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RESEARCH ARTICLE

Nematicidal activity of *Lagenandra toxicaria* Dalz and *Kaempferia rotunda* L. rhizome extracts against root-knot nematode, *Meloidogyne incognita* (Kofoid and White) Chitwood and burrowing nematode, *Radopholus similis* Cobb

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Abstract
The plant parasitic pests such as the root-knot nematode *Meloidogyne incognita* and burrowing nematode, *Radopholus similis* are considered as devastating pathogens that are responsible for huge economic loss worldwide. The development of huge resistance, high costs and non-targeted effects of synthetic nematicides made control of these pests'extremely challenging. In this scenario the present study evaluated the nematicidal potential of the rhizome extracts of two plants viz. *Lagenandra toxicaria* and *Kaempferia rotunda* against these pests. Ethyl acetate and ethanol extracts of both plants were showing strong nematicidal properties against *M. incognita* and *R. similis*. An increase in the exposure time significantly increased the mortality of nematodes. The ethyl acetate extract of *K. rotunda* (KrEA) at 8 mg/mL concentration reduced the survival rate of *M. incognita* and *R. similis* to 3.57 ± 3.5% and 9.67 ± 4.27% respectively after 72 h treatment. Similarly, the ethanolic extract (KrOH) at the same concentration reduced the survival of *M. incognita* and *R. similis* to 5.36 ± 5.1% and 13.83 ± 5.38% respectively following 72 h treatment. Ethyl acetate extract (LtEA) and ethanolic extract (LtOH) of *L. toxicaria* at 8 mg/mL, reduced the survival rate of *M. incognita* to 9.37 ± 8.08% and 18.75 ± 8.19% respectively. On the contrary, water extracts of both plants were least effective against both these parasites. The LC_{50} values calculated using probit analysis highlighted the potential of ethyl acetate and ethanol extracts of both plants against *M. incognita* and *R. similis*. Based on LC_{50} values, the water extract of both plants was recorded to be less toxic against the tested nematodes.

Keywords Carbosulfan · Carrot-disc culture · Phytochemicals · Rhizome extract · Synthetic pesticides

Introduction

Meloidogyne incognita (root-knot nematode) and *Radopholus similis* are two major parasitic species affecting many crops (Abd-Elgawad and Askary 2015; Wiratno et al. 2009). They have a wide range of hosts and cause severe damages to many crops which leads to annual economic losses estimated at $173 billion (Elling 2013). *M. incognita* infested roots undergo morphological changes including galls or swollen appearance associated with frequent cracking and splitting (Seenivasan and Senthilnathan 2018). Since the nematode infection uplifts the destruction of root tissues, it ultimately reduces the water and mineral uptake, leading to reduced plant growth and yield (Davide and Marasigan 1985; Jonathan and Rajendran 1998; Sarah 2000). In India, root-knot nematodes, *M. incognita* are considered one among the chief enemies for green house crops that induces severe yield losses under fields and protected cultivation system (Jain et al. 2007; Kashyap and Siddiqui 2020; Singh et al. 2021). Various controlling methods have been adopted to prevent/reduce nematode infections nowadays, and foremost among them is the usage of synthetic chemicals as nematicidal agents (Seenivasan 2017). Although these synthetic chemicals have been very successful in down regulating the nematodes, they have also been found to have certain environmental downsides (Mei et al. 2021; Sill 1982). One such is, it raises concern of the occurrence of residues in

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the vegetables that are cultivated for fresh consumption affecting human health (Elbadri et al. 2008). Furthermore, high costs, parasite resistance and unpredictable outputs associated with synthetic nematicides have augmented the importance of alternative, safe methods for managing plant-parasitic nematodes (Mei et al. 2021; Viaene et al. 1998).

