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Chemical constituents and biological activities against *Tribolium castaneum* (Herbst) of the essential oil from *Citrus wilsonii* leaves

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Abstract: The essential oil obtained from *Citrus wilsonii* Tanaka leaves by hydrodistillation was investigated by GC and GC–MS. The main components of the essential oil were identified to be citronellol (16.94 %), neryl acetate (10.35 %), *γ*-terpinene (9.85 %), citronellal (9.36 %) and *β*-pinene (6.72 %). These four compounds, predicted with a bioactivity-test to be the active constituents, were isolated and identified. It was found that the essential oil of *C. wilsonii* leaves and the isolated compounds possessed fumigant and contact toxicity against *Tribolium castaneum* adults. The essential oil and *γ*-terpinene showed strong fumigant toxicity against *T. castaneum* (LC_{50} = 8.18 and 4.09 mg L^{-1}, respectively). The repellency of the crude oil and the active compounds was also determined. Citronellol, neryl acetate and *β*-pinene were strongly repellent (100, 86 and 92 %, respectively, at 78.63 nL cm^{-2}, after 2 h treatment) against *T. castaneum*. The essential oil and citronellol exhibited the same level of repellency compared with the positive control, *N*,*N*-diethyl-*meta*-toluamide (DEET, *N*,*N*-diethyl-3-methylbenzamide). The results indicate that the essential oil of *C. wilsonii* leaves and its active compounds had the potential to be developed as natural fumigants, insecticides and repellents for the control of *T. castaneum*.

Keywords: fumigant toxicity; contact toxicity; repellency; neryl acetate; *γ*-terpinene.

INTRODUCTION

The red flour beetle, *Tribolium castaneum* Herbst is one of the most widespread and destructive primary insect pests of stored cereals.[1] Control of stored product insects relies heavily on the use of synthetic insecticides and fumigants,

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which has led to several adverse effects, such as water and soil contamination, insect resistance and toxicity to non target species.[2] Especially, fumigants play a very important role in the elimination of insect pests in stored products because of their ability to kill a broad spectrum of pests and because of their easy penetration into the commodity while leaving minimal residues.[3] Currently, phosphine and methyl bromide (MeBr) are the commonly used fumigants in store houses. Of the two fumigants, methyl bromide is an ozone-depletor and is being phased out as agreed through the Montreal Protocol.[3] The other fumigant, phosphine, has some issues with insecticide resistance, requires air-tight conditions, and is associated with environmental and human safety concerns.[4,5] Recent focus of attention among alternative fumigants has been directed toward biofumigants, which reflects the growing interest received by biopesticides or biorational pesticides.[6,7] The use of essential oils or their constituents could effectively prevent and/or suppress insect pests especially in storage,[6] and in some cases, have proven themselves to be more effective than traditionally used organophosphorus pesticides.[7–9] During a screening program for new agrochemicals from local wild plants and Chinese medicinal herbs, the essential oil from *Citrus wilsonii* Tanaka leaves was found to possess fumigant/insecticidal/repellent activity towards *T. castaneum*.

C. wilsonii is a good frost-resistant stock for citrus plants.[10] Through a survey on the Anguo Medicinal Material Trading Market, *C. wilsonii* was discovered to be the main origin plant of Fructus Citri. Fructus Citri, one of the traditional Chinese medicines, is the dried mature fruits of *Citrus medica* L. or *C. wilsonii* that belongs to genus Citrus, family Rutaceae. *C. wilsonii* containing naringin and essential oil is used for regulating vital energy, relieving cough and resolving phlegm, and alleviating stomach pain, emesis and coughs.[11] A literature survey showed that there are no reports on fumigant/contact/repellency activity of the essential oil of *C. wilsonii* leaves against *T. castaneum*. Thus, it was decided to investigate the chemical constituents and fumigant/contact/repellency activity of the essential oil of *C. wilsonii* leaves against *T. castaneum* for the first time and to isolate any biologically active compounds from the essential oil.

