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Full Length Research Paper

Insecticidal properties of *Citrus hystrix* DC leaves essential oil against *Spodoptera litura* fabricius

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Chemical analyisis by gas chromatography (GC) and gas chromatography-mass spectroscopy (GC-MS) revealed presence of 29 compounds in the essential oil fraction of kaffir lime, *Citrus hystrix* fresh leaves. Beta-citronellal was the major compound present with 66.85% of total oil followed by beta-citronellol (6.59%), linalool (3.90%) and citronellol (1.76%). Insecticidal properties of *C. hystrix* leaves essential oil was investigated against tobacco army worm, *Spodoptera litura* using topical application bioassay on uniform weighted second instar larvae in the laboratory. Essential oil was effectively in killing the larvae and showed that the LD_{50} is 26.748 µL/g. Insect development and growth index observations showed that the essential oil had antifeedant properties resulting in severe growth inhibition of *S. litura*.

Key words: *Citrus hystrix*, *Spodoptera litura*, essential oil, botanical insecticides.

INTRODUCTION

Vegetable comprises about 15% of the daily food intake of the Malaysian population (Ding et al., 1981, in Jipanin et al., 2001). From the observations, almost all the commercial vegetable farmers are using pesticides as the main resource to manage their vegetable pest problems since they are easily available, simple and cheap to apply, less labor-intensive and "highly" effective. However, approximately 85 to 90% of applied agricultural pesticides never reach target organisms, but disperse through the air, soil, and water (Moses et al., 1993). Such environmental mobility can cause contamination of several environmental compartments (Mansour, 2008). Regulatory environment and public health needs are creating opportunities for the use of botanicals in industrialized countries in situations where human and animals health are foremost. It is necessary to find out more potential natural products in agriculture pest regulation. Among the plants investigated to date, one showing enormous potential is the citrus family (Rechcigl and Rechcigl, 2000). The methanolic extract of leaves of kaffir lime is known to inhibit the herpes virus (Fortin et al., 2002, in Chowdhurry et al., 2009) and also used as mosquito repellent (Tawtsin et al., 2001). Hence, the present study was conducted to: (i) evaluate the toxicity of *Citrus hystrix* essential against the *Spodoptera litura* larvae and (ii) study the effects of *C. hystrix* essential oil on development and antifeedant reaction in *S. litura*.

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MATERIALS AND METHODS

Plant materials and preparation of extracts

Fresh leaves of kaffir lime were obtained from Local market, Selangor, Malaysia. The essential oil fraction was extracted from fresh leaves by hydrodistillation method (Maisonneune and Saint-Ruffine, 1975). The essential oil was stored in glass vial and put in refrigerator (-4°C) for further experiment.

Identification of chemical constituents

Qualitative and quantitative data were determined by GC and GC-MS, respectively. The identification of the chemical constituents was assigned on the basis of comparison of their mass spectra using Shimadzu NIST/EPA/NIH+ mass spectral database (225-04793-92), which produces standard bar graphs for direct comparison with published spectra.

Gas chromatography (GC)

The oil was injected onto a Shimadzu GC-17A system, equipped with an AOC-20i autosampler and a split/splitless injector. The

column used was BPX 5, 30 m, 250 µm i.d., 0.25 µm df, coated with 5% phenymethylsilane, operated with the following oven temperature programme: 60°C, held for 3 min, rising at 20°C/min to 100°C, rising at 7°C/min to 240°C, held for 5 min; injection temperature and volume, 250°C and 1.0 µL, respectively; injection mode, split; split ratio, 28:1; carrier gas, helium at 29.6 cm/s linear velocity and inlet pressure 27.0 kPa; detector temperature, 280°C; column flow rate, 0.7 mL/min; sampling rate, 0.50 s. Data was acquired by means of GC solution software (Shimadzu).

Gas chromatography-mass spectroscopy (GC-MS)

Shimadzu QP 5050 A mass spectrometer system equipped with a capillary column BPX 5 (30 m x 250 µm, 0.25 µm film thickness) was used. Helium was used as the carrier gas. The MS operating conditions were: ionization was induced by an electron impact (EI) at 70 eV, ion source 250°C. The compounds present were identified by conducting a library search using Shimadzu NIST/EPA/NIH+ mass spectral database. The operating parameters were identical with those of GC analysis described above.