Currently, natural plant derived products are obtaining more consideration due to their easily degradable, cheaper and eco-friendly nature (Ansari et al. 2020). Many plant species are naturally resistant to parasitic nematodes, pathogens, and insect pests by their own defence mechanisms (Elbadri et al. 2008). Some important reports in this direction include marigolds (Targets spp.), rattlebox (*Crotalaria spectabilis*), chrysanthemums (*Chrysanthemum* spp.), garlic (*Allium sativum*), cinnamon (*Cinnamomum verum*), neem (*Azadirachta indica*), mint (*Mentha spicata*), *Eruca sativa* and *Sinapis alba* (Aissani et al. 2015; Caboni et al. 2013; Duke 1990; Kong et al. 2007; Lee et al. 2001; Park et al. 2005; Satti et al. 2003; Satti et al. 2006; Shalaby et al. 2021). Many plants effectively resist parasite infection through the production of secondary metabolites. These plant-derived chemicals are generally non-persistent under field conditions as they are readily transformed by light, oxygen, and microorganisms into non-toxic/fewer toxic products (Ujvary 2001). The random isolation, identification, and studying the nematicidal capacity of such compounds may lead to the discovery of novel pesticides with relatively less non-targeted effects. These herbal products provide a practical solution to the non-targeted effects and environmental issues caused by synthetic pesticides (Cetintas et al. 2018; Kim et al. 2005).

Lagenandra toxicaria Dalz. (Family- Araceae; vernacular name- Neerchengazhi) is a semi-aquatic herb endemic to southwestern part of India. The rhizome extracts of this plant has been widely utilized as an important ingredient in the folklore medicinal formulations (Chopra et al. 1994) in different parts of the country. Another plant, *Kaempferia rotunda* L., commonly known as Indian crocus (*Bhumi champa* in Sanskrit; Family Zingiberaceae) is a perennial aromatic herb with a tuberous rhizome, distributed throughout India and cultivated in countries like Indonesia, Vietnam and Malaysia for medicinal purposes (Lim 2016; Voravuthikunchai et al. 2007). The medicinal property of *K. rotunda* was evaluated and well documented by many previous studies (Atun et al. 2013; Jagadish et al. 2016; Kabir et al. 2013; Krishnakumar et al. 2021; Lotulung et al. 2008). The present study was executed to test the nematicidal potentials of ethyl acetate, ethanol and water extracts of *L. toxicaria* and *K. rotunda* rhizomes against the root-knot nematode, *M. incognita* and burrowing nematode, *R. similis*. To the best of our knowledge this is the first report on the efficacy of these plants against these nematode parasites.

Materials and methods

Collection of plant materials

Lagenandra toxicaria and *Kaempferia rotunda* plants were collected from Vellikulangara area of Thrissur district of Kerala, India (10°20′20.2"N 76°27′45.5"E), and the taxonomic identification was made and herbarium specimens (*L. toxicaria* 7001 and *K. rotunda* 7002) are kept at the Department of Botany, University of Calicut, Kerala, India. Rhizomes of both plants were separated and dried at 45 °C and powdered mechanically. The powdered rhizome was extracted with ethyl acetate, ethanol, and water separately in a soxhlet apparatus and was concentrated using a rotary evaporator at 45 °C for approximately 4 h and stored in amber-colored bottles at 4 °C. The stock was made by dissolving 100 mg of this crude extract in 1000 µl DMSO.

Preparation of nematode inoculum

For *M. incognita* the extraction of nematode eggs was executed according to the method proposed by Hussey and Barker (1973) with slight modifications. Briefly, *M. incognita* culture was initiated with a single egg mass that was surface-sterilized in 1% sodium hypochlorite for about 1 min. This was followed by washing in sterile distilled water and inoculated into a pot containing 3 weeks old tomato plants (*Solanum lycopersicon* L.) grown in sterilized soil. The temperature was maintained at 25 ± 3 °C and humidity at 70% for about 3 months. Post 3 months growth, the root-knot nematode eggs were extracted from the roots of these infested tomato plants. The eggs collected in a mesh sieve (20 µm) were incubated at 28 °C in hatching chambers. The second-stage juvenile *M. incognita* (J2) with a maximum age of 48 h after hatching were taken for identification using standard procedures (Hartman and Sasser 1985). Post-identification, the *M. incognita* (J2) were used for nematicidal assays. The *R. similis* second-stage juveniles (J2) were extracted according to the maceration and filtration technique proposed by Southey (1986) with slight modifications. The nematodes were extracted from the roots of black pepper (*Piper nigrum*) which were previously infested. For this, roots were cut into small pieces and submerged in sterile water in a Petri-plates and kept for 48 h at 25 °C. The water containing infiltrated *R. similis* were then cultured on carrot disks (about 6–7 week old disks). The second stage juveniles of *R. similis* for the mortality assay were collected from the cultures maintained on carrot disks and identified using the method proposed by Huettel and Rebois (1985).