EXPERIMENTAL

Insect

The *T. castaneum* was obtained from laboratory cultures maintained for the last 2 years in the dark in incubators at 29±1 °C and 70–80 % relative humidity. The insects were reared in glass containers (0.5 L) containing wheat flour at 12–13 % moisture content mixed with yeast (wheatfeed/yeast, 10:1, *m*/*m*). The adults used in all the experiments were about 7±2 days old.

Plant material and essential oil extraction

Leaves (3.0 kg) of *C. wilsonii* were collected in May 2013 from Suzhou City (31.97° N and 120.49° E), Jiangsu Province, China. The leaves were air-dried for one week and ground to powder. The species was identified according to the voucher specimen (BNU-CMH--Dushushan-2013-05-25-007) deposited at the Herbarium (BNU) of College of Resources Science and Technology, Beijing Normal University, China. The ground powder of *Citrus wilsonii* Tanaka leaves was subjected to hydrodistillation using a modified Clevenger-type apparatus for 6 h and extracted with *n*-hexane. Anhydrous sodium sulfate was used to remove the water after extraction. The essential oil was stored in airtight container in a refrigerator at 4 °C.

Gas chromatography–mass spectrometry

GC–MS analysis was performed on a Thermo Finnigan Trace DSQ instrument equipped with a flame ionization detector and an HP-5MS (30 m×0.25 mm×0.25 μm) capillary column. The column temperature was programmed at 50 °C for 2 min, then increased at 2 °C min^{-1} to the temperature of 150 °C and held for 2 min, and then increased at 10 °C min^{-1} until the final temperature of 250 °C was reached, where it was held for 5 min. The injector temperature was maintained at 250 °C and the volume injected was 0.1 mL of 1 % solution (diluted in *n*-hexane). The carrier gas was helium at flow rate of 1.0 mL min^{-1}. The MS spectra were scanned from 50 to 550 *m*/*z*. Most constituents were identified by comparison of their retention indices with those reported in the literature. The retention indices were determined in relation to the retention times of a homologous series of *n*-alkanes (C_{10}–C_{36}) obtained under the same operating conditions. GC retention time and their mass spectra that are stored in NIST 05 and Wiley 275 libraries or from the literature were used to identify the essential oil components.[12]

Isolation and characterization of four constituent compounds

The crude essential oil (5 mL) was chromatographed on a silica gel (Qingdao Marine Chemical Plant, Shandong province, China) column (30 mm i.d., 500 mm length) by gradient elution with *n*-hexane first, then with *n*-hexane–ethyl acetate, and last with ethyl acetate to obtain 20 fractions. Based on contact toxicity, fraction 2, 7 and 14 were chosen for further fractionation. With PTLC, four purified compounds were obtained. The isolated compounds were elucidated by their NMR spectra. The NMR experiments were performed on a Bruker Avance DRX 500 instrument using $CDCl_3$ as the solvent with TMS as the internal standard.

Fumigant toxicity bioassay

The fumigant activity of the essential oil/pure compounds against *T. castaneum* adults was tested as described by Liu and Ho.[1] Serial dilutions of the essential oil/compounds (1.33–2.75 % for *γ*-terpinene, 1.98–10.00 % for the oil and *β*-pinene, five concentrations) were prepared in *n*-hexane. The 10-μL dilution was placed onto Whatman filter paper disks of 2.0 cm diameter. Each filter paper disk was then air-dried for 20 s and placed on the underside of the screw cap of a glass vial (25 mL). Ten insects were placed into each vial (5 replicates per dose) before the cap was screwed tightly and the lid was sealed with Parafilm. *n*-Hexane was used as the control. The mortality of insect was noted 24 h after treatment, and the LC_{50} values were calculated using Probit analysis.[13]

