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Research Article

Toxic and oviposition deterrence activities of essential oils from *Citrus sinensis* (L.) Osbeck and *Citrus paradisi* (Macfarlane) fruit peel against adults of *Tribolium castaneum* (Herbst)

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Abstract: The red flour beetle, *Tribolium castaneum* (Herbst), is one of the most destructive pests attacking stored grain products all over the world. Serious problems assossiated with using synthetic chemical insecticides have strongly demonstrated the need for applying alternative safe compounds such as plant essential oils. The present experiment was conducted to evaluate fumigant toxicity of essential oils from the fresh fruit peel of two *Citrus* species namely, *Citrus sinensis* (L.) Osbeck and *Citrus paradisi* (Macfarlane) against 1 to 7-days-old adults of *T. castaneum* under laboratory conditions. Moreover, oviposition deterrence activity of sublethal concentrations of the oils were assessed on the female beetles. All experiments were carried out at 27 ± 1 °C and 65 ± 5% r. h. in darkness. Findings indicated the high fumigant toxicity of both essential oils. According to probit analysis, there was no significant differences between *C. sinensis* (LC_{50} = 7.27 µl.l^{-1} air) and *C. paradisi* (LC_{50} = 7.70 µl.l^{-1} air) essential oils. Also, oviposition deterrence activity of the essential oils was significantly increased as concentrations of the oils were increased from 500 to 2500 ppm. In general, the results of our study demonstrated the high efficacy of *C. sinensis* and *C. paradisi* oils against *T. castaneum*.

Keywords: *Tribolium castaneum*, *Citrus sinensis*, *Citrus paradisi*, fumigant toxicity, oviposition deterrency

Introduction

The red flour beetle, *Tribolium castaneum* (Herbst), is a cosmopolitan and destructive beetle in the family Tenebrionidae which mainly attacks stored grain products such as flour, cereals meal, beans, seeds, and even dried museum specimens (Weston and Rattlingourd, 2000).

During the last decades, using chemical pesticides for the control of agricultural pests has been a conventional practice. Regarding the fact that many of common pesticides can adversely affect the environment, non-target organisms, and human health; needs for safer devices of pest management have become crucial. As a consequence, these problems led researchers to look for safer natural compounds such as essential oils and plant extracts. Botanical derivatives especially, plant essential oils which are obtained through steam distillation of herbs and aromatic plants have been used traditionally as medicine, flavor in dishes and drinks, perfume, and as insecticides in many countries (Pushpanathan *et al*., 2006). These compounds tend to have low mammalian toxicity, little

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environmental mal-effects and wide public acceptance (Isman, 2000). There are numerous reports dealing with the efficacy of essential oils against stored-product insects and in some cases, the examined oils have indicated strong insecticidal activity toward the target pests (Kim *et al*., 2013; Ziaee *et al*., 2014; Kim and Lee, 2014; Ghasemi *et al*., 2014; Nwachukwu and Asawalam, 2014; Fatiha *et al*., 2014; Aref *et al*., 2015; Kheirkhah *et al*., 2015).

The genus *Citrus* L. belonging to family Rutaceae contains a large number of species (more than 400) along with innumerable varieties, cultivars etc. All cultivated species probably derive from plants native to tropical and subtropical areas of Southeast Asia (Tutin *et al*., 1968). Most species of *Citrus* are medically valuable because of their high content of vitamin C. It is shown that essential oil from the fruit peel of several *Citrus* plants contain chemicals that exhibit insecticidal and antifungal activity (Sharma and Tripathi, 2008; Siskos, 2008; Singh *et al*., 2010; Saeidi *et al*., 2011, 2014).