Mortality test of nematode juveniles

The test was conducted in 6 well microtiter plates. 100 juvenile nematodes were placed in each well in 0.5 ml water. The serial concentration of each plant extract in the total volume of 0.5 ml in 0.5% DMSO was added to make a final concentration of 8, 4, 2, 1, and 0.5 mg/mL together with water containing the worms. Negative and positive controls were run with 0.5% DMSO and 0.1% carbosulfan respectively. Each test concentration was replicated 6 times and the experiment was performed thrice. Nematode mortality was assessed after 24 h, 48 h, and 72 h of exposure using a stereomicroscope. The toxicity of the rhizome extracts was assessed as the mean percentage of the dead nematodes. Dead nematodes were scored based on a complete lack of motion. The nematodes were poked with a needle when their status remained uncertain (Cayrol et al. 1989).

Statistical analysis

The data collected are expressed as mean ± SE. The survival rate of the parasites after treatment at different time points was calculated and the Kaplan–Meier survival curve was plotted using Graph pad Prism software version 5. Extract concentration required to induce 50% (LC_{50}) mortality to the juvenile nematodes was calculated using probit analysis in SPSS version 24.0.

Results

Bioactivity of extracts against *Meloidogyne incognita*

The nematicidal activity of ethyl acetate, ethanol and water extracts of *K. rotunda* and *L. toxicaria* rhizome extracts against *M. incognita* revealed that the ethyl acetate extract of *K. rotunda* exhibited pronounced mortality against the root-knot nematode. Figure 1A shows the survival curve of *M. incognita* (J2) juveniles when treated with different concentrations of KrEA. At the highest test concentration (8 mg/mL) the survival rate decreased to 28.57 ± 8.53% following 24 h treatment. This was further decreased to 7.14 ± 4.86% and 3.57 ± 3.5% respectively after 48 h and 72 h treatment. Similarly, in the alcoholic extract (KrOH) treatment the survival rate of nematodes decreased to 48.27 ± 9.27%, 10.72 ± 7%, and 5.36 ± 5.1% after 24 h, 48 h and 72 h respectively (Fig. 1B). Water extract treatment showed the lowest nematicidal activity against the *M. incognita* (J2) juveniles (Fig. 1C) with 66.35 ± 7.37 percent survival even at 8 mg/mL concentration. The data obtained by calculating the LC_{50} value post 24 h also revealed the high toxicity of KrEA and KrOH extracts against the root-knot nematode. The KrEA extract showed the lowest LC_{50} value (3.10 mg/mL) during the initial 24 h (Table 1). This was followed by KrOH extract that showed 24 h LC_{50} of 4.64 mg/mL. The KrWT was less toxic to the nematode as the LC_{50} values were beyond the maximum concentration tested (> 8 mg/mL) even after 72 h post treatment (Table 1).

Among the three extracts tested for *L. toxicaria*, LtEA exhibited pronounced mortality to the juveniles of *M. incognita* (Fig. 1D). At the highest test concentration of 8 mg/mL, LtEA treatment reduced the survival to 50 ± 9.44% following 24 h of treatment. After 48 h, the survival rate further reduced to 18.75 ± 9.26%. The LtOH treatment also showed considerable mortality to the *M. incognita* juveniles (Fig. 1E). At the highest test concentration (8 mg/mL), it showed the lowest survival rate of 18.75 ± 8.1 percent post 72 h of treatment. Same time the water extract of *L. toxicaria* (LtWT) showed the least mortality with 84.74 ± 4.68% of the nematodes surviving post 24 h treatment. Even though the survival rate decreased to 78.22 ± 5.63 and 75.33 ± 6.12 percent respectively after 48 h and 72 h, it was statistically insignificant (Fig. 1F). Carbosulfan (0.1%) was used as a reference for the assay and DMSO (0.5%) was used as vehicle control. Carbosulfan treatment reduced the survival to 9.52 ± 6.4 percent after 24 h which reached 100 percent after 48 h of treatment. Whereas no significant decrease in mortality was observed in the DMSO-treated animals. The initial 24 h LC_{50} values of all the three *L. toxicaria* rhizome extracts against the plant parasitic nematode *M. incognita* exits the maximum test concentration limit (> 8 mg/mL). So that the 48 h and 72 h LC_{50} values were calculated using probit analysis to determine the toxicity of *L. toxicaria* extracts. Here also the data obtained by calculating the LC_{50} values post 48 h also revealed the high toxicity of LtEA extract followed by LtOH extract (Table 1). The 48 h LC_{50} value of LtEA and LtOH extracts were 1.076 mg/mL and 2.140 mg/mL respectively. The LtWT was less toxic to the nematode as the LC_{50} values were greater than the maximum concentration used (> 8 mg/mL) even after 72 h of treatment (Table 1).

