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BIOPESTICIDE POTENTIAL OF (7*R*)-*TRANS*, *TRANS*-NEPETALACTONE AND *CIS*-LACHNOPHYLLUM ESTER IN CONTROL OF MUSTARD APHID, *LIPAPHIS ERYSIMI* (KALT.)

Vinod Kumar[a*], Chandra Shekhar Mathela[a], Geeta Tewari[a], Ajay Kumar Pandey[b]

[a]Department of Chemistry, Kumaun University Nainital 263 002, Uttarakhand, India
[b]Department of Entomology, College of Agriculture, G. B. Pant University of Agriculture and Technology, Pantnagar 263145, Uttarakhand, India

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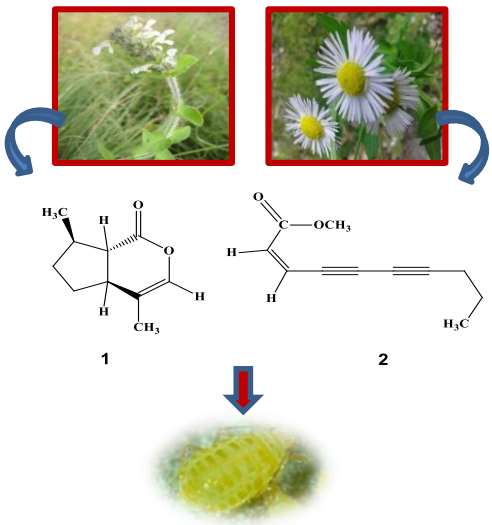
*Corresponding author
kvinod_85@yahoo.com

Graphical abstract



Abstract

(7*R*)-*trans, trans*-nepetalactone; a monoterpene iridoid **(1)** and acetylenic compound named *cis*-lachnophyllum ester **(2)** were isolated from the essential oils of *Nepeta elliptica* and *Erigeron annuus,* respectively and characterized using a combination of their spectral data (1D-and 2D-NMR, MS, IR). Compound **1** and **2** were tested for biopesticide activity against mustard aphid, *Lipaphis erysimi* (Kalt.). Compound **1** exhibited high insecticidal activity towards *L. erysimi* with LC_{50} values of 2.18 and 2.73 mg/mL; LT_{50} values of 15.24 and 17.18 h. Compound **2** also displayed significant insecticidal activity having LC_{50} values of 0.85 and 4.70 mg/mL; LT_{50} values of 13.25 and 26.2 h. The activity of compounds **1** and **2** were comparable with synthetic pesticide, monocrotophos used as positive control and thus has potential as natural pesticides for use in economically important crops.

Keywords: (7*R*)-*trans, trans*-Nepetalactone; *cis*-Lachnophyllum ester; *Lipaphiserysimi* (Kalt.), Biopesticide; Fumigant toxicity; Repellent activity

Abstrak

(7*R*)-*trans, trans*-nepetalakton iaitu iridoid monoterpena **(1)** dan sebatian ester asetilenik *cis*-laknofillum **(2)** telah di asingkan daripada minyak pati *Nepeta elliptica* dan *Erigeron annuus.* Struktur sebatian **1** dan **2** dicirikan menggunakan teknik NMR 1D dan 2D. Sebatian tersebut diuji untuk aktiviti biopestisid terhadap mustard afid, *Lipaphis erysimi* (Kalt.). Sebatian **1** menunjukkan aktiviti antiserangga yang tinggi terhadap *L. erysimi* dengan nilai LC_{50} 2.18 dan 2.73 mg/mL serta nilai LT_{50} 15.24 dan 17.18 h. Sebatian **2** juga menunjukkan aktiviti antiserangga yang signifikan dengannilai LC_{50} 0.85 dan 4.70 mg/mL serta nilai LT_{50} 13.25 dan 26.2 h. Aktiviti sebatian **1** dan **2** menyamai racun perosak sintetik monokrotofos yang digunakan sebagai kawalan positif, justeru menunjukkan potensi seperti racun perosak semulajadi untuk pertanian yang mempunyai kepentingan ekonomi.

