Research Article

Evaluation of Nematocidal Action against *Caenorhabditis elegans* of Essential Oil of Flesh Fingered Citron and Its Mechanism

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Essential oils from flesh fingered citrons were obtained by mechanical pressing extraction under optimal conditions. Thirty-three components, representing 78.25% of the total oil, were identified by gas chromatography-mass spectrometry (GC-MS). In this study, we explored the toxicity of the essential oil of flesh fingered citrons and the associated regulatory mechanism using *Caenorhabditis elegans* as a model organism. The mortality experiment showed that the LD_{50} value is 1.48 mg/ml. Oxidative stress experiments showed that essential oils of flesh fingered citrons can reduce the antioxidation of nematodes and can shorten their lifespan. In survival rate experiments for verification, d-limonene had a nematocidal effect by destroying the waterproof protective layer on the surface of the worm. β-Pinene can interfere with the normal growth of nematodes according to behavioral experiments, and reproduction experiments showed that α-pinene has very strong nematocidal properties. γ-Terpinene also has good nematocidal activity. The results demonstrated that the essential oil of flesh fingered citron has great potential to act as a new nematicide.

1. Introduction

Controlling insect pests and nematodes by synthetic nematicides is expensive and environmentally harmful. The search for naturally occurring, cheaper, and more environmentally friendly biocontrol agents is ongoing throughout the world [1]. Currently, plant chemicals are being increasingly used as effective botanical nematicides in the form of extracts and essential oils because synthetic nematicides cause many negative effects on the environment [2]. Therefore, we have explored natural plant sources for pest management agents. Due to the infrequent presence and inconsistent efficacy of synthetic pesticides, plant nematicides may be suitable alternatives to disrupt the physiological processes of target nematodes in addition to direct mortality [3, 4]. Because of their nematocidal toxicity, essential oils are considered a possible alternative [5]. These oils have been found to have significantly negative impacts

on nematode survival, reproduction, development, behavior, and metabolic pathways [6, 7] and have been studied as nematocidal, larvicidal, and repellent agents [8].

Essential oils are plant-based economically important secondary metabolites [9]. Citrus essential oils are extracted from citrus peels [10] that produce characteristic odors and have been widely applied to the food (beverage, sweets, and so on), cosmetics, and pharmaceutical industries mainly because of their fragrance and flavor [11]. The essential oils of the flesh fingered citron (*Citrus medica* cv. sarcodactylis) are used as an expectorant to treat cough, asthma, and inflammation, which is generally produced by mechanical extraction like all citrus oils [12]. Flesh fingered citron essential oil can be divided into two fractions, with the volatile fraction (93%–96%) containing monoterpenes and sesquiterpenes, such as d-limonene, α- and β-pinene, γ-terpinene [13], and other oxygenated components (4%–7%). It is an essential oil of intermediate to low odor and flavor intensity

[14]. Terpenoids that come from this essential oil are important nematocidal substances and have strong biological activity. Thus, this essential oil has been used in agricultural nematicide research. Different components like monoterpenoids (e.g., α-pinene) are responsible for nematocidal properties and physiological disruptions of nematodes [15, 16]. These compounds can be used as antifeedants, digestion and metabolism breakers, acetylcholinesterase inhibitors, and cell death inducers by acting as oxidizing agents, which can cause ionic leakage of cells and degeneration of mitochondria and proteins [17–22]. Limonene, a monocyclic monoterpene, is famous for its citrus-like olfactory properties [23]. Limonene is also an important precursor for many herbal and pharmaceutical compounds such as perillyl alcohol, carvone, and menthol [24, 25]. D-Limonene is the most widely used isomer in spices, medicines, and pesticides [26]. As a nematicide, it can break the waterproof protective layer of the worm's body surface and has strong penetrating properties so that worms die by suffocation, reducing their survival rate.