Bioactivity of the extracts against *Radopholus similis*

The survival rate of *R. similis* treated in the highest concentration of KrEA (8 mg/mL) significantly decreased to 42.3 ± 6.85%, 12.08 ± 4.6% and 9.67 ± 4.27% respectively after 24 h, 48 h and 72 h of treatment (Fig. 2A). Following KrEA, the ethanolic extract (KrOH), at the aforementioned concentration reduced the survival rate of *R. similis* to 45.28 ± 6.83%, 12.35 ± 4.68% and 10.29 ± 4.33% at similar time points (Fig. 2B). Water extract (KrWT) showed the lowest activity against *R. similis* (Fig. 2C). Probit analysis calculated the LC_{50} of *K. rotunda* rhizome extracts against *R. similis*. The data obtained by calculating the LC_{50} value after

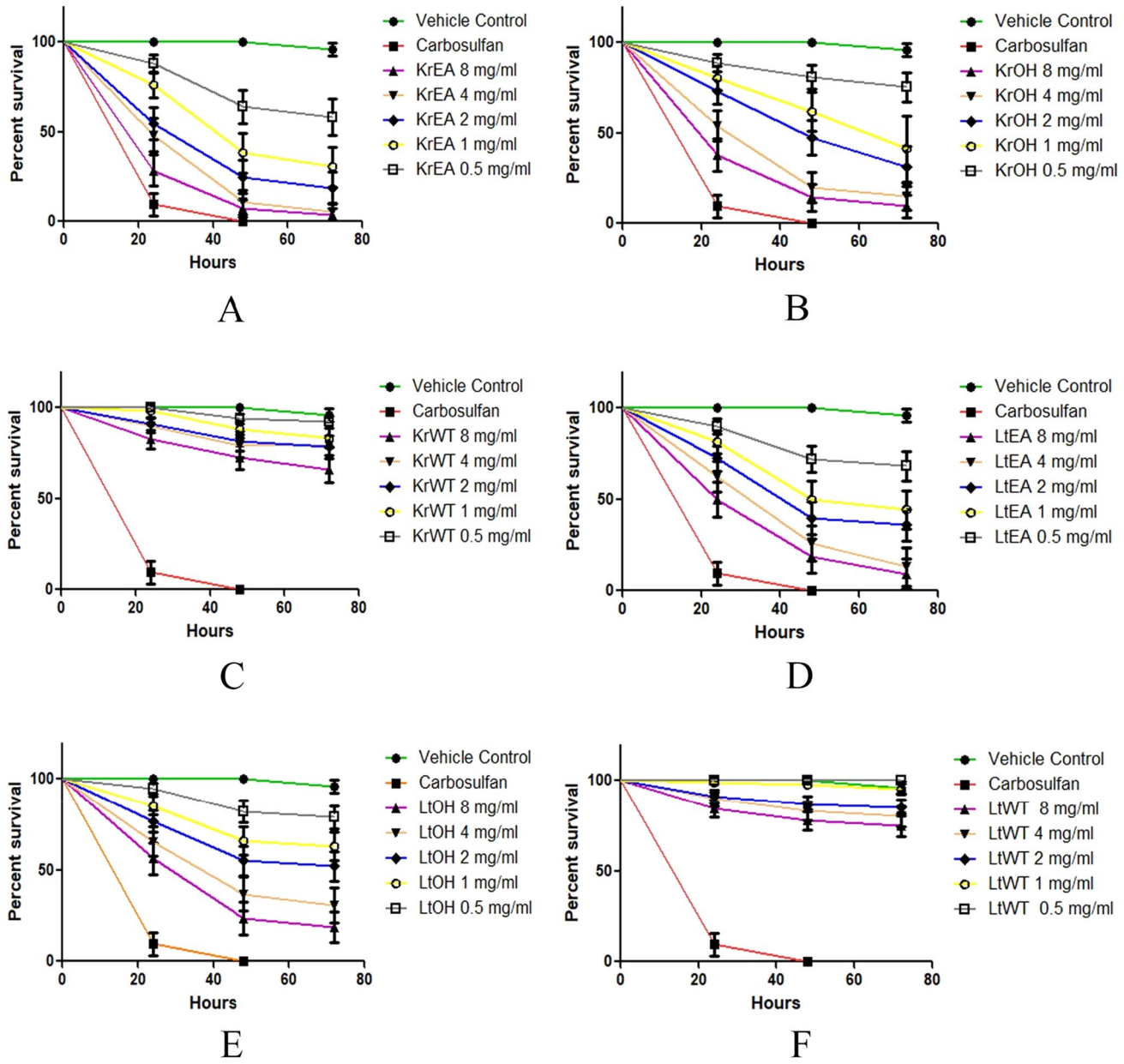

Fig. 1 The survival rate of *M. incognita* (J2) juveniles when treated with different concentrations of **A** Ethyl acetate (KrEA), **B** Ethanol (KrOH), and **C** Water (KrWT) extracts of *K. rotunda* and **D** Ethyl acetate (LtEA), **E** Ethanol (LtOH) and **F** Water (LtWT) extracts of *L. toxicaria*

24 h showed toxicity of KrEA and KrOH extracts against *R. similis*. The KrEA extract showed the lowest LC_{50} value (5.352 mg/mL) during the initial 24 h (Table 2). This was followed by KrOH extract with a 24 h LC_{50} of 6.823 mg/mL. On the other hand, even after 72 h the LC_{50} value of KrWT extract was calculated to be > 8 mg/mL.