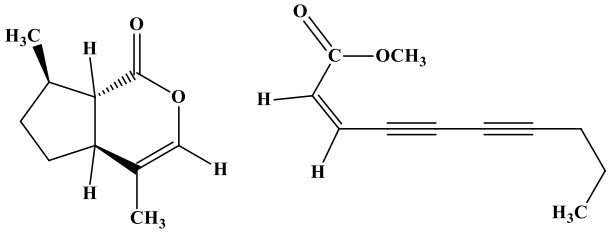
Kata kunci: (7*R*)-*trans, trans*-Nepetalakton, ester *cis*-Laknofillum, *Lipaphis erysimi* (Kalt.), Biopestisid; Ketoksikan Fumigant, Aktiviti penolak

1.0 INTRODUCTION

Oilseed cruciferous crop (*Brassica juncea*) is attacked by one of the serious pests of cruciferous crops aphid (*Lipaphis erysimi* Kalt.) [1]. Both nymphs and the adults feed entire surface of the flower buds and shoots. In order to keep this oilseed crop free from pest attack, various synthetic pesticides have been used, however their repeated use has disrupted natural biological control system and resulted the outbreak of resistant pests to various insecticides. They also caused undesirable effects on non-target organisms, show high toxicity andresidues in soil and water affecting human as well as animal health [2].

The environmental friendly alternative of synthetic pesticides needs to be developed. Natural products are one of the sustainable alternatives to synthetic pesticidesas they offer a large number of compounds that exhibit Insecticidal, larvicidal, adulticidal and repellent activities [3-4]. Botanical pesticides have many advantages, such as low mammalian toxicity, biodegradable, providing novel modes of action against insects that can reduce the risk of cross-resistance and offering new leads for design of target-specific molecules [5-6].

Essential oils (EOs) of *Nepeta* and *Erigerons* pecies usually possess biologically active monoterpene iridoid and acetylenic compounds besides various mono- and sesquiterpenoids [7-8]. Preliminary studies show the acetylenic compounds and (7R)-*trans*, *trans*-nepetalactone has strong activity against soil born phytopathogenic fungi [9-10]. As a part of future strategies for safer pest management and crop protection, this communication describes bioassay aimed at assessing the potential of monoterpene iridoid **(1)** and acetylenic compounds **(2)** as insecticides against *Lipaphis erysimi*.



1 **2**

2.0 EXPERIMENTAL

2.1 Plant Materials and Essential Oil (EO) Extraction

The plants were collected from sub-alpine Himalayan region of Uttarakhand, India (7000-10000 ft elevation) in June-September 2012 at flowering stage and identified from Botanical Survey of India, Dehradun (*N. elliptica* Acc. No. 113974 and *E. annuus* Acc. No. 113641).

The fresh aerial parts of species (2-4 kg) were subjected to steam distillation [9] and organic phase was separated with *n*-hexane. The distillate was further treated with dichloromethane to ensure complete extraction of chemical constituents. The *n*-hexane and dichloromethane extracts were combined together, dried over anhydrous Na_2SO_4 and the solvent was distilled off in a rotary vacuum evaporator to obtain residual oil which was stored in a dark vial at 4°C until use (oil yield: 0.1 - 0.6 % v/w).

2.2 GC-EIMS Analysis

Qualitative and quantitative analysis of EOs from *N. elliptica* and *E. annuus* were done by gas chromatography (GC) and gas chromatography/ mass spectrometry (GC/MS). The GC was carried out on a Nucon 5765, India GC- equipped with Rtx-5 non-polar fused silica capillary column (30 m × 0.32 mm, film thickness: 0.25 μm). The oven temperature (60-210°C) was programmed at 3°C/ min and N_2 as the carrier gas at 4 kg/ cm^2. The injector and detector temperatures were 210°C each and the injection volume was 0.5 μL of 10 % solution of the oil in *n*-hexane. The GC/MS was conducted on a Thermo Quest Trace GC 2000 (Thermo Quest/ Finnigan, Germany) fitted with Rtx-5 non-polar fused silica capillary column (30 m × 0.25 mm, film thickness: 0.25 μm) and interfaced with a Finnigan MAT Polaris Q Ion Trap mass spectrometer. Helium was used as the carrier gas at 1.0 mL/min. The injection volume was 0.10 μL and the split ratio was 1:40. The MS were taken at 70 eV with a mass range of 40-450 amu. Other operating parameters were the same as for GC. Characterization of components of the EOs was done by comparing their retention indices (RI), relative to a series of *n*-alkanes (C_8-C_{24}) indices on the Rtx-5 non-polar fused silica capillary column, compared with published data and NIST and WILEY MS library searches. The relative percentage of the oil constituents was expressed as percentage by FID response in GC.

