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Research Article

Toxicity, developmental and histological effects of monoterpenes on peach fruit fly, *Bactrocera zonata* (Diptera: Tephritidae)

Samir A. M. Abdelgaleil[1*], Mohamed A. AL-Eryan[2], Abdelaziz M. EL-Minshawy[2], Gadelhak G. Gadelhak[2] and Rafiaa A. Rabab[2]

1. Department of Pesticide Chemistry and Technology, Faculty of Agriculture, Alexandria University, Egypt.
2. Department of Applied Entomology, Faculty of Agriculture, Alexandria University, Egypt.

Abstract: In the present study, the toxicity of three oxygenated monoterpenes, viz., (*R*)-carvone, (*R*)-camphor and (1*R*, 2*S*, 5*R*)-menthol was evaluated against the adults of the peach fruit fly, *Bactrocera zonata* (Saunders). Effects of these monoterpenes on oviposition, adult longevity and ovarian development have also been studied. The tested compounds revealed strong toxic effect against the adults of *B. zonata.* Nevertheless, (-)-carvone showed higher insecticidal activity than (1*R*, 2*S*, 5*R*)-menthol and (*R*)-camphor. Moreover, the tested monoterpenes induced significant reduction in female longevity at 20 and 50 mg/kg compared with control, with (-)-carvone being the most effective monoterpene. In addition, egg deposition was completely inhibited when the adults were treated with (-)-carvone at 20 and 50 mg/kg, and (1*R*, 2*S*, 5*R*)-menthol and (*R*)-camphor at 50 mg/kg. Histological studies indicated that monoterpenes induced delay in the development of ovarioles of females that fed on artificial diet treated with 20 and 50 mg/kg of compounds. Changes, such as empty egg chambers; constricted germarium, vacant spaces between ovarioles and failure of oocyte formation were observed.

Keywords: *Bactrocera zonata*, monoterpenes, toxicity, fecundity, ovarian development

Introduction

Monoterpenes are widely distributed in plants as the major constituents of essential oils. They are responsible for specific odors and flavors of numerous plants due to their high volatility and characteristic scents. Monoterpenes are synthesized from acyclic C10 intermediate, geranyl pyrophosphate of the isoprenoid pathway. Monoterpenes have a great variety of structures with two major groups: oxygenated monoterpenes and monoterpene hydrocarbons. Each group can be subgrouped into three categories: acyclic, monocyclic, and bicyclic structures (Templeton, 1969; Windholz *et al.,* 1983). Monoterpenes are known for their numerous biological activities with important applications in chemical ecology, pharmaceutical industry and food chemistry. Monoterpenes are also involved in several plant ecological roles, such as attraction of pollinators, allelopathy and protection against microbial diseases and herbivores (Langenheim, 1994; Schewe *et al.,* 2011). Monoterpenes have been described to show numerous biological properties, such as insecticidal, herbicidal, fungicidal and bactericidal activities (Cantore *et al.,* 2009; Liska *et al.,* 2010; Marei *et al.,* 2012; Gouda *et al.,* 2016). These biological activities prove monoterpenes as leading compounds for the

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* **Corresponding author**, e-mail: samirabdelgaleil@gmail.com
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discovery of novel insecticides (Isman *et al.,* 2011).

Bactrocera zonata (Saunders) (Diptera: Tephritidae), is a harmful insect that attacks numerous fruit and vegetable crops worldwide. This insect can reduce the fruit production and quality resulting in huge economic loss to growers (Agarwal *et al.,* 1999; EPPO, 2005). It was discovered first in South and South-East Asian countries and then has been introduced into Persian Gulf region and Egypt (Carroll *et al.,* 2006).

Egyptian growers applied large amounts of synthetic insecticides to overcome the production loss caused by fruit flies, including *B. zonata.* However, extensive use of conventional insecticides has augmented resistance development and disturbed ecological systems. Moreover, the growing public awareness about harmful effects of traditional insecticides on the environment and public health has encouraged the developing of safer and ecofriendly strategies for insect control. Among the most common components of new strategies for integrated pest management are plant extracts, essential oils and plant secondary metabolites.