Many studies have employed the nematode *Caenorhabditis elegans* as an animal model to study the effects of essential oils [27]. *C. elegans* is also used as an epidemiological model for stress and aging because of its small size, relatively short lifecycle, and the clear impact of genetic and environmental factors that affect the lifespan [28].

In this study, we used *C. elegans* as an *in vivo* model to determine the mortality effect of essential oils. We examined the mechanism of action of essential oils and the main individual components on *C. elegans* mortality and tested our hypotheses through a series of experiments on oxidative stress, lifespan, reproduction, and survival rate.

2. Materials and Methods

2.1. Plant Materials and C. elegans. Mature fruits of the flesh fingered citron were harvested in November 2017 from a local orchard in Jinhua City, Zhejiang Province, P.R. China. Wild-type *Caenorhabditis elegans* (N2) and food *E. coli* OP50 strains were obtained from the *Caenorhabditis* Genetics Center.

2.2. Chemicals. D-Limonene (95%), α-pinene (98%), β-pinene ($\geq$95%), and γ-terpinene (95%) were obtained from Macklin (China). Cholesterol, calcium chloride, sodium chloride, potassium dihydrogen phosphate, disodium phosphate, magnesium sulfate, and absolute ethanol were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). Agar powder was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Potassium dichromate ($K_2Cr_2O_7$), dimethyl sulfoxide (DMSO), and 5-fluoro-2-deoxyuridine (FUDR) were purchased from Sigma (USA). Peptone (LP0037), tryptone (LP0042), and yeast extract (LP0021) were purchased from OXOID (UK). All other chemicals and solvents used were of analytical grade.

2.3. Essential Oil Preparation. The mechanical pressing method is suitable for the extraction of easily burnt raw materials such as citrus and lemon because it is easy to maintain the structure and function of essential oils. Flesh fingered citron fruits were first screened and peeled, saturated in 3% (w/v) calcium chloride or calcium hydroxide for a specified time (4 h) to remove pectin, and then cleaned and leached with distilled water. The soaked flavedo (50 g) was weighed and turned into juice using a juice extractor. The juice was supplemented with sodium chloride (0.25%) and stirred until the sodium chloride completely dissolved. After the solution was separated into layers, the oil-water mixed liquid obtained after filtering was centrifuged, and the upper oil layer of the mixture was separated and dried with anhydrous sodium sulfate [29]. The extraction rate of essential oils was about 0.3%.

The purification was carried out using a molecular distillation method carried out by a laboratory rubbed molecular distillation device (Pope, USA). At 45°C melted in the water bath, essential oil was drawn into a feed bottle and degassed using a short-path evaporator. The distillation temperature, distillation pressure, wiping roller speed, feed flow rate, and cooling water temperature were set to 70°C, 50 Pa, 200 rpm, 4 mL/min, and 10°C, respectively. Then, the feeding was turned on, and the degassed feed liquid immediately became a very thin film and spread onto the evaporation surface. A different number of volatile components evaporated as the liquid flowed down the wall through the heating zone. More volatile components concentrated onto the closely positioned internal condensing surface and became the distillates, while the less volatile components flowed down along the cylinder and became the residues. After distillation, the distillate and residue fractions were weighed and transferred to the containers. They were stored at 4°C until analysis [30]. The purification rate of essential oils was about 85%.

2.4. GC and GC-MS Analysis. A fused silica capillary column (HP-5MS) was used for GC analysis with a Hewlett-Packard 6890 gas chromatograph (5% phenyl-methylsiloxane, 60 m × 0.25 mm, film thickness 0.25 μm, Agilent Technologies, USA). The oven temperature was set to rise from an initial temperature of 50–100°C at a rate of 10°C/min and held there for 10 min and then raised to 140°C at a rate of 3°C/min. It was kept at 140°C for 10 min and finally heated to 230°C at a rate of 2°C/min and held there for 10 min. The split injection mode with a split ratio of 1 : 10 was employed. Quantification of components in the essential oil was obtained based on the GC peak areas using both the internal standard method and relative response factors. GC/MS analysis was performed on a Hewlett-Packard 6890 gas chromatograph coupled with a Hewlett-Packard mass-selective detector 5973N quadrupole mass spectrometer (MS) (Agilent Technologies, USA). All GC experimental parameters were the same as described for GC-FID analysis. The MS was operated in the electron impact ionization (EI) mode with an ionization energy of 70 eV. The temperatures of the MS source and MS quadrupole were 280 and 150°C, respectively. The constituents of the essential oil were identified based on computer matching with the Wiley/NIST

