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Aliphatic Ketones from *Ruta chalepensis* (Rutaceae) Induce Paralysis on Root Knot Nematodes

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ABSTRACT: This paper reports on the use of *Ruta chalepensis* L. extracts as a potential nematicide against root knot nematodes *Meloidogyne incognita* and *Meloidogyne javanica*. The essential oil (REO) and methanol extract (RME) of *R. chalepensis* were tested against second-stage juveniles, with REO inducing paralysis in both species ($EC_{50/1d}$ = 77.5 and 107.3 mg/L) and RME being selective for *M. incognita* ($EC_{50/1d}$ = 1001 mg/L). Chemical characterization of extracts was done by means of GC-MS and LC-MS, revealing mainly aliphatic ketones and coumarins, respectively. The first-ranking volatile nematicidal component in terms of individual activity against both species was 2-undecanone (EC_{50} = 20.6 and 22.5 mg/L for *M. incognita* and *M. javanica*, respectively). This fact together with its high concentration in the most active extract found in this study, namely, REO (2926 mg/kg), categorizes 2-undecanone among the nematicidal principles of *R. chalepensis*. On the contrary, coumarins rutin and 8-methoxypsoralen were not found to be nematicidal at concentrations of ≤500 mg/L. Interestingly, *M. incognita* was found more sensitive than *M. javanica*.

KEYWORDS: GC-MS, LC-MS, *Ruta chalepensis*, ketones, 2-undecanone, *Meloidogyne incognita*, *Meloidogyne javanica*

INTRODUCTION

Most apomictic root knot nematodes (RKN; *Meloidogyne* spp.) have host ranges that encompass the majority of flowering plants.[1] Chemical nematicides, although significantly effective, have been worldwide severely restricted due to safety and environmental concerns.[2] Methyl bromide has been the most effective multipurpose soil fumigant, and since its ban, due to its destructive potential to the stratospheric ozone, no available chemical has equally replicated its role in crop protection. At present, secondary metabolites originating both from microorganisms and from plants are widely used as biocontrol agents to reduce ng nontarget exposure to hazardous pesticides and to face resistance development.[3,4] More than 90 nematicidal toxins includiquinines, alkaloids, terpenoids, peptides, pyrans, furans, and naphthalenes have been investigated.[5] Alcohols, acids, esters, ketones, and lipids are the principles of the mycofumigation effects caused by the endophytic fungus *Muscodor* sp. and soil bacteria and act among others also against phytonematodes.[5,6] Similarly, plant-derived aldehydes have been recently found to exhibit strong nematicidal fumigant activity against *Meloidogyne incognita*.[7] This fumigant effect is of most importance for nematode control because it helps reach the target inside the soil even in protected areas where nematodes reside.[8] *Ruta chalepensis* L. is a perennial herb growing wild in dry, often rocky regions of the Mediterranean area with stems 30−70 cm high and long lanceolate leaves. The inflorescence is lax and sepals are glabrous, whereas petals are oblong and the fruit is a glabrous capsule. It flowers from April to July and yields intriguing natural scents, encompassing volatile organic sulfur-containing constituents, ketones, alkaloids, flavonoids, phenols, amino acids, furocoumarins, and saponins.[9−11] The closely related species, *Ruta graveolens* L., known as common rue, is cultivated for commercial production of rue oil,[9,12] whereas in Italy rue leaves are sometimes added to grappa to obtain the so-called "grappa alla ruta". *R. chalepensis* L. (Rutaceae) is mentioned in "De Materia Medica" by Dioscorides and represents one of the most cited species of local origin found in prescriptions of traditional and local medicine of the Byzantine tradition in the eastern Mediterranean region,[13] whereas some *Ruta* species have been introduced in Chinese medicine.[11] Some of the biological activities exhibited by *R. chalepensis* L. are the insecticidal activity against insect vectors[14,15] and pests, with no detrimental effects on parasitoids;[16] activities against bacteria, fungi, and certain cancer cell lines,[17−21] an anti-inflammatory effect;[22−25], an anthelmintic activity;[26] and a depressant effect on the central nervous system.[12] Within the framework of our ongoing research on natural nematicides of plant origin, we have now tested *R. chalepensis* against two root knot nematode species. The aim of this work was to study for the first time (1) the chemical characterization of the *R. chalepensis* essential oil (REO) as well as the methanol extract (RME) by means of GC-MS and LC-MS and (2) the paralysis activity of *R. chalepensis* extracts and their constituent compounds against *M. incognita* and *javanica*.