Contact toxicity by topical application

The contact toxicity of the essential oil/pure compounds against *T. castaneum* adults was measured as described by Liu and Ho.[1] Aliquots of 0.5 μL of the essential oil and four isolated compounds at different concentrations (0.00, 2.96, 4.44, 6.67, 10.00 or 15.00 % of oils or compounds diluted with *n*-hexane) were applied topically to the dorsal thorax of the insects (10 insects per replicate, five replicates per dose). Insects treated with *n*-hexane alone were used as controls. Both treated and control insects were then transferred to glass vials (10 insects per vial) with culture media and kept in incubators. The insect mortality was checked after 24 h, and the LD_{50} values were calculated using Probit analysis.[13] The positive control, pyrethrins (pyrethrin 1: 24 %; pyrethrin 2: 13 %; cinnerin 1: 2 %; cinnerin 2: 2 %; jasmolin 1: 1 %; jasmolin 2: 1 %), were purchased from Dr Ehrenstorfer GmbH, Germany.

Repellent test

The repellent activity of the essential oil/pure compounds to *T. castaneum* adults was tested using the area preference method.[14] The essential oil/compounds were diluted in *n*-hexane so that different final concentrations (78.63, 15.73, 3.15, 0.63 and 0.13 nL cm^{-2}) will be achieved at the paper, and *n*-hexane was used as the control. A filter paper (9 cm in diameter) was cut in half. 500 μL of a treatment solution was placed on one half of the filter paper and allowed to dry for 30 s. The other half was treated with 500 μL of *n*-hexane. The treated side was then joined to the control side by tape and placed in glass Petri dishes (9 cm in diameter). Twenty insects were released in the center of each filter paper disk, and a cover was placed over the Petri dish. Five replicates were used. Counts of the insects present on each strip were made after 2 and 4 h. The percent repellency (*PR*) of each volatile oil/compound was then calculated using the equation:

$$PR(\%) = 100\frac{(N_c - N_t)}{(N_c + N_t)} \quad (1)$$

where N_c is the number of insects present in the negative control half and N_t is the number of insects present in the treated half. Analysis of variance (One-Way ANOVA and GLM Univariate) and Tukey's test were conducted by using SPSS 20.0 for Windows 2007. Percentage mortality data were subjected to arcsine square-root transformation before analysis of variance. The commercial repellent *N*,*N*-diethyl-*meta*-toluamide (DEET, *N*,*N*-diethyl-3-methylbenzamide) was purchased from the National Center of Pesticide Standards (Shenyang, China) and used as a positive control.

RESULTS AND DISCUSSION

Chemical constituent of essential oil

The yield of *C. wilsonii* leaves essential oil was 0.55 % (*V*/*m*) with a density 0.87 g mL^{-1}. GC–MS analysis of the essential oil of *C. wilsonii* leaves led to the identification and quantification of a total of 15 major components, accounting for 84.12 % of the total components present (Table I). The main constituents of *C. wilsonii* leaves essential oil were citronellol (16.94 %), neryl acetate (10.35 %), *γ*-terpinene (9.85 %), citronellal (9.36 %) and *β*-pinene (6.72 %).

There is only one report on the extraction of the essential oil from *C. wilsonii* leaves. *γ*-Terpinene (27.1 %), *p*-cymene (10.3 %), limonene (8.4 %), neral (7.5 %), *β*-pinene (5.2 %), nerol (4.8 %), ocimene (4.4 %) and citronellal (4.3 %) were the main components of the essential oil of *C. wilsonii* leaves obtained from Georgian SSR.[10] However, there are a few reports about the extraction of essential oil from other parts of *C. wilsonii*. For example, the essential oil of Xiangyuan (*C. wilsonii*) peel collected from the Jiangsu Province contained limonene (50.53 %), *p*-ocimene (16.40 %), *γ*-terpinene (8.75 %), *β*-ocimene (5.03 %), *β*-pinene (3.35 %), *α*-pinene (2.66 %) and *β*-myrcene (2.30 %).[15] The essential oil of Fructus Aurantii Immaturus of *C. wilsonii* collected from Shaanxi Province contained D-limonene (58.09 %), *γ*-terpinene (23.76 %), *β*-cubebene (2.83 %) and *α*-pinene (2.00 %). Furthermore, D-limonene (65.32 %), *γ*-terpinene (17.36 %), 1-methyl-5-methylene-8-(1-methylethyl)-1,6-cyclodecadiene (1.80 %) and *α*-terpineol (1.43 %) were the major compounds of the essential oil of the Fructus Aurantii of *C. wilsonii*.[16]