library and comparisons of retention indices with those reported in the literature [31].

2.5. C. elegans Strains, Handling, and Mortality.

The nematodes used in this study were Bristol wild-type N2 [32]. *C. elegans* was grown and assayed on NGM agar plates carrying *E. coli* OP50 at 20°C. The phytochemicals were dissolved in 0.1% DMSO. All chemicals in the NGM plate and liquid are expressed in the final concentrations.

To investigate the nematocidal effect of the flesh fingered citron essential oil, a survival assay was deployed. In this assay, the worms were treated with essential oils at varying concentrations of 0, 0.5, 1, 1.5, and 2 mg/ml. *C. elegans* mortality was determined after 24 h exposure, and LD_{50} was calculated [33].

2.6. Mechanism Experiments of Essential Oil Nematicide in C. elegans

2.6.1. Oxidative Stress Resistance Assays for C. elegans.

Oxidative stress assays were performed essentially as previously described [34]. Wild-type N2 *C. elegans* was synchronized to the L4 period and pretreated with 0, 0.5, 1, 1.5, and 2 mg/ml essential oil and 0.1% DMSO as a solvent control for 48 h, then exposed to $K_2Cr_2O_7$ (8 µl, 10 mmol/L), and finally cultured for 48 h. Worms were observed every 4 h, and their survival was determined by the touch-provoked movement [35]. At least three independent biological replications were performed.

2.6.2. Lifespan Assays on C. elegans.

L4 larval *C. elegans* (wild-type N2) were transferred to 96-well plates with S-medium to inhibit progeny development in the presence of 0, 0.5, 1, 1.5, and 2 mg/ml essential oil of flesh fingered citron dissolved in 0.1% DMSO. We evaluated whether the essential oil influenced the lifespan of *C. elegans* by comparing the lifespans of untreated (control) and essential oil-treated wild-type N2 *C. elegans*. Live and dead *C. elegans* was counted every other day (from the first day of adulthood) until all *C. elegans* had died as determined by the touch-provoked movement. At least three independent biological replications were performed.

2.6.3. Survival Rate of C. elegans.

As a verification experiment, L1 stage larvae were treated with the essential oil solution for 3 days to the L4 stage and exposed to air for half an hour, and then the survival rate was determined. We performed at least three independent biological replications.

2.6.4. Behavioral Experiments on C. elegans.

Body bends and head swings indicate alternating muscle contractions. Synchronized L4 adults were transferred to an empty NGM board and allowed to be free for at least 1 min. The number of body flexions within 20 sec and the number of head swings with a drop of distilled water within 1 min were determined. We performed at least three independent biological replications.

2.6.5. Reproductive Assays of C. elegans.

The effect of essential oil on fecundity was assessed using age-synchronized N2 wild-type worms [36]. The total progeny yield of each worm after exposure to essential oil (0, 0.5, 1, 1.5, 2 mg/ml) was assessed according to standard protocols. Essential oil-treated/untreated L4 larvae were transferred to fresh treatment/control plates until reproduction terminated. Three days later, the number of nematodes in the two plates was determined. We performed at least three independent biological replications.

2.7. Mortality Rate Inflicted by Four Key Components in Essential Oil.

The experiments were similar to those in Section 2.5. Four major components, D-limonene, γ-terpinene, α-pinene, and β-pinene (5 mM each), were used to treat *C. elegans*. At least three independent biological replications were performed using at least 100 *C. elegans* strains.