MATERIALS AND METHODS

Chemicals. Standards of 2-nonanone, 2-nonanol, 2-decanone, octyl acetate, 2-undecanone, 2-dodecanone, 2-tridecanone, rutin, 8-methoxypsoralen, and angelicin of >98% purity, as well as Tween 20, were obtained from Sigma-Aldrich (Milan, Italy). Methanol and ethanol used were of HPLC grade, and the Water was Milli-Q purified.

Extraction and Chemical Characterization. *Plant Materials.* The aerial parts of *R. chalepensis* were collected in November 2010 (before flowering) from an area called "Calamosca" in Cagliari (Italy) and were dried in the absence of light at room temperature. Then they

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Figure 1. (Left) Paralyzed second-stage *M. incognita* juvenile; (right) tomato roots infested with *M. javanica*.

were sealed in paper bags and stored at room temperature kept in the dark, until use. Voucher specimens were deposited in the Department of Life and Environmental Sciences (Botany and Botanical Garden Division, Herbarium CAG, Sardinian Section, voucher specimen 272, University of Cagliari, Italy) for species identification.

RME. Dried aerial plant parts (100 g) were ground and extracted with MeOH (1:10 w/v) in a sonicator apparatus for 15 min, filtered through a Whatman no. 40, and centrifuged for 15 min at 13000 rpm. The extract was then dissolved in acetone and analyzed for component identification by means of GC-MS and LC-MS. The yield was determined on average over three replicates.

REO. The dried aerial plant parts were subjected to hydrodistillation for 4 h in a semi-industrial stainless steel apparatus. The REO obtained was dried over anhydrous Na_2SO_4 and stored in dark glass vials at −20 °C until use. The yield was determined on average over three replicates.

GC-MS Analysis. The chromatographic separation for REO component identification purposes was performed on a Hewlett-Packard 5980 series II (Hewlett-Packard, Avondale, PA) coupled with a HP 5971 A mass spectometer and a HP 7673 autosampler, in a split−splitless mode. Data were elaborated using MS HP ChemStation v. C.00.07. The column was fused silica capillary DB-5MS (5% phenylmethylpolysyloxane, 30 m × 0.25 mm; film thickness = 0.25 μm, J&W Scientific Fisons, Folsom, CA). Injector and interface temperatures were kept at 200 and 280 °C, respectively. The oven temperature was programmed as follows: from 60 to 180 °C (3 °C/min) and kept at this temperature for 15 min. The carrier gas was helium with a flow of 0.9 mL/min; the sample (1 μL) was injected in split mode (1:20). The MS conditions were as follows: ionization voltage, 70 eV; scan rate, 1.6 scan/s; mass range, 40−500; transfer line, 180 °C. The components of *R. chalepensis* were identified by (a) comparison of their relative retention times and mass fragmentation with those of authentic standards and (b) computer matching against NIST98 (99%), as well as retention indices as calculated according to Kovats, for alkanes C9−C24 compared with those reported by Adams.[27] Quantitative analysis of each component was carried out with an external standard method.

LC-MS Analysis. A Varian tandem mass spectrometer (Palo Alto, CA) consisting of a ProStar 410 autosampler, two ProStar 210 pumps, and a 1200 L triple-quadrupole mass spectrometer equipped with an electrospray ionization source was used. Varian MS workstation version 6.9 software was used for data acquisition and processing. The column used was a 150 × 2.0 mm, 5 μm, Varian Pursuit XRS. The mobile phase consisted of (A) bidistilled water containing 0.1% formic acid and (B) methanol. The elution started at A/B, 80:20 (v/v), arriving at 100% B in 10 min. The mobile phase, previously degassed with high-purity helium, was pumped at a flow rate of 0.3 mL/min, and the injection volume was 10 μL. The electrospray ionization source mass spectrometer was operated in ion switching mode. The electrospray capillary potential was set to 75 V, whereas the shield was at 575 V. Nitrogen at 50 mTorr was used as drying gas for solvent evaporation. The atmospheric pressure ionization (API) housing and drying gas temperatures were kept at 54 and 375 °C, respectively. The scan time was 1 s, and the detector multiplier voltage was set to 1450 V, with an isolation width of m/z 1.2. Quantitative analysis of each component was carried out with the external standard method. Peak areas were obtained by extraction of the characteristic ion in total ion mode. For calibration curves the calibration coefficients were between 0.9990 and 0.9998. For the establishment of the limit of quantification (LOQ) and determination (LOD), 1000 mg/L standard solutions of compounds of interest were gradually diluted with methanol. Each individual standard was injected three times. The LOD (S/N = 3) for each standard was 0.1 mg/L, whereas the LOQ (S/N = 10) was 1.2 mg/L for all compounds.