TABLE I. Chemical composition of the essential oil of *Citrus wilsonii* Tanaka leaves; *RI* – retention index as determined on a HP-5MS column using the homologous series of *n*-hydrocarbons

Compound	*RI*	Content, %
(+)-*α*-Pinene	931	1.41
β-Pinene	981	6.72
4-Cymene	1024	4.66
(*S*)-(–)-Limonene	1029	1.28
β-Phellandrene	1031	2.41
γ-Terpinene	1057	9.85
Linalool	1094	3.97
D-Citronellal	1152	9.36
Citronellol	1226	16.94
3-Methyl-3-(4-methyl-3-pentenyl)-2-oxiranecarboxaldehyde	1234	1.33
2,6-Dimethylocta-2,6-diene	1338	4.89
Neryl acetate	1362	10.35
(–)-Spathulenol	1577	4.04
Caryophyllene oxide	1584	3.06
Phytol	2119	3.85
Total		84.12

Structure confirmation of isolated compounds

On further isolation, four purified compounds were obtained that were analyzed by several NMR techniques including ^{1}H- and ^{13}C-NMR. Combining all the NMR spectra data, the four isolated compounds were finally recognized as citronellol (0.44 g),[17–19] *γ*-terpinene (0.22 g),[17,20] neryl acetate (0.23 g)[17] and *β*-pinene (0.15 g).[21]

Fumigant toxicity

The essential oil of *C. wilsonii* leaves showed strong fumigant toxicity against *T. castaneum* adults with an LC_{50} value of 8.18 mg L^{-1} (Table II). The isolated compounds *γ*-terpinene and *β*-pinene also exhibited strong fumigant toxicity against *T. castaneum* adults with LC_{50} values of 4.09 and 15.22 mg L^{-1}, respectively (Table II).

The crude essential oil was almost five times less toxic to *T. castaneum* adults compared with MeBr (LC_{50} = 1.75 mg L^{-1}).[22] However, as most commercial fumigants (*e.g.*, phosphine and MeBr) are synthetic insecticides and highly toxic to humans and other non-target organisms, the fumigant activity of the essential oil of *C. wilsonii* leaves and *γ*-terpinene were quite promising. Compared with the commercial fumigant MeBr, *γ*-terpinene exhibited an almost two times lower fumigant toxicity against *T. castaneum* adults. Nevertheless, compared with other essential oils reported in the literature, the crude essential oil had a stronger level of fumigant toxicity towards *T. castaneum* adults than, for example, the essential oils of *Illicium difengpi* (LC_{50} = 16.22 mg $L^{–1}$),[23] *I. pachyphyllum* (LC_{50} = 15.08 mg $L^{–1}$),[24] *Zanthoxylum schinifolium* seeds (LC_{50} = = 11.77 mg $L^{–1}$),[25] *Perovskia abrotanoides* (LC_{50} = 11.39 μL $L^{–1}$),[26] *Citrus reticulata* (LC_{50} = 19.47 μL $L^{–1}$) and *Schinus terebenthifolius* (LC_{50} = 20.50 μL $L^{–1}$),[27] but a lower toxicity than the essential oil of *Carum carvi* (LC_{50} = 2.53 mg $L^{–1}$).[28]

TABLE II. Fumigant toxicity of the essential oil of *Citrus wilsonii* Tanaka leaves and its main components against *Tribolium castaneum* adults