2.8. Statistical Analysis.

For the oxidative stress assays and lifespan assays, we used GraphPad Prism (GraphPad Software). Survival curves resulting in p values <0.05 were considered significantly different relative to untreated controls. Statistics were analyzed using SPSS Statistics Software (SPSS). Results are expressed as the mean-± standard error of the mean (SEM). Univariate analysis of variance was performed using the ANOVA algorithm, while the LSD test was used for minimally significant difference analysis. *Post hoc* tests to determine the significance and interaction between the factors of the lifespan of *C. elegans* were performed with a two-factor variation analysis (two-way ANOVA). Differences were considered significant at the $P < 0.05$, $P < 0.01$, and $P < 0.001$ levels [35].

3. Results and Discussion

3.1. GC-MS.

Qualitative and quantitative analytical results of the chemical composition of essential oil of flesh fingered citron by GC/MS are listed in Table 1. Quantification of analytes was carried out by peak area normalization and expressed in terms of the percentage of the total peak area to facilitate comparison with data reported in the literature. As shown in the table, 33 compounds representing 78.25% of the total peak area were identified. The essential oil consisted mainly of monoterpene hydrocarbons, and the main ones were d-limonene (43.39%), γ-terpinene (18.57%), α-pinene (2.88%), β-pinene (2.69%), β-myrcene (1.77%), (Z)-β-ocimene (1.46%), and α-thujene (1.31%). The components of EO in the present study had a composition similar to that in the previous reports about other species of citrus fruits [37, 38].

3.2. Essential Oils of Flesh Fingered Citron Kill Wild-Type C. elegans.

The lethal doses (LD_{50}) were used to compare the effects of the essential oil exposure in contact toxicity assays [15]. In residual toxicity bioassays, mortality differences were found according to the concentration. The mortality rate of the essential oil of flesh fingered citron to *C. elegans* is

TABLE 1: Major components from the essential oil of fingered citron.

No.	Constituent	Retention index[a]	Percentage[b]
	Monoterpene hydrocarbons		
1	α-Thujene	919	1.31
2	α-Pinene	931	2.88
3	β-Pinene	980	2.69
4	α-Phellandrene	1005	0.26
5	δ-3-Carene	1015	0.30
6	α-Terpinene	1019	0.23
7	D-Limonene	1032	43.39
8	γ-Terpinene	1066	18.57
9	Terpinolene	1087	1.44
10	β-Myrcene	987	1.77
11	(Z)-β-Ocimene	1045	1.46
12	2-Carene	1018	0.31
13	β-Phellandrene	964	0.38
	Sesquiterpene hydrocarbons		
14	β-Caryophyllene	1425	0.19
15	(S)-β-Bisabolene	1485	0.46
16	α-Bergamotene	1437	0.33
17	Germacrene D	1485	0.032
	Oxygenated monoterpenes		
18	Linalool	1095	0.13
19	trans-Limonene oxide	1139	0.12
20	Terpin-4-ol	1183	0.28
21	α-Terpineol	1196	0.43
22	Neral	1235	0.23
23	Nerol	1229	0.051
24	Geranial	1263	0.28
25	Neryl acetate	1349	0.039
26	Geranyl acetate	1368	0.032
27	Geraniol	1252	0.28
28	cis-Limonene oxide	1138	0.079
29	(Z)-Carveol	1222	0.061
30	Thymol	1292	0.017
	Oxygenated sesquiterpenes		
31	Caryophyllene oxide	1612	0.13
32	Spathulenol	1604	0.061
	Nonisoprenoid components		
33	Decanal	1200	0.032
	Total		78.25