The insecticidal activity of monoterpenes, ((*R*)-carvone, (*R*)-camphor and (*1R*, 2*S*, 5*R*)-menthol,) has been studied against some economic insects. For example, menthol was reported to show mosquitocidal activity against *Aedes aegypti* (L.), *Culex quinquefasciatus* (Say), and *Anopheles tessellatus* (Theobald) (Samarasekera *et al.,* 2008), and larvicidal and ovicidal properties against *Triatoma infestans* (Klug) (Laurent *et al.,* 1997). Similarly, (*R*)-carvone was toxic against *Rhyzopertha dominica* (Fabricius), *Sitophilus oryzae* (Linnaeus), and *Cryptolestes pusillus* (Schönherr) (Lopez *et al.,* 2010). (*R*)-Camphor was effective toxicant against *Blattella germanica* (Linnaeus) and *Tribolium castaneum* (Herbst) (Jung *et al.,* 2007; Liska *et al.,* 2010). Moreover, in our earlier studies, the three monoterpnes displayed marked contact and fumigant toxicities against *Culex pipiens* (Linnaeus) and *Spodoptera littoralis* (Boisduval) (Abdelgaleil, 2010; Zahran and Abdelgaleil, 2011). Otherwise, menthol and carvone were reported to decrease adult emergence and pupation of *Ostrinia nubilalis* (Hübner) (Lee *et al.,* 1999) and *C. pipiens* (Zahran and Abdelgaleil, 2011).

In the literature, as far as we know, there are no studies on the biological activities of monoterpenes against the adults of *B. zonata.* However, in our previous study, monoterpenes showed toxic and developmental male effects on the larvae of *B. zonata* (El-Minshawy *et al.,* 2018). Thus, the current study was undertaken to examine the toxicity of (*R*)-carvone, (1*R*, 2*S*, 5*R*)-menthol and (*R*)-camphor on the adults of *B. zonata.* The effects on female longevity, fecundity and reproductive system induced by monoterpenes were also evaluated.

Materials and Methods

Test monoterpenes

Technical grade of(*R*)-carvone (98%), (*1R*, 2*S*, 5*R*)-menthol (98%) and (*R*)-camphor (98%) were obtained from Sigma-Aldrich Chemical Co., Steinheim, Germany.

Insect collection and rearing

The peach fruit fly (PFF), *B. zonata*, was reared at Department of Applied Entomology, Faculty of Agriculture, Alexandria University, Egypt under controlled conditions at 25 ± 2 ºC, 70 ± 5 % R. H. and a photoperiod of 14:10 (L: D). Insect adults were originally obtained from infested guava, *Psidium guajava* L., fruits collected from house backyards in Agamy district at the Western North coast, Egypt during the year 2015. Insects were reared for three generations on guava fruits in the laboratory for adaptation, followed by rearing for six generations on semi-artificial diet on small scale in an insect rearing cabinet (2 × 3 × 3m).The adults were fed on diet containing beef extract and sugar (3: 1). The larvae were fed on diet containing soybean flour (15%), gelatin (12.6%), sugar (8.9%), corn oil (1.4%), nipagen (0.15%), sodium benzoate (0.15%), citric acid (1.7%) and water (60%) (Rabab *et al.,* 2016).

Toxicity bioassay

The insecticidal activity of monoterpenes was evaluated on the newly emerged flies of *B.*

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zonata. The tested compounds were first prepared in acetone and mixed with semi-artificial diet to give a series of concentrations ranged between 20 and 100 mg/kg. The diet was mixed with acetone in control treatment. Ten adults were transferred gently from the rearing cages to glass vials containing 10g of diet placed inside small cups and covered with a piece of cloth. Five replicates were used in each treatment. The treated and untreated insects were maintained at the same rearing conditions. The mortality percentages were recorded after 24, 48 and 72h of treatment. The lethal concentration causing 50% mortality (LC_{50}) expressed as mg/kg was calculated from log-concentration mortality regression lines (Finney, 1971).

Fecundity and adult female longevity test

Effect of monoterpenes on fecundity and adult female longevity of *B. zonata* was evaluated on the newly emerged flies. Female is characterized with three segmented ovipositor. The adults were fed on diet treated with 20 and 50 mg/kg of monoterpenes for three days. Afterwards, the survived adults were fed on untreated diet until completing their life cycle. The insects were daily examined and the egg number deposited by each female (fecundity), and female longevity (days) were recorded.