Till now, many publications have been documented on the biological activity of essential oils from plant species belonging to the genus *Citrus* against stored-product pests (Don-Pedro, 1985, 1996a; Moravvej and Abbar, 2008; Moravvej *et al*., 2010; Abbas *et al*., 2012; Zia *et al*., 2013; Saeidi *et al*., 2014). Some experiments have been specifically done on the efficacy of *Citrus* oils against *T. castaneum*. Safavi and Mobki (2012) reported the fumigant toxicity of *Citrus reticulata* Blanco peel essential oil against *T. castaneum* (Herbst). In another study, Campolo *et al*. (2013) assessed the fumigant bioactivity of five *Citrus* essential oils against *T. confusum*. In almost all cases, it has been proved that the toxicity of *Citrus* essential oils is largely attributed to limonene as the major component of their oils (Mansour *et al*., 2004; Papachristos *et al*., 2009). In this case, Saeidi *et al*. (2014) reported that limonene is the main compound (> 70% of the total constituents) of essential oils from *C. reticulata*, C. *aurantium*, and *C. limon* cultivated in Iran. The similar results were also published for different varieties of *C. sinensis*, C. *aurantium*, and *C. limon* cultivated in Greece (Papachristos *et al*., 2009), and for *C. sinensis*, *C. aurantium*, *C. reticulate* Blanco, *C. limon*, and *C. bergamia* (Risso and Poiteau) cultivated in Italy (Campolo *et al*., 2013). Nevertheless, no report has yet been published on the fumigant toxicity of *C. sinensis* (L.) Osbeck and *C. paradisi* (Macfarlane) against *T. castaneum*. This work is the first study on toxicity of essential oils taken from the fresh fruit peel of *C. sinensis* and *C. paradisi* as fumigant against adults of *T. castaneum*. We report here bioassay results of the oils as well as their oviposition deterrence activity on the females of subjectd beetle at sublethal concentrations.

Materials and Methods

Insect culture

Adults of *T. castaneum* were obtained from an insectarium and reared in plastic containers (25 cm length, 15 cm width, and 10 cm height) containing wheat flour. The cultures were kept in a growth chamber set at 27 ± 1 °C and 65 ± 5% r.h. in darkness and all experiments were conducted in the same conditions. Only 1 to 7-days-old adult beetles were used for fumigant toxicity tests.

Plant materials and extraction of the essential oils

Fruits of *C. sinensis* and *C. paradisi* were collected from Noshahr and Namak Abrood cities in Iran during 2013-2014. Essential oils were extracted from their fresh fruit peels using a modified Clevenger-type apparatus (Negahban *et al*., 2007). Conditions of the oil extraction were: 50 g of fresh fruit peels, 500 ml distilled water and 4 h distillation. After extraction, anhydrous sodium sulfate was used to eliminate water. Extracted oils were placed in sealed glass tubes and stored at 4 °C for bioassay tests.

Fumigant toxicity bioassays

To determine lethal concentration values (LC_{10}, LC_{30}, LC_{50} and LC_{90}) of tested oils, ten 1 to 7-days-old adults of *T. castaneum* were put into 500 ml glass bottles with screw lids and then were treated with random concentrations of the oils. After preliminary dose-setting experiments,

the final concentrations of the oils causing 5-95% mortality were obtained based on logarithmic distance (Robertson *et al*., 2007). The calculated concentrations of the oils were infused on the filter paper (Whatman No. 1, cut into 2 cm diameter pieces) and then were attached to the caps of glass vials. Oils were applied as pure using microapplicator. The caps of vials were sealed tightly with parafilm. Control insects received no oil. Each concentration was replicated five times. Number of dead and alive insects in each vial was counted 24 h after commencement of exposure to the oils. When no leg or antennal movements were observed, insects were considered dead. Percentage insect mortality was calculated using the Abbott correction formula for natural mortality in untreated controls (Abbott, 1925).