Among the *L. toxicaria* rhizome extracts, LtEA and LtOH were moderately active against *R. similis*. Whereas LtWT extract failed to show any considerable nematicidal potential when compared with the 0.5% DMSO (vehicle control). Figure 2D showed the percent survival of *R. similis* treated in different concentrations of LtEA. At the highest test concentration (8 mg/mL) the survival rate decreased to 37.47 ± 9.89 percent following 72 h of incubation. In the case of LtOH extract at 8 mg/mL concentration, the survival rate decreased to 43.9 ± 10.34 percent after 72 h (Fig. 2E). Same time post 72 h, 86.45 ± 5.34 percent of nematodes survived the treatment in 8 mg/mL concentration of LtWT (Fig. 2F). Also, at lower concentrations of LtWT (2–0.5 mg/mL) no mortality was observed. In carbosulfan treated groups the survival rate decreased to 41.66 ± 10.06 percent after 24 h and eventually decreased to zero percent after

Table 1 LC_{50} values in mg/mL (LCL—UCL) for *M. incognita* after 24 h, 48 h and 72 h of exposure to *K. rotunda* and *L. toxicaria* extracts using probit analysis

Extract used	Duration of treatment		
	24 h	48 h	72 h
KrEA	3.10 (2.550–3.894)	–	–
KrOH	4.64 (3.677–6.303)	–	–
KrWT	> 8	> 8	> 8
LtEA	> 8	1.092 (0.626–1.601)	–
LtOH	> 8	2.140 (1.775–2.592)	–
LtWT	> 8	> 8	> 8

48 h of treatment (Fig. 2A–F). Same time no mortality was observed in the DMSO treated groups (Fig. 2A–F) even after 72 h after treatment. Table 2 showed the LC_{50} values of *L. toxicaria* against *R. similis*. The initial 24 h LC_{50} values of all the three extracts of *L. toxicaria* exceeded the maximum test concentration used in the current study (> 8 mg/mL). The 48 h LC_{50} of LtEA and LtOH extracts were 3.96 mg/mL and 6.367 mg/mL respectively. On the other hand, 72 h LC_{50} value of LtWT extract was greater than the maximum concentration of the extract used in the present study.