Histological studies on ovary

Ovaries of *B. zonata* adult females from both treatments and control were taken for histological examinations after 15 days of feeding for three days on artificial diet treated with 20 and 50 mg/kg of monoterpenes. The insect ovaries were separated out in 0.9% NaCl solution and then fixed in aqueous Bouin's solution for 24h. The ovaries were then moved to ethyl alcohol solution (70%). The individual ovarioles were separated and embedded in paraffin wax (58-60 °C). Longitudinal sections were cut at 7μ thicknesses using rotary microtome. The sections were deparaffinised and stained in Heidenhain's hematoxylin and counterstained with eosin (Drury and Wallington 1980) and stained sections were examined (Hedaya 1990).

Statistical analysis

The percentages of mortality were analyzed by probit program (Finney, 1971) to obtain the LC_{50} values, using SPSS 12.0 (SPSS, Chicago, IL, USA). The LC_{50}values were considered significantly different if the 95% confidence limits did not overlap. Number of eggs/female and female longevity were subjected to one-way analysis of variance followed by Student-Newman-Keuls test (Cohort software Inc., 1985) to determine significant differences among mean values at the probability level of 0.05.

Results

Effect of monoterpenes on the mortality of *B. zonata* adults

Table 1 shows the LC_{50} values (mg/kg) for the three monoterpenes after 24, 48 and 72h of treatment on the adults of *B. zonata*. Based on LC_{50} values, the tested monoterpenes showed remarkable insecticidal activity. The toxic effect of the three tested monoterpenes enhanced significantly with increasing the time of treatment. (-)-Carvone revealed the highest toxicity to the adults after 24 and 48h of treatment. Also, (-)-carvone and (*R*)-camphor were more toxic than (*1R*, 2*S*, 5*R*)-menthol after 72h of treatment as their LC_{50} were less than 20 mg/kg.

Effect of monoterpenes on female longevity

The results of the effect of monoterpenoidal compounds on female longevity are given in Table 2. In general, the monoterpenes produced obvious reduction in female longevity. At 20 mg/kg, (-)-carvone induced the highest reduction in female longevity, followed by (*R*)-camphor and (1*R*, 2*S*, 5*R*)-menthol. The treatment with (-)-carvone at 20 mg/kg for 72 h reduced female longevity to 25.67 days compared to 51.0 days in control. The tested monoterpenes caused higher reduction in female longevity at 50 mg/kg than 20 mg/kg without significant differences among the compounds.

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Effect of monoterpenes on fecundity

Table 3 shows the effect of three monoterpenes at concentrations of 20 and 50 mg/kg on the adult fecundity of *B. zonata*. The tested compounds significantly reduced the number of laid egg per female. The reduction of adult fecundity was greater at 50 mg/kg than 20 mg/kg. Insect fecundity was completely inhibited by the three monoterpenes at concentration 50 mg/kg.

Histological changes in ovary

The effects of tested monoterpenes on growth of ovary and development of ovarioles are shown in Fig. 1.

Table 1 Comparative toxicity of monoterpenes against *Bactrocera zonata* adults after different exposure times.

Compound	Time (h)	LC_{50} (95% Confidence limits) (mg/kg)	Slope ± SE	Intercept ± SE	x^2
(+)-Camphor	24	71.55 (60.33-89.08)	2.10 ± 0.30	-3.97 ± 0.62	0.26
	48	33.52 (26.30-40.46)	1.96 ± 0.26	-2.99 ± 0.45	0.14
	72	< 20	-	-	-
(-)-Menthol	24	65.77 (61.48-66.59)	5.78 ± 0.68	-10.51 ± 1.26	1.61
	48	44.43 (39.36-49.53)	2.91 ± 0.29	-4.79 ± 0.50	3.49
	72	31.81 (27.59-35.86)	3.10 ± 0.35	-4.84 ± 0.57	1.50
(-)-Carvone	24	< 50	-	-	-
	48	25.31 (20.04-30.24)	2.42 ± 0.28	-3.40 ± 0.47	1.87
	72	< 20	-	-	-

Table 2 Effect of monoterpenes on female longevity of *Bactrocera zonata* adults treated with 20 and 50 mg/kg.