2.3 Isolation and Characterization of Compounds

Monoterpene Iridoid Compound

The EO (1 mL) of *N. elliptica* was subjected to silica gel column chromatography (230-400 mesh, Merck, 20 g) with hexane:diethyl ether (Et_2O) (99:1-92:8) as eluent and fifteen fractions (Fr.1-Fr.15) were collected and screened by thin layer chromatography (TLC) and gas chromatography (GC). TLC of fractions was performed on silica gel plate 60 F_{254} (Merck, Germany) using hexane:Et_2O (80:20) solvent system. Plates were sprayed with anisaldehyde-H_2SO_4, followed by heating at 110°C. The fractions showing similar TLC pattern were mixed and further analyzed by GC for their purity. Fr. 3 which possessed the major compound **1** [eluted in 97:3 (hexane:Et_2O)] purified by HPLC [Thermoelectron quadrupole High Performance Liquid Chromatography, Nucleosil non-polar column (25 cm × 0.5 mm internal diameter), Macherey-Nagel GmbH & Co. KG (Sigma Aldrich), 5 μm particle size; Injection volume: 2 μL; Flow rate: 1 mL/min; Detector was used, Refractive index (RI-150) and ultraviolet (UV-1000)}] were used as detectors. The isolated compound **1** (80 mg; >97% purity) was

identified as (7R)-*trans*, *trans*-nepetalactone by Mass, ^{1}H and ^{13}C-NMR spectral data [7, 10].

Acetylenic Compound

The EO (2 mL) of *E. annuus* was subjected to silica gel column chromatography (230-400 mesh, Merck, 20 g) with hexane: diethyl ether (99:1 to 85:15) as eluent. Twenty five fractions (Fr.1-Fr.25) were collected and screened by TLC and GC. TLC of fractions (Fr.1-Fr.25) was performed on silica gel plate 60 F_{254} (Merck, Germany) using hexane:Et_2O (80:20) and sprayed with anisaldehyde-H_2SO_4, followed by heating at 110°C. The fractions showing similar TLC pattern were mixed and further analyzed by GC for their purity. Afterwards, Fr. 13-16 were mixed and run on a separate silica gel column (230-400 mesh, Merck, 20 g) with hexane: Et_2O (99:1-95:5) as eluent to obtain pure compound **2** (90 mg; >98% purity). It was characterized as *cis*-lachnophyllum ester on the basis of its MS, ^{1}H and ^{13}C-NMR spectra and comparison with reported data [9,11].

2.4 Insect Cultures

Lipaphis erysimi were collected together with the infested leaves and flowers of *Bracicca juncea* from the oilseed crop field located at Crop Research Centre, GBPUA&T, Pantnagar, India and maintained on *B .juncea* plants grown in polyhouse. Adults of same size of 4-5 days old were used in all bioassay which were carried out at 20 ± 2 ° C.

