstrated that both ethanolic and aqueous extracts of *T. bellirica* fruit had good nematicidal activity on J_2s and eggs of *M. incognita* across all tested concentration. These effects increased proportionally with both the concentration of the extracts and the duration of exposure. Results reported herein revealed that ethanolic extract showed more suppressive effects in eggs hatchability as well asjuvenile mortality in comparison to the aqueous extract leading to 91.6% J_2 mortality and 76.4% hatch inhibition at 20% concentration after 72 h exposure.There was small difference was noticed between eggs hatch inhibition and juvenile mortality at the same concentration (400 ppm) of aqueous extract.

The greater efficacy of the ethanolic extract may be attributed to its higher content of active secondary metabolites. As *T. bellirica* fruit is being reported for the first time in the biological control of plant-parasitic nematodes, the findings of this study may open new avenues for developing plant-based nematicides

5. Conclusion

In conclusion, our study demonstrates that *T. bellirica* fruit has promising potential as a biocontrol agent against *M. incognita*. Such an approach could help reduce the risks and environmental hazards associated with synthetic nematicides, particularly in vegetable crops intended for fresh consumption. However, more information is needed on secondary metabolite identifications, mode of action, mechanism involve in nematode suppression by compounds presents in *T. bellirica* fruit.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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