Compound	Time (h)	Female longevity (Mean ± SE) (day)	
		20 mg/kg	50 mg/kg
Control	24	51.00 ± 3.46a	51.0 ± 3.46a
	48	51.00 ± 5.34a	51.0 ± 5.34a
	72	51.00 ± 0.0a	51.0 ± 0.00a
(+)-Camphor	24	39.00 ± 1.0b	33.0 ± 1.73b
	48	39.00 ± 1.0b	33.0 ± 1.73b
	72	38.00 ± 1.0b	33.0 ± 1.73b
(-)-Menthol	24	53.33 ± 2.03a	35.0 ± 2.89b
	48	47.33 ± 4.81a	35.0 ± 5.78b
	72	40.33 ± 2.34b	35.0 ± 2.89b
(-)-Carvone	24	28.00 ± 1.0c	27.0 ± 4.04b
	48	26.33 ± 0.66c	27.0 ± 4.04b
	72	25.67 ± 0.66c	25.0 ± 2.89b

Mean values with different letters are significantly different (Student–Newman–Keuls test, P < 0.05).

Table 3 Effect of monoterpenes on fecundity of *Bactrocera zonata* adults treated with 20 and 50 mg/kg.

Compound	Time (h)	Deposited eggs			
		20 mg/kg		50 mg/kg	
		No. of eggs/female	Reduction (%)	No. of eggs/female	Reduction (%)
Control	24	55.00 ± 5.01a	0.0	55.00 ± 5.01a	0.0
	48	50.00 ± 0.0a	0.0	50.00 ± 0.00a	0.0
	72	53.33 ± 3.34a	0.0	53.33 ± 3.34a	0.0
(+)-Camphor	24	16.67 ± 3.34b	69.69	0b	100
	48	3.33 ± 2.96c	93.34	0b	100
	72	0c	100	0b	100
(-)-Menthol	24	6.67 ± 1.66c	87.87	0b	100
	48	6.67 ± 1.66c	86.66	0b	100
	72	6.67 ± 2.03c	87.49	0b	100
(-)-Carvone	24	0c	100	0b	100
	48	0c	100	0b	100
	72	0c	100	0b	100

Mean values with different letters are significantly different (Student-Newman-Keuls test, P < 0.05).

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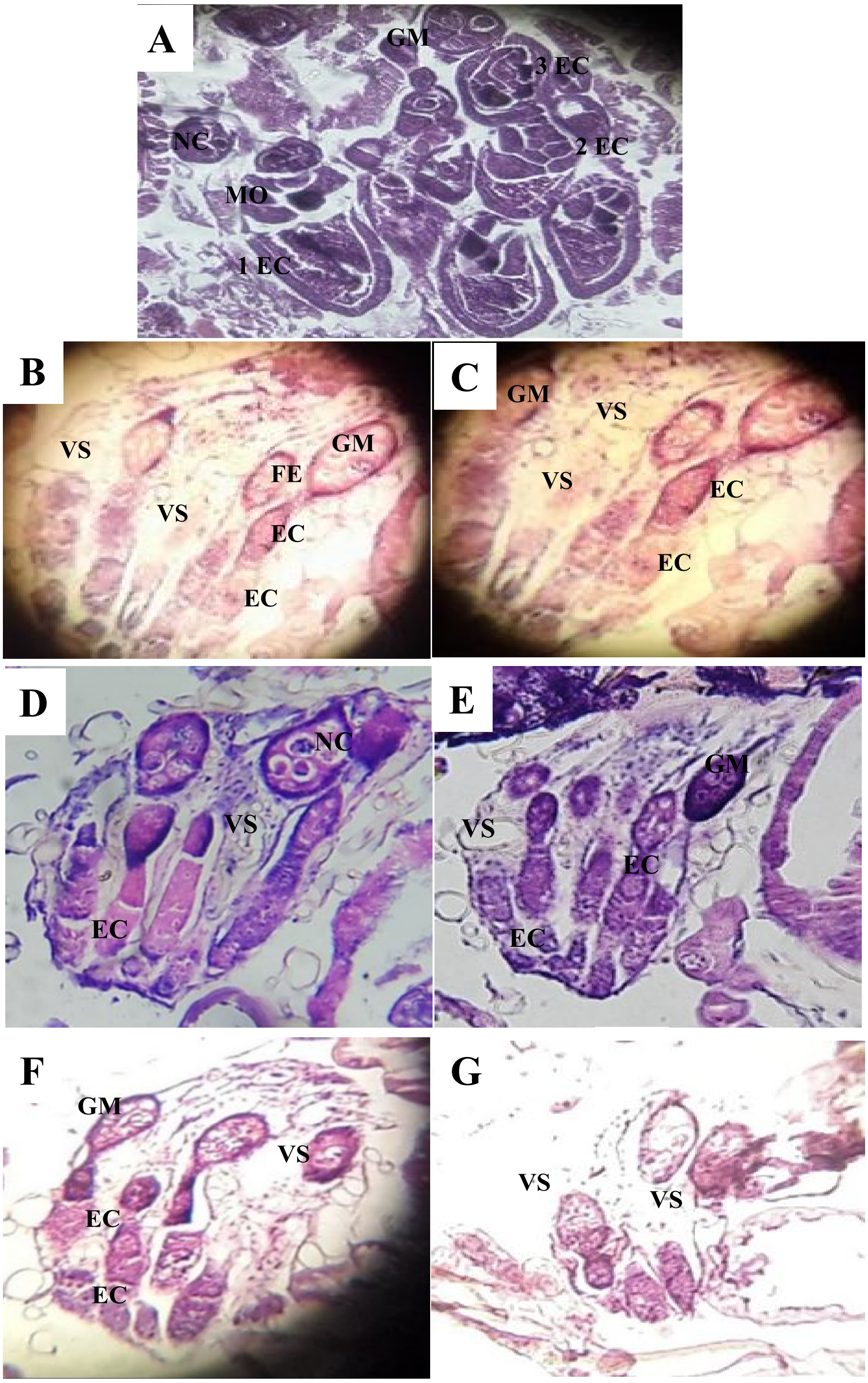