Another experiment was designed to estimate lethal time values (LT_{50} and LT_{90}) of the *C. sinensis* and *C. paradisi* oils at different concentrations. According to the method mentioned above, ten 1 to 7-days-old adults of *T. castaneum* were placed into 30 ml glass vials and treated with concentrations of 17, 34, 50, 67 and 83 µl. l^{-1} air of the oils. Mortality was determined 3, 6, 9, 12, 15, 18, 21 and 24 h after initial exposure to the oils. Control insects were kept under the same conditions without any oil. Each concentration and time exposure were replicated three times. When no leg or antennal movements were observed, insects were considered dead. All experiments were carried out at 27 ± 1 °C and 65 ± 5% in darkness.

Oviposition deterrence tests

Effect of the sublethal concentrations of studied oils on oviposition rate of female *T. castaneum* was assessed according to the method of Huang *et al*. (2000). Male and female beetles were set apart from each other based on their genital organ in pupal stage. Aliquots of 500 µl of estimated concentrations (750, 1000, 1500, 2000 and 2500 ppm) of the oils in acetone were applied to black filter papers installed on the bottom of glass Petri dishes (9.0 cm) and dried for 30 minutes. Five pairs (5 males and 5 females) of adult beetles were introduced on each treated filter paper and were confined within a glass ring (5.0 cm). Five grams of wheat flour was added to the filter paper to provide food for the insects. Acetone treated filter papers were used as controls. Five replicates were prepared for each concentration and control. The oviposition rate of female was recorded after 24 h and oviposition deterrence was calculated with the following formula (Pascual-Villalobos and Robledo, 1998):

$$\%\text{Oviposition deterrence} = \left[1 - \frac{NE_t}{NE_c}\right] \times 100$$

Where NE_t is the number of eggs in treatment and NE_c is the number of eggs in control.

Data analysis

The LC values and 95% confidence limits were estimated by probit analysis (Finney, 1971) using the POLO-PC computer program (LeOra Software). Estimation of the LT values and analysis of data from oviposition deterrence assays were done using the SPSS program version 16.0. Data obtained in percentages was subjected to the Arcsine $\sqrt{\frac{\chi}{100}}$ before ANOVA. The means were grouped using Tukey's test ($\alpha = 0.05$).

Results

Oil yield

The yields of essential oils from *C. sinensis* and *C. paradisi* were 5-7% and 4-6% (v/w based on dry weight), respectively.

Fumigant toxicity tests

Results of fumigant toxicity of essential oil of *C. sinensis* and *C. paradisi* are presented in Table 1. Probit analysis showed that there was no significant differences between *C. sinensis* (LC_{50} = 7.27 µl.l^{-1} air) and *C. paradisi* (LC_{50} = 7.70 µl.l^{-1} air) essential oils (Table 1).

Estimated lethal time (LT) for 50% mortality of the pest at different concentrations of subjected oils are presented in Table 2 The median lethal time for *T. castaneum* adults after exposure to the highest concentration (83 µl.l^{-1} air) of *C. sinensis* and *C. paradisi* were calculated to be 3.31 and 3.42 hours, respectively.

Table 1 Estimated LC values of essential oils from *Citrus sinensis* and *Citrus paradisi* against *Tribolium castaneum* adults applied as fumigant.

Essential oils		LC values (µl.l^{-1} air)[1]				Pearson Goodness-of-Fit test			RMP 95% (CL)[2]
		LC_{10}	LC_{30}	LC_{50}	LC_{90}	Slope ± SE	X^2 (df)	*P*-value	
C. sinensis	450	6.05 (5.75-6.28)	6.74 (6.54-6.19)	7.27 (7.11-7.42)	8.73 (8.47-9.09)	16.14 ± 1.49	7.79 (6)	0.254	1.06 (1.02-1.10)
C. paradis	450	6.05 (5.73-6.31)	6.98 (6.74-7.18)	7.70 (7.49-7.92)	9.80 (9.38-10.40)	12.23 ± 1.04	1.39 (6)	0.966	

[1] LC values are expressed with their 95% confidence limits (CL).
[2] Relative median potency = LC_{50} of *C. paradisi* divided by LC_{50} of *C. sinensis*.