[a]Relative to C_5–C_{25} n-alkanes calculated from a nonpolar HP-5MS™ capillary column. [b]Based on FID peak area normalization.
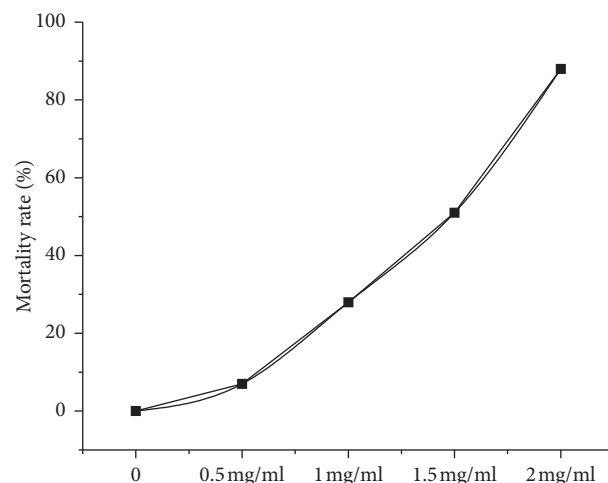

FIGURE 1: Essential oil of the flesh finger citron causes mortality of *C. elegans*. At least three independent biological replications were performed, and statistical significance of the difference between the curves was demonstrated by origin analysis.

citron in 0.1% dimethyl sulfoxide (DMSO) had a nematocidal effect on *C. elegans* via oxidative stress (Figure 2). Essential oils contain a variety of compounds that may unleash different biological activities. Moreover, higher concentrations of the extract may be toxic, and the higher the concentration, the greater the toxicity, thus reducing the antioxidation effect of *C. elegans*.

3.3.2. Essential Oil of Flesh Finger Citron Affects the Lifespan of Wild-Type C. elegans.

The *C. elegans* model system was used to assess the effects of essential oils on aging. The increase in free radical production and the destruction of the redox balance lead to oxidative stress, causing aging and age-related diseases [40]. Senescence is well illustrated in *C. elegans* and shares homologous mechanisms with primates and humans [41–43]. The experimental results (Figure 3) show that the higher the concentration of essential oil, the more obvious the nematocidal effect, and the lower the concentration of essential oil, the longer the survival of *C. elegans*. This indicates that the essential oil of flesh fingered citron can reduce the lifespan of *C. elegans*.

3.3.3. Survival Rate.

The formation of allergenic oxidation products like D-limonene oxide and dD-limonene 2-hydroperoxide has been reported [44] for oxidized citrus oil (rich in d-limonene), a frequent skin sensitizer. D-Limonene can break the waterproof protective layer of the worm's body surface and has strong penetrating properties so that worms die by suffocation. It can be seen from the graph (Figure 4) of the survival rate of *C. elegans* that the presence of essential oils of flesh fingered citron reduces the survival rate of nematodes.

3.3.4. Slowed Behavioral Activity in C. elegans.

Terpenes are the largest group of phytochemicals resulting from secondary metabolism [45]. Some components (e.g., β-pinene)

linear for each dose concentration range (Figure 1). When the concentration range of the essential oil is 1.0–1.5 mg/ml, the equation can be obtained from the linear relationship: $y = 46x - 18$, so the LD_{50} value is 1.48 mg/ml.