J2 Paralysis Bioassays. Freshly hatched J2 (24 h) (Figure 1) were extracted from tomato roots separately infested with *M. incognita* or *M. javanica* according to the method of Hussey and Barker[28] and were used for paralysis experiments. Nemathorin 150EC (ai fosthiazate 15%, Hellafarm Co.) and tap water as well as solvent carriers were used as controls for paralysis correction. The bioassays were performed in Cellstar 96-well cell culture plates (Greiner bio-one), and each treatment was represented by 25 J2 per well. Plates were covered with plastic lids and were maintained in the dark at 28 °C. Juveniles were observed with the aid of an inverted microscope (Euromex, The Netherlands) at 40× and were ranked into two distinct categories: motile or paralyzed. After the last assessment (1 day), J2 were washed with tap water through a 20 μm pore screen, to remove the excess of test substance, and were moved to plain water. Motility regain was studied by transferring J2 to tap water after the last assessment and observing again after 24 h. Paralysis results presented herein correspond to data before rinsing, because J2 never regained activity after moving to plain water. The paralysis experiments were performed twice, and every treatment was replicated per experiment six times.

RME, REO, and Constituent Compounds Activity against J2 Immersed in Treatment Wells. The RME and REO were tested against root knot nematodes at the dose ranges of 625−10000 and 20−320 mg/L, respectively. Compounds identified by GC-MS or LC-MS, namely, 2-nonanone, 2-nonanol, 2-decanone, octyl acetate, 2-dodecanone, 2-tridecanone, angelicin, 8-methoxypsoralen, and rutin, were individually subjected to dose response experiments against J2 at the dose range of 31.25−500 mg/L. Being the most active of pure substances tested, 2-undecanone was tested at the dose of 6.25−100 mg/L. As part of a study on the nematicidal activity of ketones, 2-heptanone and 3-octanone were also tested in the bioassay. Stock solutions of EO and pure