Bioassay to evaluate toxicity of extracts

The experiments were conducted using topical application bioassay. Armyworm larvae were obtained from laboratory colonies maintained by Malaysia Agriculture Research and Development Institute (MARDI), Serdang. Five doses of solvent extracts (ranging from 0 to 40 mg/g) and essential oils (ranging from 0 to 40 µL/g) were tested on one hundred larvae per dose with acetone used as a control. A 0.5-µL droplet of pesticide-acetone solution or acetone was administered to the thoracic tergum using a micro syringe (5 µL, Hamilton Series 600/700 Standard microliter syringe, Fisher Scientific).

After treatment, treated larvae were individually introduced into each Petri dish. Organic mustard leaves were applied to consume by the treated larvae. Mortality was assessed at 24 and 48 h. Larva was considered dead if it was unable to make a coordinated movement when gently prodded. Percentage of mortality was recorded for each treatment. Data were evaluated by probit analysis (PoloPlus program) to determine the LD50 (representing the dosage inµg/g insect that caused 50% mortality) along with 95% confidence intervals.

Effect of essential oil on development of *S. litura*

Based on the results of the toxicity study, the essential oil was selected to further evaluate its effects on growth and development of *S. litura* until adult emergence. The experiment was conducted by topical bioassay on 2nd instar larvae as previously described. After application, treated larvae were placed singly in disposable Petri dishes for observation until adult emergence. Larvae mortality (%), larvae head capsule length (mm), larvae weight gain (g), percentage of pupation (%), pupa weight (g), malformed pupae (%), adult emergence (%) and sex ratio were recorded. Corrected efficacy (%) of larvae mortality was calculated using the formula proposed by Abbott (1925), malformed pupae and pupae mortality were calculated by Sun-Shepard's formula (Püntener, 1981) and adult emergence was calculated by Henderson-Tilton's formula (Henderson and Tilton, 1955). Larval growth index and total developmental growth index were calculated as per Gupta and Girah (2001).

All data were analyzed using the ANOVA procedure in SAS Statistical package and means were compared by Tukey's Studentized Range (HSD) Test.

Antifeedant test

The experiment was conducted using the leaf dip method. *C. hystrix* essential oil was selected to test on the 2nd instar of *S. litura*. The essential oil (0.1 mL) was dissolved in 0.5 mL xylene (solvent) and 0.1 g Triton X-100 (surfactant) as emulsifiable concentrate and finally dissolved in 20 mL distilled water to get a clear solution. The percentages of formulation were assigned based on experimentation including trial and errors. Leaf discs, 60 mm diameter, were cut and soaked in the prepared extract, air dried and singly placed into disposable Petri dishes. Feeding tests were conducted using second instar larvae of *S. litura*. One larva per dish and a total of 50 larvae were used per treatment. Leaf disc areas were measured using a leaf area meter (LI-3100, LI-COL, USA) at 24 and 48 h after exposure. Antifeedant index (AI) was calculated as per Gupta and Girah (2001).

RESULTS AND DISCUSSION

Chemical constituents of *C. hystrix* essential oil

Twenty-nine components were identified in hydrodistillation oil of kaffir lime (Table 1). The leaf oil of kaffir lime yielded high number of oxygenated monoterpenes. The identified oxygenated compounds contained approximately 86.15% of the total oil. The major component characterized from the kaffir lime leaf oil was β-citronellal, a monoterpenoid, representing 66.85% of the total oil. This was followed by β-citronellol (6.59%), linalool (3.90%) and citronellol (1.76%). Others components were never exceeded 2% of the oil composition.

The oil from leaves of *C. hystrix* was commercially used in Malaysia as flavor and fragrance agents, perfumery and medicinal preparation (Burkill, 1935). Research paper from Ibrahim et al. (1996) showed that the leaf oils of kaffir lime at Masjid Tanah, Melaka was mainly made up of citronellal (72.4%), which is the most abundant compound in the leaf oil of kaffir lime. The results were similar to present study whereas the concentration of citronellal was the highest among all the leaf oils. Farah Fazwa et al. (2005) reported that citronellal is the major compound which comprised 40 to 81% of the total leaf oil from 5 populations of *C. hystrix* in Peninsular Malaysia, while other major chemical compounds which are present in significant amounts were citronellol (2.1 to 23.7%), linalool (1.1 to 5.2%), citronellyl propionate (0.2 to 8.9%) and (E) nerolidol (0.3 to 1.8%). Lawrence et al. (1971) studied the chemical constituents of the volatile oil from kaffir lime by steam distillation and similar results were obtained as in present investigation.

Toxicity test

Results from these experiments showed that LD_{50} for the leaf oil of *C. hystrix* were 29.254 and 26.748 µL/g at 24 and 48 h after treatment, respectively (Table 2). Review

Table 1. Composition of essential oil components in fresh leaves of *C. hystrix* (Compounds listed in order of elution from GC-MS column and identification was based on comparison with pure standards).