3.3. Mechanism Experiments

3.3.1. Essential Oil Affects Oxidative Stress Resistance of C. elegans.

It has been widely reported that many kinds of citrus essential oils from natural plants exhibit antioxidant, antiradical, and antimicrobial properties [34]. $K_2Cr_2O_7$ is an oxidative stress inducer because of its heavy metal oxidant that produces an intracellular oxidation-reduction reaction with O_2 production and is commonly used to generate intracellular oxidation in *C. elegans* [39]. Pretreatment with different concentrations of the essential oil of flesh fingered
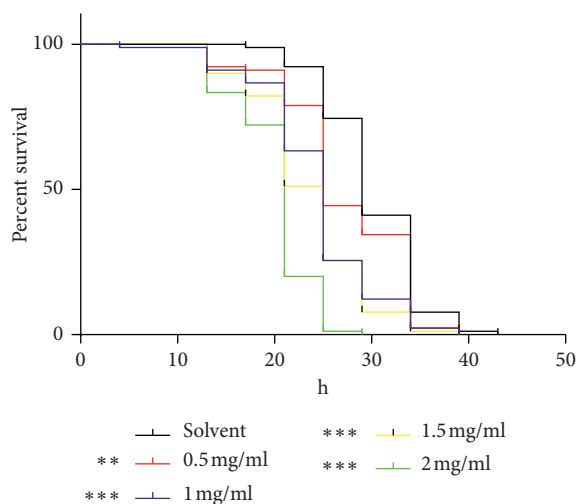
FIGURE 2: Essential oil of flesh fingered citron affects the oxidative stress resistance of wild-type *C. elegans*. At least three independent biological replications were performed, and statistical significance of the differences between curves was demonstrated by a log-rank test using Kaplan–Meier survival analysis. Error bars represent the standard error, and differences compared to the solvent (0 mg/ml, 0.1% DMSO) were considered significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ by one-way ANOVA and the LSD post hoc test.
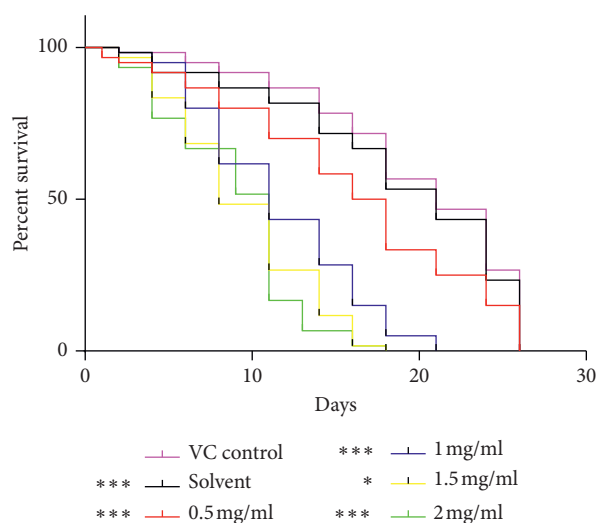

FIGURE 3: High concentrations of the essential oil of flesh fingered citron shorten the lifespan of wild-type *C. elegans*. Day 1 refers to the first day of treatment after adulthood. Results include at least three independent biological replicates. Statistical significance of the difference between the curves was demonstrated by a log-rank test using Kaplan–Meier survival analysis. Error bars represent the standard error, and differences compared to the solvent (0 mg/ml, 0.1% DMSO) were considered significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ by one-way ANOVA and the LSD post hoc test.
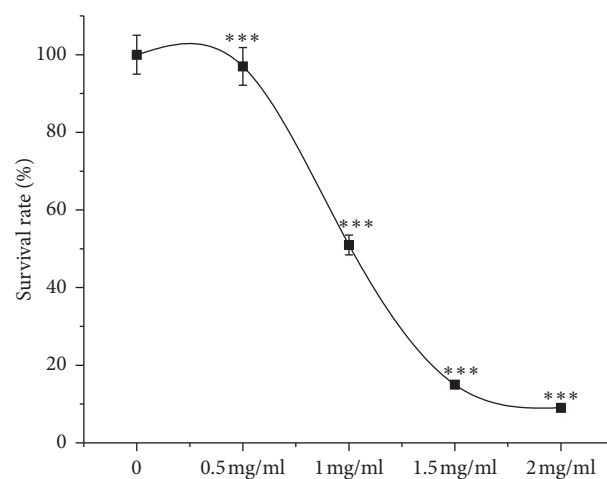

FIGURE 4: Effects of essential oils on survival rates in *C. elegans*. Results show at least three independent biological replicates. Error bars represent the standard error, and differences compared to the control (0 mg/ml, 0.1% DMSO) were considered significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ by one-way ANOVA and the LSD post hoc test.

are highly toxic and interfere with the normal growth of *C. elegans* and affect behavior. It can be seen from the results (Table 2) that different concentrations of essential oil have a strong inhibitory effect on the number of body bends of nematodes and reduce the number of head swings. The essential oil of flesh fingered citron therefore affects the behavior of nematodes in addition to its nematocidal action.