Discussion

This in vitro study exhibited the nematicidal potentials of rhizome extracts of two plant species against the juveniles of *M. incognita* and *R. similis*. Among the six extracts tested, ethyl acetate and ethanol extracts showed better activity against both parasites. Water extracts of both plants failed to induce noticeable mortality when compared with the other extracts and carbosulfan (positive control). Probit analysis exposed the concentration of extract required to induce 50% (LC_{50}) mortality to the juvenile *M. incognita* and *R. similis*. *K. rotunda* ethyl acetate extract gave the lowest LC_{50} at the shortest time point of 24 h, compared to alcohol extract which took 48 h to induce 50% mortality. On the other hand, ethyl acetate extract of *L. toxicaria* took 48 h to induce 50% mortality. Water extract of both plants failed to induce any significant mortality and was unable to calculate the LC_{50} even after 72 h of treatment. There are several previous studies which recorded superior efficacy for the organic solvent extracts when compared to aqueous extract. The least bioactivity of water extracts of both plants in the present study is in line with the previous report of Oka et al. (2001) in which the organic solvent extracts of plant materials were found to be more toxic to the J2s of parasitic nematode sp than water extracts. Earlier, Abid et al. (1997) reported a huge mortality induced by the crude ethanolic extracts of *Fumaria indica* against the J2s of *Meloidogyne javanica*. Similarly, superior efficacy of n-hexane extract of *Fumaria parviflora* against *M. incognita* compared to chloroform, ethyl acetate and methanol extracts of the same plant were reported by Naz et al. (2013). These reports clearly indicated that different solvent extracts of the same plant species can vary in their anthelmintic potentials.

The mechanism of the nematicidal action of crude extracts of *K. rotunda* and *L. toxicaria* is not clear from our studies. It is assumed that the compounds in the crude extracts may be acting synergistically for the anthelmintic action. Previously Bizimenyera et al. (2006) reported that individual compounds isolated from the extracts exhibited less activity than the crude extracts. The synergistic deed of diverse metabolites in each rhizome extract may vary, which could elucidate the differential nematicidal potential of the six extracts against these parasitic nematodes. Polyphenols were among the major secondary metabolites extracted in our study. The ethyl acetate and ethanol extracts of *K. rotunda* and *L. toxicaria* showed a high amount of phenolics compared to water extract (Table S1 & S2). The organic solvent extract gives a good cocktail of these bioactive molecules to induce mortality in the parasites. It is noteworthy that the rhizomes of both these plants under study are generally well protected from these parasitic worms.

Over the past couple of years, major concerns have been increasingly expressed regarding the possible unintended side effects of chemicals used in veterinary and agricultural practices (Lumaret and Errouissi 2002). The problems associated with carbosulfan and other synthetic chemicals on non-target organisms have been studied and proved previously. A previous study on the lethal concentration and toxicity stress of carbosulfan, glyphosate (Roundup), and atrazine to freshwater fish *Channa punctatus* proved that carbosulfan insecticide was more toxic to *C. punctatus* than glyphosate and atrazine herbicides (Nwani et al. 2010). Certain other studies exposed the carbosulfan induced mitotic aneuploidy in the yeast *S. cerevisiae* (Wiedenmann et al. 1990) and have mutagenic and genotoxic effects on non-target animals (Nwani et al. 2010). The acute effects of carbosulfan on the larval pest of *Sphodroxia maroccana* (targeted organism) and on two (non-targeted) beetles *Pimelia platynota* and *Pachychila* sps (Fegrouche et al. 2014) were also added the severity of the side effects caused by synthetic agents. An alternative for this issue is using plant-derived compounds, which are comparatively less toxic to non-target organisms and the environment. Results of the current study highlighted the possibility of using the organic solvent extracts of *K. rotunda* and *L. toxicaria* as an environmentally safe remedy against the plant parasitic nematodes *M. incognita* and *R. similis*. Nevertheless more studies are required to elucidate the mechanisms of action of these crude extracts