Table 2 Estimated LT_{50} (h) and LT_{90} (h) values of different concentrations of the essential oil from *Citrus sinensis* and *Citrus paradisi* against the adults of *Tribolium castaneum.*

Essential oils	Concentration (µl.l^{-1} air)	n	LT_{50} (h)[1]	LT_{90} (h)[1]	Slope ± SE	X^2 (df)
C. sinensis	17	240	13.62 (11.43-16.49)	50.04 (34.93-96.27)	2.26 ± 0.36	15.22 (22)
	34	240	6.85 (5.53-8.08)	19.94 (16.36-26.58)	2.76 ± 0.35	14.11 (22)
	50	240	4.44 (3.71-5.11)	8.22 (7.06-10.13)	4.79 ± 0.65	9.36 (22)
	67	240	3.67 (2.81-4.41)	8.01 (6.73-10.17)	3.78 ± 0.56	11.87 (22)
	83	240	3.31 (2.46-4.02)	7.09 (5.92-9.12)	3.87 ± 0.63	3.31 (22)
C. paradisi	17	240	13.34 (11.26-15.92)	45.79 (32.90-82.82)	2.39 ± 0.37	16.20 (22)
	34	240	8.28 (7.01-9.49)	20.12 (17.02-25.50)	3.32 ± 0.39	10.19 (22)
	50	240	6.24 (5.38-7.04)	11.57 (10.13-13.80)	4.77 ± 0.55	10.97 (22)
	67	240	5.27 (4.46-6.03)	10.15 (8.78-12.33)	4.49 ± 0.55	10.23 (22)
	83	240	3.42 (2.96-3.89)	5.04 (4.33-6.76)	7.60 ± 1.59	2.50 (22)

[1] LT_{50} and LT_{90} values are expressed with their 95% confidence limits (CL).

The lowest concentration of the oils (17 µl.l^{-1} air) yielded 66.6 and 70% mortality of *T. castaneum* after 24 h of exposure, respectively (Fig. 1). Increasing the oils concentration to 34 µl.l^{-1} air resulted in 93.3% mortality after 24 h. At concentration of 50 µl.l^{-1} air of both oils, mortality of the beetles was more considerable and 100% mortality was achieved only 15 h after commencement of exposure. At the highest concentration (83 µl.l^{-1} air), total mortality of *T. castaneum* by *C. sinensis* and *C. paradisi* oils was obtained after 12 and 9 h of exposure, respectively.

Oviposition deterrence tests

Oviposition deterrence activity of various concentrations of the oils on the females of *T. castaneum* are shown in Fig. 2. It was proved that oviposition rate of treated females decreased as concentration of the oils was increased from 500 to 2500 ppm (Fig. 2). Also, the oils' oviposition deterrence activity significantly increased as concentrations of the oils were increased (F = 50.57, df = 5, P < 0.0001). In case of *C. sinensis* oil, treating female beetles with concentrations of 500, 1000, 1500, 2000, and 2500 ppm resulted in a 8.84, 17.69, 20.35, 50.44, and 67.25% decline in the oviposition rate, respectively. Similarly, treating female beetles with concentrations of *C. paradisi* caused a 6.19, 21.23, 29.20, 56.63, and 70.79% decline in the oviposition rate, respectively (Fig. 2).

Discussion

Essential oils synthesized by aromatic plants play an important role in protecting plants against insect pests. These compounds affect insects via insecticidal, repellent, deterrent, and antifeedant activities (Isman, 2006). Our results clearly indicated that *C. sinensis* and *C. paradisi* oils can be considered efficient insecticides against *T. castaneum*, causing high mrtality in the laboratory even at low concentrations.