Treatment	LC_{50} / mg L^{-1} air[a]	Slope±*SE*	*df*	χ^2	*P*
C. wilsonii	8.18 (6.44–9.32)	5.44±1.07	23	11.71	0.975
Citronellol	>150.75	–	–	–	–
γ-Terpinene	4.09 (3.74–4.32)	10.24±1.64	23	5.13	1.000
Neryl acetate	>157.75	–	–	–	–
β-Pinene	15.22 (13.69–16.96)	4.04±0.44	23	20.93	0.585
MeBr[b]	1.75	–	–	–	–

[a]95 % lower and upper measurement limits are shown in parentheses; [b]data from Liu and Ho[1]

The development of natural fumigants would help to decrease the negative impact of synthetic fumigants, such as residues, resistance and environmental pollution. In this respect, natural fumigants may be effective, biodegradable, and less harmful to the environment. In the present study, the crude essential oil, *γ*-terpinene and *β*-pinene showed strong fumigant toxicities against *T. castaneum*. Based on these findings, these or other essential oil may serve as viable alternatives to synthetic insecticides.

Contact toxicity

The essential oil of *C. wilsonii* leaves showed contact toxicity against *T. castaneum* adults with LD_{50} values of 48.49 μg per adult (Table III). Compared with the positive control pyrethrins the crude essential oil demonstrated 186 times lower toxicity against the red flour beetle because the pyrethrins have acute contact toxicity to *T. castaneum* with an LD_{50} value of 0.26 μg per adult. The isolated compounds citronellol, *γ*-terpinene, neryl acetate and *β*-pinene also exhibited contact toxicity against *T. castaneum* adults with LD_{50} values of 35.89, 35.59, 25.84 and 22.10 μg per adult, respectively (Table III). Among the four isolated compounds, *β*-pinene demonstrated a stronger contact toxicity against *T. castaneum* than the other three isolated compounds.

TABLE III. Contact toxicity of essential oil of *Citrus wilsonii* Tanaka leaves and its main components against *Tribolium castaneum* adults

Treatment	LD_{50}[a] / μg adult^{-1}	Slope±*SE*	*df*	χ^2	*P*
C. wilsonii	48.49 (44.19–54.03)	5.39±0.67	23	21.30	0.563
Citronellol	35.89 (32.50–39.93)	4.45±0.47	23	12.12	0.969
γ-Terpinene	35.59 (32.57–39.07)	5.33±0.56	23	16.12	0.850
Neryl acetate	25.84 (23.49–28.33)	4.75±0.49	23	15.13	0.890
β-Pinene	22.10 (16.10–27.05)	2.55±0.42	23	20.24	0.627
Pyrethrins	0.26 (0.22–0.30)	3.34±0.32	23	13.11	0.950

[a]95 % lower and upper measurement limits are shown in parenthees

Repellent activity

The results of the repellency assays for the essential oil and isolated compounds against *T. castaneum* adults are presented in Figs. 1 and 2. The essential oil from *C. wilsonii* leaves at a dose of 78.63 nL cm^{-2} showed 98 and 96 % repellency against *T. castaneum* adults 2 and 4 h after exposure, respectively. At the lowest concentration (0.13 nL cm^{-2}), the essential oil still showed strong repellency (78 and 72 %) against *T. castaneum* at 2 and 4 h after exposure, respectively (Figs. 1 and 2). Among the four constituents of the crude essential oil, citronellol produced strong repellency (100 % at 78.63 nL cm^{-2}, after both 2 and