Component	Formula	Mol. weight	Retention time	% in total oil
Unknown	C_7H_8	92	3.486	4.74
3-Hexen-1-ol	$C_8H_{14}O_2$	142	5.592	0.03
Sabinene	$C_{10}H_{16}$	136	6.609	0.20
β-Myrcene	$C_{10}H_{16}$	136	6.801	0.08
2,6-Dimethyl-5-heptenal	$C_9H_{16}O$	140	8.018	0.24
(E)- furanoid linalool oxide	$C_{10}H_{18}O_2$	170	8.366	0.27
Cis-Linalool oxide	$C_{10}H_{18}O_2$	170	8.673	0.24
Linalool	$C_{10}H18O$	154	8.857	3.90
Tetrahydro-4-methyl-2-(2-methyl-1-propenyl)-2*H*-pyran	$C_{10}H18O$	154	9.083	0.05
(*E*)-2,5-Dimethyl-1,6-octadiene	$C_{10}H_{18}$	138	9.382	0.08
β-Citronellal	$C_{10}H_{18}O$	154	9.954	66.85
Isopregol	$C_{10}H_{18}O$	154	10.216	0.70
Isopulegol	$C_{10}H_{18}O$	154	10.411	0.18
Terpinen-4-ol	$C_{10}H_{18}O$	154	10.618	0.34
2-Methyl-7-oxabicyclo-heptane	$C_7H_{12}O$	112	10.795	0.13
α-Terpineol	$C_{10}H_{18}O$	154	10.906	0.11
3-Undecanol	$C_{11}H_{24}O$	172	11.176	1.04
β-Citronellol	$C_{10}H_{20}O$	156	11.322	6.59
Citronellol	$C_{10}H_{20}O$	156	11.685	1.76
Geraniol	$C_{10}H_{18}O$	154	11.803	0.42
5,9-Dimethyl-1-decanol	$C_{12}H_{26}O$	186	12.038	4.96
Methyl citronellate	$C_{11}H_{20}O_2$	184	12.979	1.90
4-Methyl-6-hepten-3-ol	$C_8H_{16}O$	128	13.464	0.26
Cis-2,6-Dimethyl-2,6-octadiene	$C_{10}H_{18}$	138	13.565	0.33
2-(2-Hydroxy-2-propyl)-5-methyl-cyclohexanol	$C_{10}H_{20}O_2$	172	13.679	0.96
Geranyl acetate	$C_{12}H_{20}O_2$	196	13.894	1.80
1,8-Terpin	$C_{10}H_{20}O_2$	172	14.275	0.95
4,8-Dimethyl-1,7-nonadien-4-ol	$C_{11}H_{20}O$	168	14.359	0.60
Nerolidol	$C_{15}H_{26}O$	222	15.500	0.04

Table 2. LD_{50} of *C. hystrix* essential oil on *S. litura* 2nd instar larvae.

Treatment	Hours	Extract	Slope ±SE	x^2	df	LD_{50}	95% confidence interval (Lower to Upper)
C. hystrix	24	Essential oil	3.896 ± 0.493	2.565	3	29.254 µL/g	26.115 to 33.527
(leaves)	48	Essential oil	3.647 ± 0.443	1.955	3	26.748 µL/g	23.803 to 30.611

study reported that plant essential oils extracted from Rutaceae family, from fruit peel of orange (*Citrus sinensis* L.) and lemon (*Citrus limon* L.) exhibited strong toxicity against mosquito larvae of *Culex pipiens* with the LC_{50} values ranging from 30.1 (lemon) to 51.5 mg/L (orange) depending on citrus species and their composition (Michaelakis et al., 2009). Beta-citronellal (66.85%) was the most abundant compound in the leaf oil of *C. hystrix,* it might be toxic to the test larvae. However, further investigation will be needed.

Effect of essential oil on growth and development of *S. litura*

Results showed that kaffir lime essential oil possessed inhibiting properties against *S. litura* (Table 3). Effect of essential oil on insect development was evaluated daily until adult emergence. Corrected efficacy of larvae mortality (%) treated with kaffir lime oil was 48.31% compared to those treated with acetone only (control). Efficacy of the leaf oil on malformed pupae, pupae

Table 3. Corrected efficacy (%) of *C. hystrix* essential oil on larval mortality, malformed pupae, pupal mortality and adult emergence of *S. litura*.