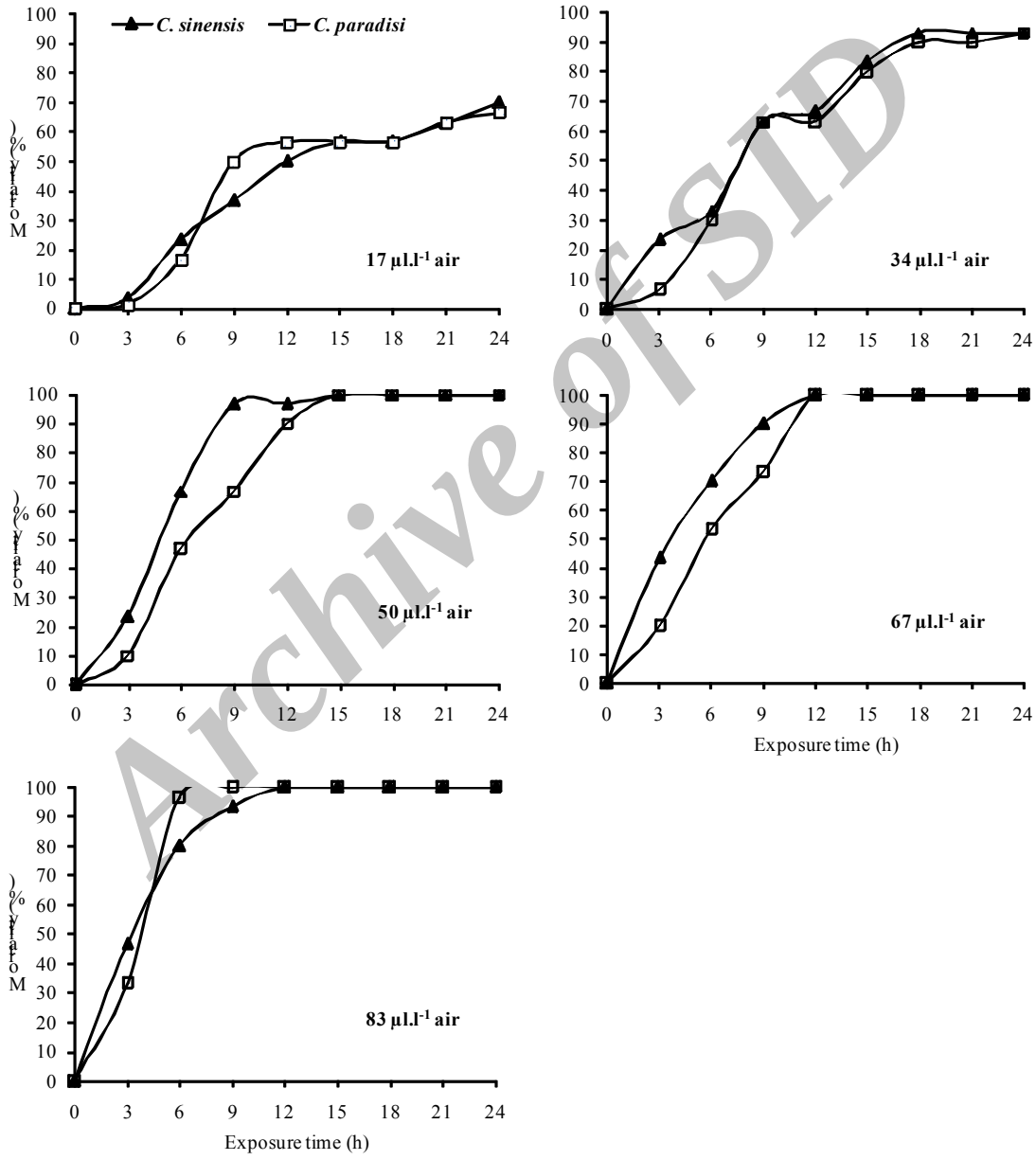

Figure 1 Percentage mortality of *Tribolium castaneum* adults exposed for various periods of time to the different concentrations of essential oils from *Citrus sinensis* and *Citrus paradise*.