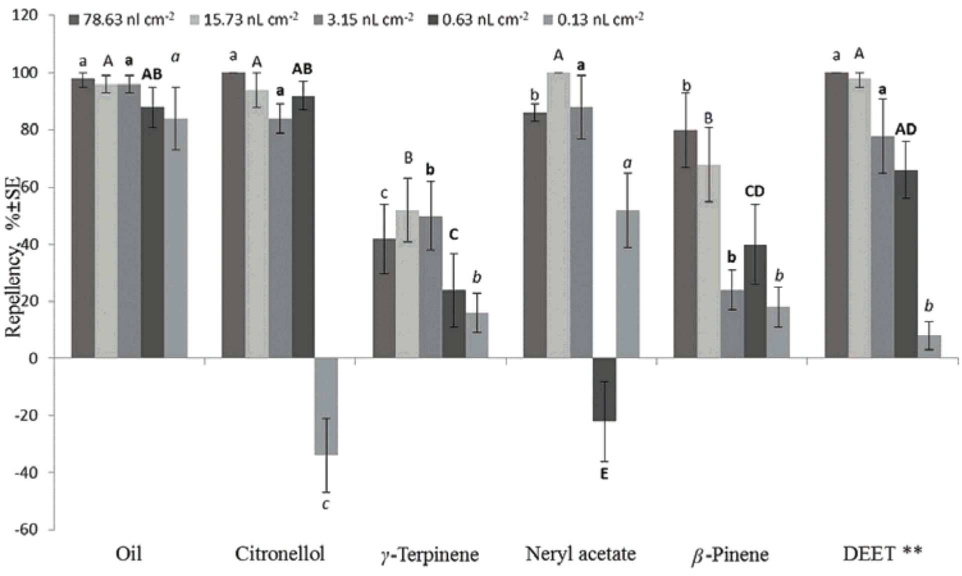

Fig. 1. Percentage repellency (*PR*) of the essential oil from *Citrus wilsonii* Tanaka leaves and its constituents against *Tribolium castaneum* at 2 h after exposure; means in the same column followed by the same letters do not differ significantly (*P* > 0.05) in ANOVA and Tukey's tests. The *PR* values were subjected to an arcsine square-root transformation before the ANOVA and Tukey's tests; ** – positive control.

4 h treatment). Citronellol at dose of 0.63 nL cm^{-2} still showed strong repellency (92 and 94 %, respectively) against *T. castaneum* at 2 and 4 h after exposure (Figs. 1 and 2). Neryl acetate and *β*-pinene also showed obvious repellency (>68 %) at dose of 78.63 and 15.73 nL cm^{-2} after 4 h treatment. However, compared with the other three constituents, *γ*-terpinene produced less repellency (42 and 26 %, respectively, at 78.63 nL cm^{-2} after 2 and 4 h treatment).