Parameter	Treated (%)	Control (%)	Corrected efficacy (%)
Larvae mortality	54	11	48.31
Malformed pupae	15.22	13.48	25.29
Pupae mortality	20.51	16.88	31.99
Adult emergence	79.49	83.12	4.37

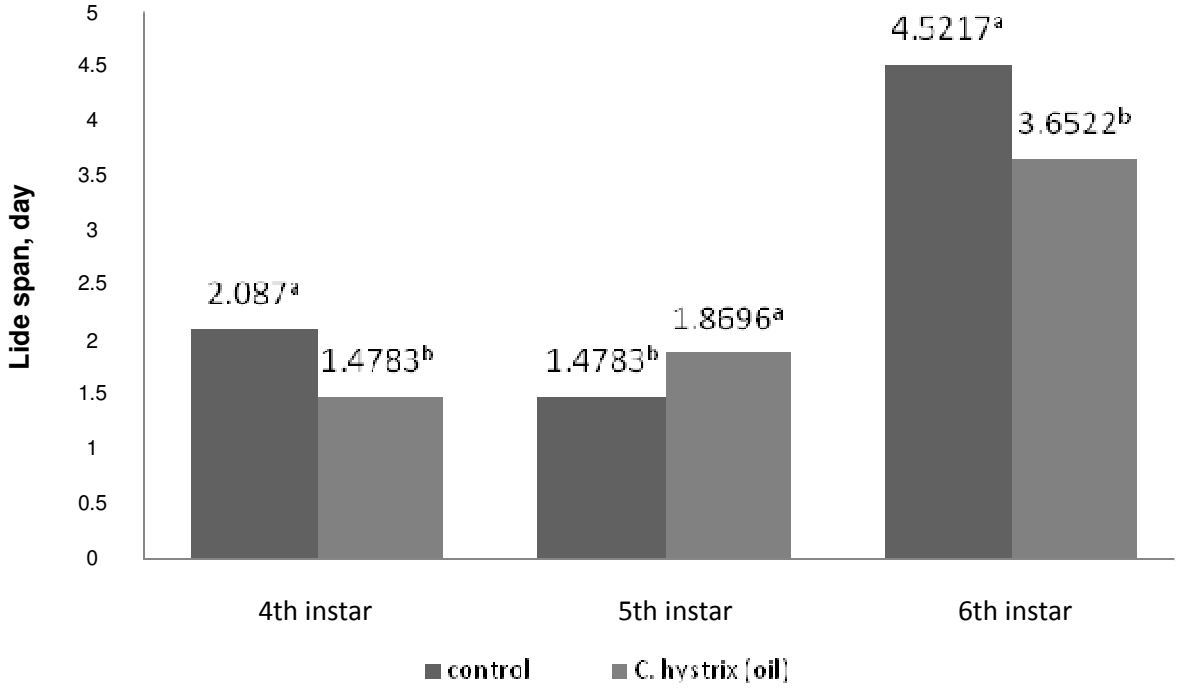

Figure 1. Effect of essential oil of leaves of *C. hystrix* on life span and head capsule measurement of *S. litura* larvae. Tukey's Studentized Range (HSD) Test for larvae longevity. *Means with the same letter are not significantly different within the same instar ($p < 0.05$).

mortality and adult emergence were 25.29, 31.99 and 4.37, respectively.

The results on physical development showed growth of the head capsule in 4th, 5th and 6th instar larvae was 1.076, 1.761 and 2.620 mm, respectively. The life span for 4th instar larvae treated by control treatment was significantly different compared to kaffir lime oil, which were 2.087 and 1.4783 days, respectively. Life span of 5th instar larvae treated with kaffir lime oil was significantly longer (1.8696 days) than in the control treatment (1.4783 days). However, the longevity of 6th instar larvae in the control treatment was significantly longer (4.5217 days) compared to the *C. hystrix* treatment (3.6522 days) (Figure 1). Total larval period (1st and 6th instar) for the control treatment was 16 days while it was 15 days in the kaffir lime oil treatment, which was 1 day shorter than the control (Table 4).

Pupation periods in the kaffir lime oil and control treatments were 7.8 and 9.3 days, respectively. Total development period of *S. litura* (1st instar until adult emergence) in contact with kaffir lime oil and control treatment were 22.8 and 25.3 days, respectively. The essential oil exhibited a lower larval growth index (LGI) and a lower total development growth index (TDGI) of 2.95 and 1.36, respectively as compared to the control treatment (which were 5.54 and 2.53, respectively).