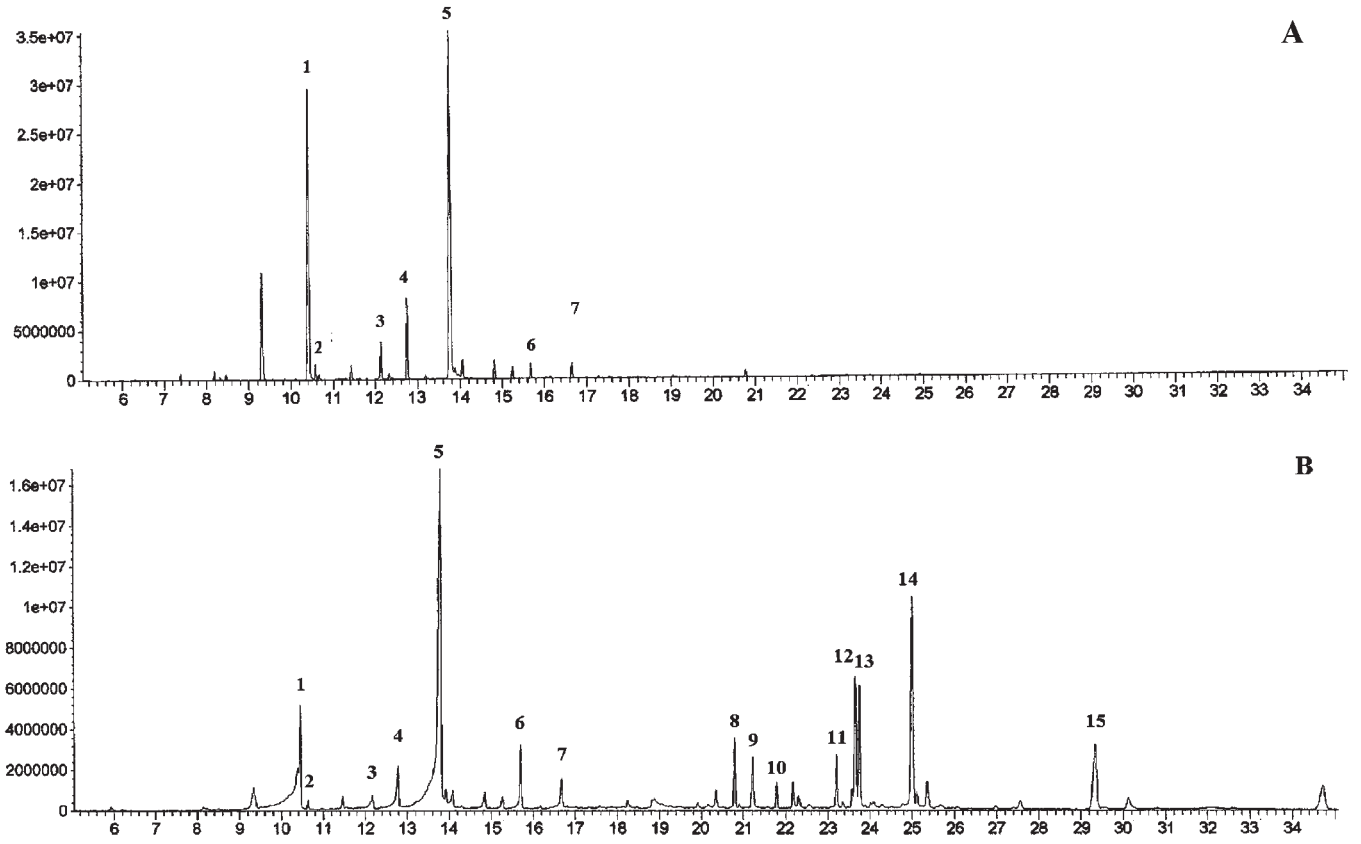

Figure 2. GC-MS chromatograms of (A) *R. chalepensis* essential oil extract and (B) *R. chalepensis* methanol extract. Peaks: (1) 2-nonanone, (2) 2-nonanol, (3) 2-decanone, (4) octyl acetate, (5) 2-undecanone, (6) 2-dodecanone, (7) 2-tridecanone, (8) unknown, (9) angelicin, (10) hexadecanoic acid methyl ester, (11) unknown, (12) phytol, (13) 8-methoxypsoralen, (14) unknown, (15) unknown.

Table 1. Constituent Compounds of RME and REO Determined by GC-MS Analysis, Listed in Order of Elution[a]

compound	KI	mol wt	EI-MS characteristics *m/z* (amu) (abundance)	RME constituents (mg/kg)	REO constituents (mg/kg)
2-nonanone	1091	142.24	58 (100%); 71 (35%)	75.8	2374
2-nonanol	1098	144.25	45 (100%); 57(25%); 69 (30%)	6.5	222
2-decanone	1192	156.27	58 (100%); 71 (45%); 45 (10%)	11.8	275
octyl acetate	1211	172.27	56 (100%); 70 (90%); 84 (85%)	32.7	nd
2-undecanone	1291	170.30	58(100%); 71(50%); 85 (10%)	208.2	2926
2-dodecanone	1369	184.31	58 (100%); 71 (45%); 85 (10%)	23.2	110
2-tridecanone	1392	198.34	58 (100%); 71 (40%); 85 (10%)	20.1	137

[a] Identification was done by comparison of mass spectra of commercial standards with the respective data of NIST Library in total ion current (TIC) and the literature, as well as retention indices as calculated according to Kovats for alkanes C9−C24 compared with those reported by Adams.[27] KI, Kovats index; nd, not detected.

compounds were prepared in ethanol and were successively diluted in distilled water containing the polysorbate surfactant 20 (Tween 20). The stock solution of the methanol extract was prepared in water, and similarly were the dilutions made. Final concentrations of ethanol and Tween 20 in treatment wells never exceeded 1 and 0.3% (v/v), respectively. Assessments were made 1 h and 1 day post bioassay start. In all cases motility recovery was assessed as well, as described previously.

Fumigant Activity Against J2. nematodes juveniles were immersed in water, in wells adjacent to the treatment wells with the test solution (RME, REO and pure compounds). For every treatment well four adjacent ones were used, and in each plate only one treatment dose. Paralysis percentages recording the fumigant activity in the four adjacent to the treatment wells, served as an experiment's treatment replication with 100 J2 instead of 25. Assessments were made 1 h (1 h) and 1 day (1d) after experiments start.