3.3.5. Reproduction Rate Reduced in *C. elegans*.

As an essential oil component, γ-terpinene has good insecticidal activity [46] and is highly toxic. α-Pinene is a strong repellant [47], has nematocidal characteristics, and induces physiological changes in some nematodes, and through the production of oxidizing compounds, it acts as an antifeedant, digestive and metabolic disruptor, acetylcholinesterase inhibitor, and cell death inducer, leading to the ionic leakage of cells and the degeneration of mitochondria and proteins [6]. It can be seen from the fertility rate (Figure 5) that because of reduced synthesis of DNA, the reproduction of nematodes treated by the essential oil was lowered.

TABLE 2: Number of body bends and head swings.

Essential oil/0.1% DMSO (mg/ml)	0	0.5	1	1.5	2
Bending bodies/20 s	9.0 ± 1.63	8.8 ± 0.22	8 ± 0.65	6.6 ± 0.24	6.3 ± 0.26
Swinging heads/1 min	157 ± 4.32	147 ± 2.94	139 ± 5.35	126 ± 2.94	110 ± 2.87

Note. Data are expressed as the average value $\pm$ standard deviation ($n = 3$).
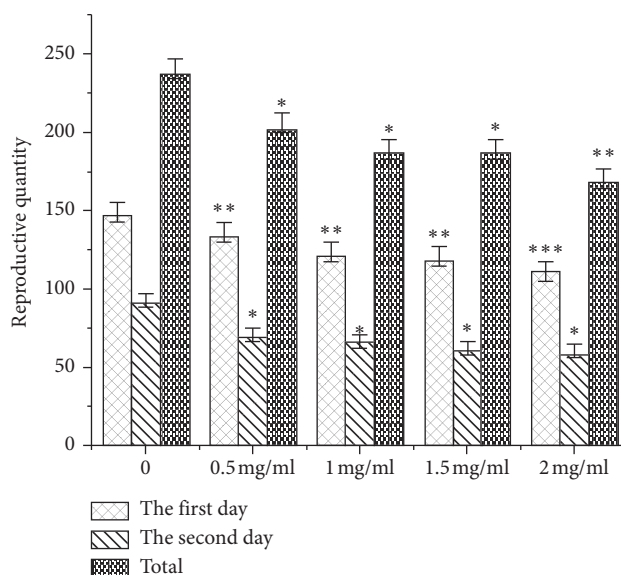