2.5 Insecticidal Activity

Insecticidal activity was determined against *L. erysimi* by the direct spray and indirect spray. A series of dilutions of tested samples (0.50-10.00 mg/mL) were prepared using dimethyl sulphoxide (DMSO) as solvent with addition of Tween-20 (0.05%) as emulsifier. In the direct spray method, *L. erysimi* was released on 4-6 weeks old *B. juncea* plants using camel hairbrush and after 24 h, 5 mL of each solution was sprayed separately using atomizer. Mean while in the indirect spray method, *L. erysimi* was released after 1 h of spray. Negative controls were treated with DMSO and Tween-20, and monocrotophos a synthetic insecticide used as positive control. Ten same size adults of *L. erysimi* were used for each concentration and positive control, and the experiment was replicated three times. Mortality was recorded after 24 h of the treatment and calculated by using Abbott's formula [12], and the LC_{50} value were calculated according to Finney [13]. Insects incapable of moving after slight touch with fine brush were considered as dead. Another experiment was designed in order to determine the toxicity of oils and the exposure time required to kill 50% insects (LT_{50}). Replication and other conditions were the same as described for the previous experiment. Mortality was recorded after 8, 24, 30 and 48 h of exposure to the samples. Time-mortality data for each experiment were analyzed via the method developed by Finney [13].

2.6 Fumigant Activity

Fumigant toxicity of samples was calculated against *L. erysimi* based on Pascual-Villalobos and Robledo [14]. *L. erysimi* (10 same size adults) were transferred from stock colony to mustard leaf with petioles (warped with moist cotton) placed in petridice (9 cm diameter) and allowed to settle for half an hour before being exposed to sample. Aliquots of 0.20 mL of the sample dilutions (0.50-10.00 mL/L air) were applied on filter paper (3cm^2, Whatman #1) and air dried for half an hour. The impregnated filter paper was then attached to the undersurface of the petridice. Petridices were then sealed with parafilm, each of which contained ten adults of *L. erysimi*. Each treatment was replicated thrice. Negative control having no tested sample was also used. All the petridices were kept under the identical experimental conditions. Mortality data were recorded after 2, 4, 6 and 24 h of exposure to the samples. Mortality data were subjected to Probit analysis [13], to calculate the LC_{50} & LT_{50} values.

2.7 Repellent Activity

Two-leaf choice and no choice bioassay methods were used to evaluate repellency of *L. erysimi* by the samples. In no choice method mustard leaf (3 cm^2) was treated with 0.20 mL acetonic solution of each concentration (0.50-10.00 mg/mL) of samples. Leaf treated with acetone without any sample was used as control. Treated and control leaves were placed in separate petri plates over wet Whatman #1 filter paper. Twenty adults of same size were released into each petri plate after half an hour for solvent evaporation. Three replicates were maintained for each concentration. Control and treated leaves were placed in the same petri plate and the test aphids were placed at an equal distance from either leaf. After 24 h, number of insects were counted on treated and control leaf separately and insects that did not settle on any leaves were discarded from calculation in both assay. The percentage repellency (PR) was calculated as follows: PR = [(C-T)/(C+T)] × 100, where C= numbers of insects on control leaf and T= numbers of insects on treated leaf [14-15].

2.8 Statistical Analysis

Data were subjected to one-way Analysis of Variance (ANOVA) and compared by Duncan Multiple Range tests at a level of significance of $p< 0.05$. Probit analysis (Finney, 1971) was conducted to estimate the lethal concentration (LC_{50}) and lethal time (LT_{50}) with their 95% fiducial limit. Analysis was done using SPSS 16.0 statistical software.

3.0 RESULTS AND DISCUSSION

3.1 Insecticidal Activity

The insecticidal activity of the compounds isolated from *Nepeta* and *Erigeron* species was evaluated against *L. erysimi* using two different spray bioassays and compared with the known insecticide such as

monocrotophos which served as positive control shown in Table 1 and 2. The comparative study between two compounds revealed that compound **1** (LC_{50} = 2.18, 2.73 mg/mL)and **2** (LC_{50} = 0.85, 4.70 mg/mL)were significantly toxic at the tested concentration (P<0.05).