Statistical Analysis. Because paralysis in solvent (ethanol, Tween 20) did not differ significantly from that observed in distilled water, the percentages of paralyzed J2 were corrected by eliminating the natural death/paralysis in the water control (≤5% of total number of J2) according to the Schneider Orelli formula:[29] corrected % = [(mortality % in treatment − mortality % in control)/(100 − mortality % in control)] × 100. Experiment results were combined over time and were then analyzed (ANOVA) for the study of any significant treatment by time interaction. Because no such indication was evident, means of experiments over

time replication were averaged. Corrected percentages of paralyzed J2 treated with tested compounds were subjected to nonlinear regression analysis using the log−logistic equation proposed by Seefeldt et al.:[30] $Y = C + (D - C)/\{1 + \exp[b(\log(x) - \log(EC_{50})]\}$, where C = the lower limit, D = the upper limit, b = the slope at EC_{50}, and EC_{50} = the test substance's concentration required for 50% death/paralysis of nematodes after elimination of the control (natural death/paralysis). In the regression equation, the test substance's concentration was the independent variable (x) and the paralyzed J2 (percentage increase over water control) was the dependent variable (y). The mean value of the six replicates per test substance concentration and immersion (or exposure to vapors) period was used to calculate the EC_{50} value. Mean data values are presented with respective standard deviations (Figure 3).

RESULTS AND DISCUSSION

R. chalepensis yields (expressed as dry material) in REO and RME were 0.36 ± 0.1 and 1.41 ± 0.05% (w/w), respectively. Among ketones identified in *R. chalepensis* extracts, 2-undecanone, 2-dodecanone, and 2-decanone were the most active, followed by 2-nonanone and 2-tridecanone, whereas the coumarins rutin, angelicin, and 8-methoxypsoralen did not provoke paralysis in J2. Interestingly, the ester octyl acetate was found to exhibit high nematicidal activity equivalent to that of the most active ketones.

Specifically, according to the GC-MS analysis (Figure 2), both REO and RME afforded 2-nonanone, 2-nonanol, 2-decanone, octyl acetate, 2-undecanone, 2-dodecanone, and 2-tridecanone (Table 1), but the concentration levels in REO were higher than those contained in RME because EOs are in general very concentrated extracts. As a result of the GC-MS analysis, seven compounds accounting for 82.6% of the total oil were identified. When REO was tested against *M. incognita* and *M. javanica*, clear dose response relationships and significant paralysis of J2 were evident after 1 day of exposure for both species (Figure 3). Specifically, the $EC_{50/1day}$ values were calculated as 107.3 and 77.5 mg/L for *M. javanica* and *M. incognita*, respectively. These values are rather low considering the activities of other EOs against RKN, such as the 27 EOs tested by Oka and co-workers at 1000 mg/L,[31] and similar to the ones of *Pimpinella anisum* and *Foeniculum vulgare* ($EC_{50/1\ h}$ = 125 and 109 mg/L, respectively).[32] On the other hand, the LC-MS analysis of RME afforded mainly coumarins (Table 2) and specifically angelicin, 8-methoxypsoralen, and rutin. When RME was tested against RKN, it exhibited paralysis on *M. incognita* but failed to afford any effect on *M. javanica* (Figure 3). The $RME_{M.\ incognita}$ EC_{50} value was calculated as 1001 mg/L after 1 day of J2 immersion in test solutions. This activity is similar to the one exhibited by *Inula viscosa*, a plant species of notable nematicidal activity against RKN.[33,34] The selectivity of RME against *M. incognita* could be attributed to the enhanced resistance of *M. javanica*, as demonstrated by the EC_{50} values of pure compounds (Figure 3). Specifically, when ketones were tested individually at the dose range from 500 to 31.25 mg/L, clear dose response relationships were established against both species, but the EC_{50} values calculated against *M. incognita* were in all cases lower than the ones against *M. javanica*. Similar differences in these two nematode species susceptibility in paralysis induction are also reported by Al-Banna and co-workers.[35] Interestingly, neither RME nor REO showed any fumigant effect (data not shown).