Figure 1 Histological changes on the ovaries of 18 days old females of *Bactrocera zonata* fed for three days, after emergence, on diet treated with monoterpenes: A = Longitudinal section (LS) in ovary of female fed on untreated diet, B and C = LS in ovary of female fed on diet treated with 20 and 50 mg/kg of (-)-menthol, respectively, D and E = LS in ovary of female fed on diet treated with 20 and 50 mg/kg of (+)-camphor, respectively, F and G = LS in ovary of female fed on diet treated with 20 and 50 mg/kg of (-)-carvone, respectively. GM = germarium; MO = mature ovum; NC = nurse cell; VS = vacant spaces; EC = egg chamber, FE = follicular epithelium.

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Clear reduction in oocyte numbers per ovary and the size of the basal oocyte were observed. Advanced degeneration in ovarian contents was observed when the adults were treated with (1*R*, 2*S*, 5*R*)-menthol and (*R*)-camphor at concentration of 20 mg/kg. Most of the ovarioles appeared vacant. The numbers of oocytes were small and the nurse cells were not clear. The treatment with (*R*)-camphor and (1*R*, 2*S*, 5*R*)-menthol at 50 mg/kg produced abnormal ovarioles full of nurse cells but without oocytes. Moreover, the treatment with (*R*)-camphor and (1*R*, 2*S*, 5*R*)-menthol at 50 mg/kg induced abnormality specified by vacuated spaces (VS) between ovarioles, dark stained masses (DM) and whole vanishing of oocytes. Likewise, (-)-carvone treatment caused the maximum damage to the ovaries. The deterioration of all the ovariol contents characterized by only few patchy egg chambers and empty spaces was detected (Fig. 1).

Discussion

The results of this study indicated that the tested monoterpenes, (1*R*, 2*S*, 5*R*)-menthol, (*R*)-carvone and (*R*)-camphor revealed marked insecticidal activity against *B. zonata* adults. To the extent of our knowledge, this is the first study reported on the insecticidal activity of monoterpenes against the adults of *B. zonata.* Nevertheless, the tested monoterpenes have been mentioned to show insecticidal effects against the larvae of *B. zonata* (El-Minshawy *et al.,* 2018) and other insects, such as *S. littoralis* (Abdelgaleil, 2010), *C. pipiens* (Zahran and Abdelgaleil, 2011), *T. castaneum* (Abdelgaleil *et al.,* 2009) and *S. oryzae* (Lee *et al.,* 2003). In this study, (*R*)-carvone exhibited greater insecticidal activity than (1*R*, 2*S*, 5*R*)-menthol and (*R*)-camphor against adults of *B. zonata.* Similarly, the previous studies concluded that(*R*)-carvone was more toxic than (1*R*, 2*S*, 5*R*)-menthol and (*R*)-camphor against *C. pipiens*, *S. oryzae*, *S. littoralis* and *T. castaneum*. The toxicity studies also illustrated that the ketones ((*R*)-camphor and (*R*)-carvone) are more potent than an alcohol ((*1R*, 2*S*, 5*R*)-menthol). Likewise, it has been noticed that some monoterpenes containing ketone groups were more effective toxicants than monoterpenes containing alcohol groups against house fly, southern maize rootworm and red flour beetle (Rice and Coats, 1994).