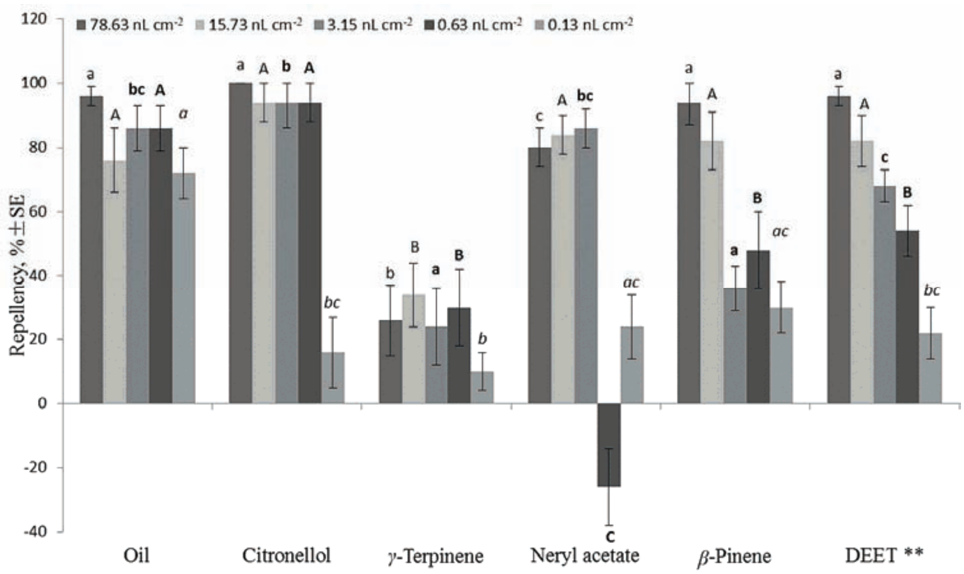

Fig. 2. Percentage repellency (*PR*) of the essential oil from *Citrus wilsonii* Tanaka leaves and its constituents against *Tribolium castaneum* at 4 h after exposure; means in the same column followed by the same letters do not differ significantly ($P > 0.05$) in ANOVA and Tukey's tests. The *PR* values were subjected to an arcsine square-root transformation before the ANOVA and Tukey's tests; ** – positive control.

Many essential oils and their constituents have been evaluated for repellency against insects.[29] For example, Zhang *et al*. reported that geraniol and citronellol exhibited stronger repellency against the red flour beetle than DEET, whereas limonene and citronella showed the same level of repellency against the red flour beetle as DEET.[14] At 0.03 mg cm^{-2}, origanum oil, linalool and *p*-cymene showed 98, 83 and 85 % repellency (after 2 h treatment) against *T. castaneum* adults, respectively.[30] However, in this paper, we report the repellency of the essential oil of *C. wilsonii* leaves for the first time. The essential oil and citronellol exhibited the same level of repellency against *T. castaneum* adults as DEET, the positive control.

To the best of our knowledge, this is the first report regarding the fumigant/insecticidal/repellent action of the essential oil of *C. wilsonii* leaves against *T. castaneum*. The results suggest that the essential oil and the four compounds show potential for development as natural fumigants, insecticides and repellents for stored product protection. However, for the practical application of the essential oil and the four compounds as novel fumigants/insecticides/repellents, further studies on the safety of the essential oil and the four compounds toward humans and on the development of formulations are necessary to improve the efficacy and stability, and to reduce cost.

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ИЗВОД

ХЕМИЈСКИ САСТАВ И БИОЛОШКА АКТИВНОСТ ЕТАРСКОГ УЉА ЛИСТОВА *Citrus wilsonii* СПРАМ БРАШНЕНОГ МОЉЦА *Tribolium castaneum* (HERBST)

HAI PING CHEN[1,2], KAI YANG[1], CHUN XUE YOU[1], SHU SHAN DU[1], QIAN CAI[2], QING HE[3], ZHU FENG GENG[3] и ZHI WEI DENG[3]

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Етарско уље листова *Citrus wilsonii* Tanaka добијено је дестилацијом воденом паром и анализирано методама GC и GC–MS. Главни састојци уља су идентификовани као цитронелол (16,94 %), нерил-ацетат (10,35 %), γ-терпинен (9,85 %), цитронеал (9,36 %) и β-пинен (6,72 %). Четири састојка су била активна, што је утврђено тестом биоактивности: цитронелол, нерил-ацетат, γ-терпинен и β-пинен. Утврђено је да етарско уље листова *C. wilsonii* и изолована једињења имају фумигантну и контактну токсичност спрам одрасле форме *Tribolium castaneum*. Етарско уље и γ-терпинен су испољили јаку фумигантну токсичност спрам *T. castaneum* (LC_{50} 8,18 и 4,09 mg L^{-1}, редом). Репелентност сировог уља и активних једињења је, такође, одређивана. Цитронелол, нерил--ацетат и β-пинен су били јако репелентни спрам *T. castaneum* (100, 86 и 92 %, редом, при 78,63 nL cm^{-2}, после 2 h третмана). Етарско уље и цитронелол су испољили исту репелентност као и позитивна контрола, DEET. Резултати указују да етарско уље листова *C. wilsonii*, као и његова активна једињења, имају потенцијал да се развију као природни фумиганти, инсектициди и репеленти за контролу *T. castaneum*.

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