FIGURE 5: Effects of essential oils on the reproduction rate in *C. elegans*. Results include at least three independent biological replicates. Error bars represent the standard error, and differences compared to the control (for each column of 0 mg/ml, 0.1% DMSO) were considered significant at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ by one-way ANOVA and the LSD post hoc test.
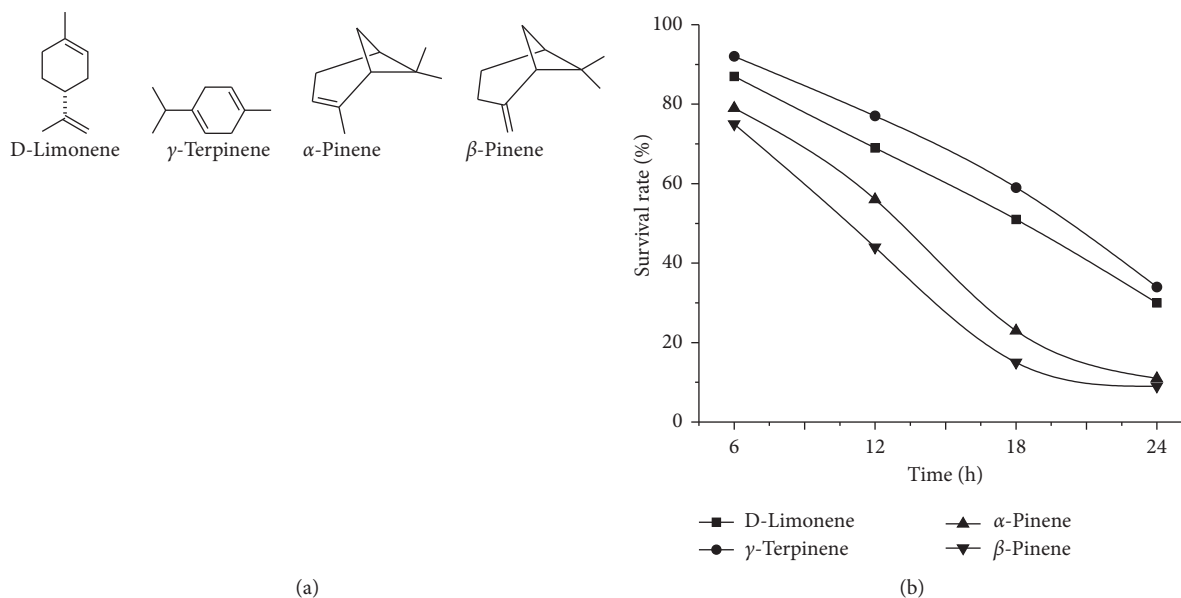

(a)

(b)

FIGURE 6: (a) Structural formulae of four components. (b) Effects of four components from the essential oil of flesh fingered citron on the mortality of *C. elegans*.

3.4. Effect on Mortality of Four Key Components in the Essential Oil.

The structural formulae of four components are shown in Figure 6(a). The survival rate of nematodes exposed to d-limonene was 30% at 24 h; it was 34% for γ-terpinene, 11% for α-pinene, and 9% for β-pinene (Figure 6(b)). The survival rate of nematodes exposed to

α-pinene was 23% and that of β-pinene was 15% at 18 h. Therefore, the components of the essential oil of flesh fingered citron have obvious nematocidal effects.

4. Conclusions

Plant essential oils and extracts directly affect nematode survival [48]. Essential oil extracted from the flesh fingered citron can be used as a plant-derived nematicide to kill C. elegans. At a dose of 2 mg/ml (about 0.2%, v/v), the mortality rate reached 88%. These concentrations are much lower than the concentrations of insecticidal and nematocidal essential oils in the literature studies [49, 50], and the LD_{50} value was 1.48 mg/ml. Furthermore, oxidative stress experiments showed that the essential oil can shorten the survival time of nematodes by reducing the antioxidant activity of C. elegans. Essential oils can also kill C. elegans by reducing its lifespan, in addition to affecting behaviors such as body bends and head swings and lowering the reproductive rate. Finally, four isolated components had a significant mortality effect on the nematodes, indicating that these four terpenes in the essential oil are responsible for its effects. Monoterpenes are the main ingredients and the key nematicides. This article uses C. elegans as a biological model to kill nematodes in the realistic laboratory, which lays an experimental basis for the subsequent development of new natural pesticides. It has a very important theoretical significance and application value. This essential oil is a candidate for partial or complete replacement of current chemically synthesized pesticides to enhance sustainability.

Data Availability

The numerical data used to support the findings of this study are included within the article.

Additional Points

Highlights. (1) A kind of plant-derived nematicide is proposed. (2) The lethal mechanism of flesh fingered citron essential oil on nematodes is studied. (3) The nematocidal effect is excellent which also can replace chemically synthesized nematicides, which is sustainable and environmentally friendly.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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