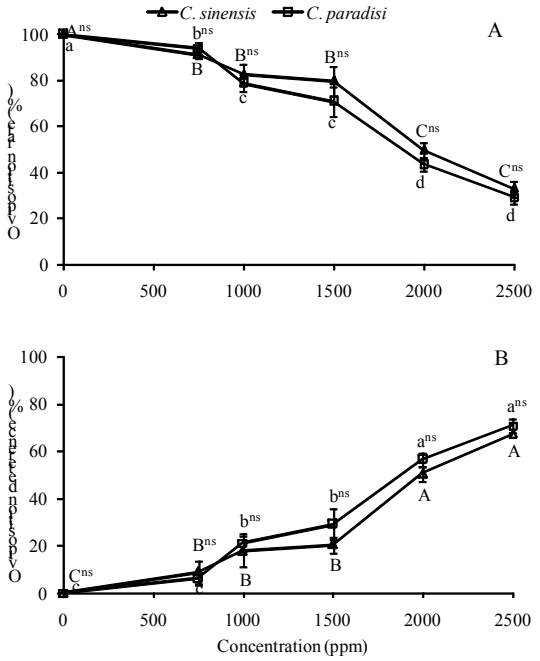

Figure 2 Effect of different concentrations of *Citrus sinensis* and *Citrus paradisi* oils on oviposition rate (A) and percentage oviposition deterrence (B) of female adults of *Tribolium castaneum* after 24 h fumigation (the mean ± SE). Means followed by the different letters for each oil (capital letters for *C. sinensis* oil and small letters for *C. paradise* oil) indicate significant differences at $p < 0.05$, Tukey's test. Means were compared for each concentration by independent student's t-test. *ns:* no significant differences.

Many plant essential oils have been screened for their insecticidal activies against *T. castanum*. Studies have not so far been reported dealing with the fumigant toxicity of *C. sinensis* and *C. paradisi* oils against adults of this insect species. Based on the estimated lethal concentration values, it is shown that essential oils from *C. sinensis* and *C. paradisi* are very toxic against adults of *T. castaneum*. In a similar study, Safavi and Mobki (2012) showed that LC_{50} value of *C. reticulata* peel essential oil was 38.2 $\mu l.l^{-1}$ air at 24 h after exposure of 1 to 7-days-old adults of *T. castaneum*. The LC_{50} of the essential oils of fruit peels and seeds of *C. reticulata* against adult of *T. castaneum* was 58.31 µl, 53.00, and 43.81 µl at 24, 48, and 72 h exposure (Saleem *et al*., 2013). Also, LC_{50} value of *Carum copticum* C. B. Clarke (Apiaceae) was estimated to be 33.14 $\mu l.l^{-1}$ air against adults (1-7-days-old) of *T. castaneum* (Sahaf *et al*., 2007). So, differenrt plant species vary in their toxicity against *T. castaneum* and according to the findings of our research it could be concluded that *C. sinensis* and *C. paradisi* essential oils are highly toxic to *T. castaneum* compared with the previously examined oils. In fact, the toxicity of essential oils against an insect species is influenced by factors such as plant species, season, ecological conditions, method of oil extraction, time of extraction, plant part used, and most importantly the chemical composition of the oil (Don-Pedro, 1996b; Lee *et al*., 2001). Limonene is the major and the most toxic monoterpenoid of *Citrus* essential oils which causes insect mortality (Mansour *et al*., 2004; Papachristos *et al*., 2009). It has been proven that monoterpenoids kill insects by interfering with acetylcholinesterase enzyme (AChE) activity (Houghton *et al*., 2006). So, it would likely seem that the high fumigant toxicity of *C. sinensis* and *C. paradisi* oils toward *T. castaneum* is linked to possible precense of high amount of limonene and more AChE inhibition. However, further studies would be required for chemical characterization of active ingredients of the oils and more comprehensive toxicity assays.