When ketones were tested individually against RKN for paralysis activity, 2-heptanone and 3-octanone were not found to provoke any paralysis on J2 (data not shown), whereas 2-undecanone was the most active ($EC_{50/1day}$ = 22.5 and 20.6 mg/L for *M. javanica* and *M. incognita*, respectively). The activity of 2-undecanone is rather high considering the one of the commercial nematicide fosthiazate ($EC_{50/1day}$ = 15.9 and 0.4 mg/L for *M. javanica* and *M. incognita*, respectively). Additionally, 2-undecanone was the only treatment in which a fumigant activity was evident (20% over untreated control at the test concentration of 100 mg/L). Interestingly, 2-undecanone has already been reported to exhibit nematicidal activity against *M. incognita* but as a constituent compound of the plant growth-promoting rhizobacteria *Bacillus megaterium* YMF3;[36] also, other naturally occurring volatiles (alcohols, aldehydes, ketones, alkenes, ethers) from soil bacteria have been found to possess nematicidal activities against the free-living *Panagrellus redivivus* and the pinewood nematode *Bursaphelenchus xylophilus*.[5] 2-Undecanone as originally derived

Table 2. Summary of LC-MS Characteristics of RME[a]

peak	MW	compound	RME constituents (mg/kg)	characteristic ions in LC-MS
1	610	rutin	45.01	610 $[M]^-$
2	186	angelicin	27.82	187 $[M + H]^+$
3	216	8-methoxypsoralen	26.99	217 $[M + H]^+$

[a] Identification and quantitation was done by co-chromatography using commercial standards.

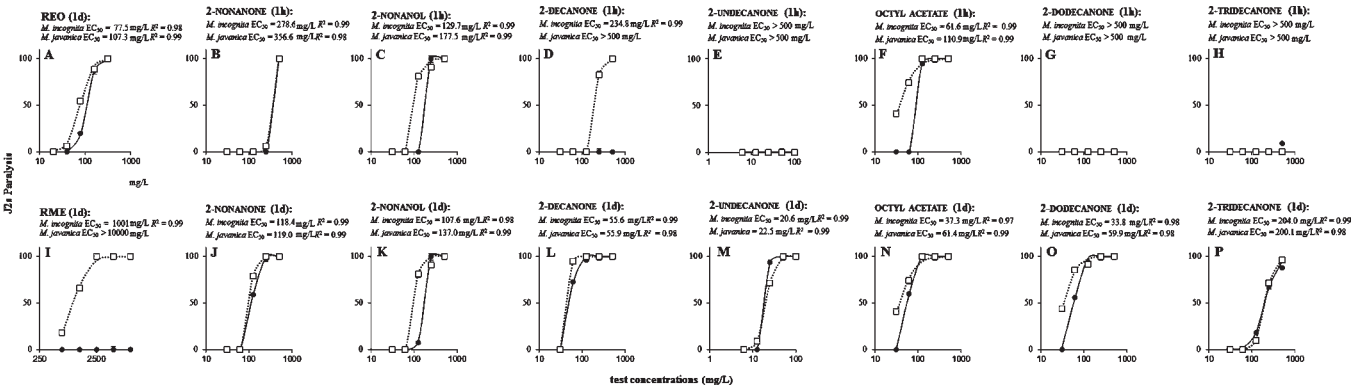

Figure 3. Regression curves of *M. incognita* and *M. javanica* paralysis following J2 immersion in solutions of REO (A), RME (I), and pure ketones (B−H, J−P) for 1 h and 1 day. Each point represents the average percent number of paralyzed J2 of six replications per treatment (two experiments replications) after elimination of natural paralysis/death observed in the control. Standard deviations of mean data values are presented as error bars.

from wild tomato plants is reported to act strongly as a repellent against *Tetranychus urticae*[37] as well as against arthropods, and therefore it is formulated in domestic-use insect repellents.[38] On the other hand, 2-nonanone has been reported to exhibit low activity against *Monilia laxa* as reported by Neri and co-workers.[39]

This is the first report of the irreversible nematicidal activity of ketones as constituents of *R. chalepensis* against *M. incognita* and *M. javanica*. According to our results 2-undecanone is a principal nematicidal constituent of REO and RME. Interestingly, coumarins (rutin and 8-methoxysalene) were not found to be nematicidal against RKN (data not shown), contrary to the findings of Liu and co-workers, who found that psoralen, as a constituent compound of *Ficus carica* L., exhibits nematicidal activity against *B. xylophilus*, *P. redivivus* and *Caenorhabditis elegans*.[40] We are currently studying the mode of action of ketones and aldehydes against root knot nematodes as found in our present and previous studies.[7]

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ABBREVIATIONS USED

GC-MS, gas chromatography−mass spectrometry; LC-MS, liquid chromatography−mass spectrometry; RME, *R. chalepensis* methanolic extract; REO, *R. chalepensis* essential oil.

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