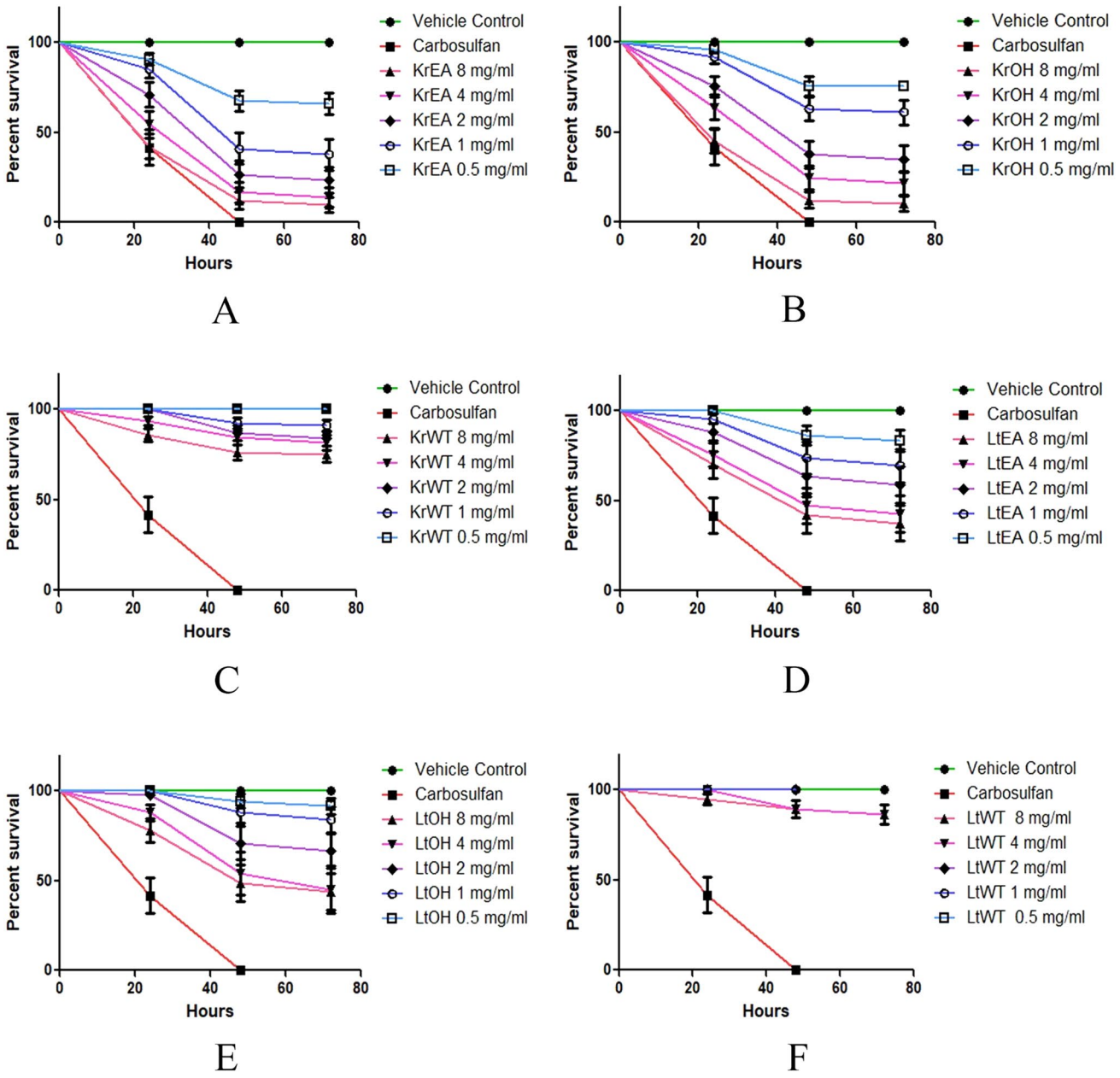

Fig. 2 The survival rate of *R. similis* juveniles when treated with different concentrations of **A** Ethyl acetate (KrEA), **B** Ethanol (KrOH), and **C** Water (KrWT) extracts of *K. rotunda* and **D** Ethyl acetate (LtEA), **E** Ethanol (LtOH) and **F** Water (LtWT) extracts of *L. toxicaria*

Table 2 LC_{50} values in mg/mL (LCL—UCL) for *R. similis* after 24 h, 48 h and 72 h of exposure to *K. rotunda* and *L. toxicaria* extracts using probit analysis

Extract used	Duration of treatment		
	24 h	48 h	72 h
KrEA	5.352 (4.217–7.377)	–	–
KrOH	6.823 (5.372–9.475)	–	–
KrWT	> 8	> 8	> 8
LtEA	> 8	3.960 (3.029–5.667)	–
LtOH	> 8	6.367 (4.896–9.207)	–
LtWT	> 8	> 8	> 8

on the tested nematodes and the chemical nature of the active compounds responsible for the observed activity.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42360-022-00527-3.

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Declarations

Conflict of interest The authors declare no conflicts of interest.

Animal welfare and ethics statement No ethical approval was required as there were no experiments performed using any vertebrate animals.

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