It was also indicated that *T. castaneum* mortality increased with increasing the oils concentration and exposure time. Findings of many studies have shown the same trend as that observed in the present research. For example in a parallel study, Zia *et al*. (2013) indicated that toxicity of essential oils extracted from peel of various *Citrus* species increased as the exposure length and concentration were increased for the subjected insect including the adults of *T. castaneum*. In another study, it was proved that the mortality of adults of *Callosobruchus maculatus* (F.) and *Sitophilus granarius* (L.) significantly increased as *Thymus daenensis* Celak EO concentration and exposure time increased (Jarrahi *et al*., 2016).

In addition to toxicity, the essential oils have been shown to possess ovipopsition deterrence

effect on the female adults of *T. castanum*. Results exhibited that the oils are effective for reducing the oviposition rate of *T. castaneum*. Similar observations on other plant essential oils have also been made. For example, Huang *et al*. (2000) showed that the essential oil of cardamom, *Elletaria cardamomum* (L.) Maton., strongly declined the number of eggs laid by female *T. castanum*.

Conclusion

There are several reports presenting the relative tolerance of *T. castanum* to essential oils of various plants (Liu *et al*., 1999; Huang *et al*., 2000; Sahaf *et al*., 2007). Nevertheless, the oils tested in this research proved to be highly toxic to this devastating beetle even at low concentrations. The hope is that *C. sinensis* and *C. paradisi* oils could be used as effective and safe compounds in storage systems after comprehensive semi-field and field evaluations.

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سمّیت و فعالیت بازدارندگی تخم‌ریزی اسانس پوست میوه پرتقال *Citrus sinensis* (L.) Osbeck و گریپ‌فروت *Citrus paradise* (Macfarlane) علیه حشرات بالغ شپشه قرمز آرد *Tribolium castaneum* (Herbst)

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چکیده: شپشه قرمز آرد *Tribolium castaneum* (Herbst) یکی از مخرب‌ترین آفاتی است که محصولات انباری را در سرتاسر دنیا مورد حمله قرار می‌دهد. مشکلات جدی حاصل از مصرف حشره‌کش‌های شیمیایی سنتز شده، نیاز به استفاده از ترکیبات ایمن جایگزین مانند اسانس‌های گیاهی را بیش از پیش مشخص کرده است. پژوهش حاضر به‌منظور ارزیابی سمّیت تنفسی اسانس پوست تازه میوه دو گونه مرکبات شامل پرتقال *Citrus sinensis* (L.) Osbeck و گریپ‌فروت *Citrus paradise* (Macfarlane) علیه حشرات بالغ یک تا هفت روزه شپشه قرمز آرد تحت شرایط آزمایشگاهی انجام شد. هم‌چنین، فعالیت بازدارندگی تخم‌ریزی غلظت‌های زیرکشنده این اسانس‌ها روی سوسک‌های ماده مورد بررسی قرار گرفت. تمام آزمایش‌ها در دمای ۱ ± ۲۷ درجه سلسیوس، رطوبت نسبی ۵ ± ۶۵ درصد و تاریکی انجام شد. یافته‌ها بیانگر سمّیت تنفسی بالای دو اسانس مورد آزمایش است و تجزیه پروبیت داده‌ها نشان داد که اختلاف آماری معنی داری میان اسانس پرتقال (LC_{50} = ۷/۲۷ میکرولیتر بر لیتر هوا) و اسانس گریپ‌فروت (LC_{50} = ۷/۷۰ میکرولیتر بر لیتر هوا) وجود ندارد. علاوه بر این، خاصیت بازدارندگی تخم‌ریزی اسانس‌ها با افزایش غلظت اسانس از ۵۰۰ پی‌پی‌ام به ۲۵۰۰ پی‌پی‌ام به‌طور معنی‌داری افزایش یافت. به‌طور کلی، نتایج این مطالعه کارایی بالای اسانس پرتقال و گریپ‌فروت علیه شپشه قرمز آرد را اثبات می نماید.

واژگان کلیدی: شپشه قرمز آرد، پرتقال، گریپ‌فروت، سمّیت تنفسی، بازدارندگی تخم‌ریزی