Table 1 Lethal concentration (mg/mL) of 1,2 and standard insecticide against *L. erysimi* after 24 h treatment

Sample	Direct spray				Indirect spray			
	LC_{50}(95% FL)	Slope ± SE	χ2	df	LC_{50}(95% FL)	Slope ± SE	χ2	df
Compound **1**	2.18 (1.06-4.28)	0.82 ± 0.23	6.64	13	2.73 (1.61-5.08)	0.96 ± 0.23	3.66	13
Compound **2**	0.85 (0.32-1.41)	1.02 ± 0.24	7.56	13	4.70 (2.84-11.27)	0.97 ± 0.24	9.69	13
Monocrotophos	0.28 (0.06-0.49)	1.72 ± 0.47	4.63	13	0.77 (0.22-1.37)	0.92 ± 0.24	7.86	13

LC: lethal concentration; FL: fiducial limit; χ2: chi square; df: degree of freedom

Table 2 Lethal time (h) of 1,2 and standard insecticide against *L. erysimi* at 10 mg/mL concentration

Sample	Direct spray				Indirect spray			
	LT_{50} (95% FL)	Slope ± SE	χ2	df	LT_{50} (95% FL)	Slope ± SE	χ2	df
Compound **1**	15.24 (10.87-19.27)	2.54 ± 0.47	10.00	10	17.18 (11.83-22.27)	2.16 ± 0.45	10.05	10
Compound **2**	13.25 (9.99-16.29)	3.24 ± 0.53	10.49	10	26.22 (21.41-36.66)	3.30 ± 0.63	10.72	10
Monocrotophos	7.19 (3.47-8.29)	7.41 ± 2.66	2.01	10	8.91 (5.74-11.60)	2.97 ± 0.56	8.92	10

LT: lethal time; FL: fiducial limit; χ2: chi square; df: degree of freedom

Mortality was affected by the essential oil concentration as well as exposure time. The lethal time values decreased significantly with increase in the concentration. The LT_{50} values at 10 mg/mL concentration of compounds are presented in Table 2, Figure 1. The LT_{50} value of compound **1** against the *L. erysimi* was 15.24 and 17.18 h, while compound **2** had LT_{50} value. 13.25 and 26.22 h. Monocrotophos(with LT_{50} of 7.1 and 8.9 h) was found to be more toxic as compared to the compounds. Present study revealed that compound **1** and **2** have comparable LC_{50} and LT_{50} values with synthetic insecticide which indicated signficant toxicity to the *L. erysimi at (P<0.05)*.

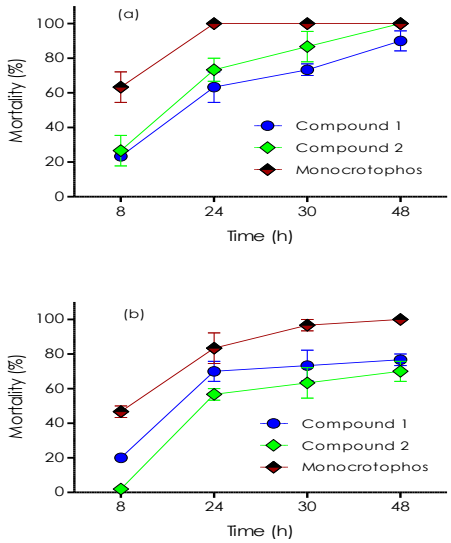


Figure 1 Time mortality response of compound **1, 2** and insecticide (10 mg/mL) against *L. erysimi* after 8-48 h. (a) Direct spray and (b) Indirect spray.

3.2 Fumigant Activity

As shown in Table 3 and 4, compound **1** and **2** were toxic to *L. erysimi* and mortality depended on nature of molecule as well as their concentration and duration of exposure. The LC_{50} values when *L. erysimi* were fumigated with purified compound **1** and **2** are presented in Table 4. Both the compounds showed slightly different insecticidal activity. The LC_{50} value for the compound **1** and **2** were 2.29 and 1.49 mL/L air, respectively.

LT_{50} values obtained from fumigant action of tested samples against *L. erysimi* are given in Table 3. Estimation of lethal time shows that when insect were fumigated with lowest concentration (0.50 mL/L air) of compound **1** and **2**, the LT_{50} values were 51.13 and 28.82 h, respectively, while at higher concentration (10 mL/L air), the LT_{50} values were 12.66 and 10.07 h, respectively (Table 3). The results revealed that compounds had significantsame fumigant toxicity at P<0.05 against *L. erysimi* as compared to commercial insecticide which has LC_{50} value 0.71 mg/mL and LT_{50} value 21.44 h at tested lowest concentration and all the results are comparable.