Average weight gains of larvae in *C. hystrix* essential oil and control treatments are presented in Table 5. Results showed that the daily weight gains of larvae treated with essential oil were significantly lower compared to the control treatment during the 1st to 5th day. The larvae weight gained very fast after the 6th day and became significantly higher as compared to control treatment on the 7th and 8th day. The mature larvae

Table 4. Effect of essential oil of *C. hystrix* on development period and growth index of *S. litura*.

Parameter	*C. hystrix* leaf oil	Control
Larval period (days)	15.0	16.0
Pupation (days)	7.8	9.3
Total development period (days)	22.8	25.3
Larval growth index, LGI	2.95	5.54
Total development growth index, TDGI	1.36	2.53

Table 5. Effects of essential oil of *C. hystrix* on larvae weight gained.

Observation day	Weight gained, g	
	Essential oil	Control
1	0.0105[b]	0.0175[a]
2	0.0372[b]	0.0519[a]
3	0.0934[b]	0.1409[a]
4	0.1187[b]	0.2391[a]
5	0.1926[b]	0.2587[a]
6	0.1834[a]	0.1713[a]
7	0.1158[a]	0.0619[b]
8	0.0767[a]	0.0268[b]

Tukey's Studentized Range (HSD) Test for larvae weight gained. *Means within rows with the same letters are not significantly different ($p < 0.05$).

Table 6. Effect of essential oil of *C. hystrix* on *S. litura* sex ratio.

Treatment	Sex ratio (male: female)
Control	1: 0.77
C. hystrix essential oil	1: 0.94

stopped feeding and pupated in a small cell in the disposable Petri dishes. After emergence, the gentle of adult moths were identified through their morphology and external genitalia. The results showed that the sex ratio (male: female) for adults emerging in contact with essential oil and control treatments were 1:0.94 and 1: 0.77, respectively (Table 6). The development of *S. litura* under laboratory conditions (26°C, 60 to 80°C RH) fed on different host plants had been studied (Xue et al., 2009). Their result showed that overall larval development of *S. litura* was significantly affected by the host plant from shortest to longest in the following order: Chinese cabbage (13.3 days), cowpea (15.8 days), sweet potato (17.5 days), and tobacco (23.2 days). The study showed that pupal development times ranged from 10.9-9.5 days when fed on different host plants. In the present study, the larval duration found in the *C. hystrix* and control treatment ranged from 15 to 16 days, which was still within the range of the 4 different hosts. But the pupal duration found in the *C. hystrix* treatment were relatively lower compared to the study by Xue et al. (2009). The sex ratio (male: female) reported by Xue et al. (2009) was 1: 0.64, was different to the present study. The sex ratio (male: female) in kaffir lime essential oil and control treatment was 1: 0.94 and 1:0.77, respectively. The present study demonstrates that the difference in development of *S. litura* could be influenced by others physical and biological factors.

Antifeedant test

Result of the growth and development study showed that weight gained of larvae treated with *C. hystrix* essential oil were lower as compared to control treatment (Table 5). The antifeedant test (leaf dip method) (Table 7) conducted on the second instar larvae showed that the antifeedant effect ranged from 15.63 to 46.01% compared to control treatment at 24 h after treatment. The antifeedant properties decreased after 48 h with

Table 7. Antifeedant Index of essential oil of *C. hystrix* leaves against second instar larvae of *S. litura*.

Concentration (mL/L)	*C. hystrix* essential oil	
	Antifeedant Index, AI	
	24h	48h
4.830	46.01	43.85
2.420	40.45	36.08
1.210	25.73	21.03
0.604	15.63	8.41

protection ranging from 8.41 to 43.85% over the control treatment.

GC-MS analysis obtained that major components characterized from *C. hystrix* leaf oil was β-citronellal (66.85%). Beta-citronella has insect repellent properties, and previous research shows that it has high repellent effectiveness against mosquitoes (Kim et al., 2005). Repellency properties of *C. hystrix* essential oil in the present investigation might also be due to β-citronellal. Others major compounds in *C. hystrix* essential oil such as β-citronellol (6.59%), linalool (3.90%) and citronellol (1.76%) might have repellent activities against the larvae.

Conclusion

C. hystrix essential oil showed good repellence properties against the *S. litura* larvae. Repellency of *C. hystrix* essential oil might be due to β-citronellal; therefore, it is suggested that further study should consider the comparison of the repellent activity of *C. hystrix* leaf oil with analytical grade β-citronella in investigating the bioactive compounds. As conclusion, *C. hystrix* essential oil is potent to be used as supplement of other control methods in sustainable agriculture practices.

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