3.3 Repellent Activity

The repellent activity of the compounds **1** and **2** was assessed against *L. erysimi* using two different methods. The study revealed that overall lower repellent activity was observed in two leaf choice method as compared to the no choice method and significantly (*P*<0.05) influenced by the concentration applied (Figure 2). In two-leaf choice method compound **1** (< 60% repellency) was more repellent than compound **2** (< 50% repellency). Overall *L. erysimi* was particularly less sensitive to both the compounds.

Table 3 Lethal time (h) of 1,2 and standard insecticide as fumigant against *L. erysimi*

Sample	LT_{50} (95% FL)				
	Concentration (mL/L air)				
	0.50	1.00	2.50	5.00	10.00
Compound **1**	51.13 (23.76-82.24)	28.09 (14.40-70.19)	31.85 (14.02-68.26)	20.62 (12.30-66.46)	12.66 (8.80-22.79)
Compound **2**	28.82 (14.98-51.66)	63.33 (25.06-83.62)	17.57 (10.29-65.67)	14.90 (10.35-27.21)	10.07 (7.29-15.90)
Monocrotophos	21.44 (10.92-36.77)	10.43 (5.18-17.39)	4.63 (3.24-7.14)	3.07 (2.06-4.04)	3.21 (2.35-4.06)

LT: lethal time; FL: fiducial limit; χ2: chi squa

Table 4 Fumigant toxicity of 1,2 and standard insecticide after 24 h exposure against *L. erysimi*

Sample	LC_{50}[a] (95% FL)	Slope ± SE	χ2	df
Compound **1**	2.29 (0.48-10.89)	0.53 ± 0.22	8.24	13
Compound **2**	1.49 (0.37-3.15)	0.66 ± 0.23	11.32	13
Monocrotophos	0.71 (0.47-0.94)	2.22 ± 0.39	8.17	13

LC: lethal concentration; FL: fiducial limit; χ2: chi square; df: degree of Freedom; [a] Concentration: mL/L air

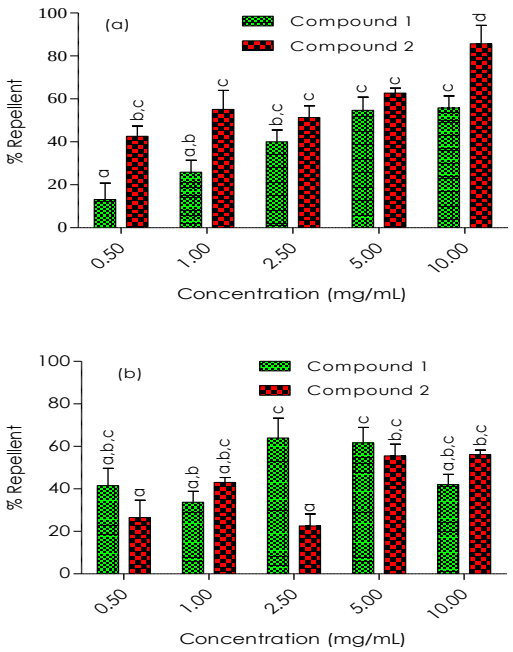


Figure 2 Repellent activity of compound **1** and **2** against *L. erysimi* after 24 h. (a) two-leaf choice and (b) no choice.Bar with different letters (a-c) are statistically different at $p<0.05$ according to Duncan test

4.0 CONCLUSION

Repellency prevents settlements of arriving insects while the toxic property kills the insects that are already present on the crop. Present results demonstrated repellency, toxicity as well as fumigant activity of monoterpene iridoid **1** and acetylenic ester **2** which were able to kill the treated pest through spray and fumigant toxicity and they acted as an insect repellent as well. These could therefore be used for aphid pest management

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