In spite of numerous studies that have described the insecticidal potential of different classes of monoterpenes, the modes of toxic action of monoterpenes on insects are not entirely elucidated. However, it has been described that monoterpenes showed inhibitory effects on acetylcholinesterases (AChEs) isolated from insects and other animal species (Ryan and Byrne 1988; Abdelgaleil *et al.,* 2009). Moreover, it has been mentioned that octopamine receptors and GABA-gated chloride ion channel may be targeted by monoterpenes (Höld *et al.,* 2000; Enan, 2001). In addition, monoterpenes have been described to have effects on cytochrome P_{450}monooxygenase and pheromone and hormone systems (De-Oliveira *et al.,* 1999; Garcia *et al.,* 2005). Thus, toxicity of monoterpenes against the adults of *B. zonata* may be due to their effects on one or more of these targets.

The results also pointed out that the tested monoterpenes reduced female longevity of *B. zonata* which indicates that the monoterpenes affected the hormonal balance of *B. zonata.* Previous studies supported these findings and clearly confirmed that monoterpenes (limonene, linalool and α-pinene) and other terpenes can cause hormetic-like effects on insects, similar to juvenile hormone and juvenile hormone mimics (Amos *et al.,* 1974; Papanastasiou *et al.,* 2017). For example, eugenol was reported to reduce female longevity of *Agrotis ipsilon* (Abd El-Aziz *et al.,* 2007). In addition, essential oils and volatile compounds (geraniol, isoeugenol and α-lonone) significantly decreased the life span of males and females of *Phthorimaea operculella* (Sharaby *et al.,* 2014).

Moreover, the tested monoterpenes completely inhibited oviposition of *B. zonata* at at 50 mg/kg. In agreement with these findings, plant extracts have been demonstrated to deter the oviposition of *B. zonata* (Siddiqi *et al.,* 2006, 2011; Khattak *et al.,* 2006; Ur-Rehman *et al.,* 2009). It has been also found that some monoterpenes affect the oviposition of other insect species. For instance, Chaubey (2012) mentioned that monoterpenes, such as α-pinene and β-caryophyllene significantly decreased adult emergence, oviposition and pupation and of *T. castaneum*. Other monoterpenes (linalool, terpinen-4-ol, R-(+)-limonene, geraniol and 1,8-cineole,) induced lower egg hatchability and fecundity of *T. confusum* (Stamopoulos *et al.*, 2007). Similarly, 1,8-cineole and terpinen-4-olhave been described to diminish eggs hatchability and fecundity of *T. confusum* and *T. castaneum* (Amos *et al.,* 1974). Carvone was also shown to cause complete suppression of *T. castaneum* egg hatching when tested at 7.22 mg/cm^2 (Tripathi *et al.,* 2003).

In this study, the tested monoterpenes induced damaging effects on ovary and ovarioles of *B. zonata* adults. These changes resulted in complete inhibition of oviposition. These findings referred to the possible use of monoterpenes as chemical sterilants in sterile insect release programs. There are few studies describing the effects of natural products and compounds on insect ovary and ovarioles. For instance, the treatment of *Trogoderma granarium* with caraway oil affected the basophilic affinity of the nuclei of germarium cells and the follicular epithelium of developing oocytes (Osman *et al.,* 2016). In addition, the treatment of *Heteracris littoralis* with garlic, mint and eucalyptus essential oils reduced oogenesis and egg laying (Sharaby *et al.,* 2012). Blends of cedarwood oil with eucalyptus, peppermint and camphor oils caused showed potent fecundity inhibition of *Corcyra cecphalonica*. The treatment with these oil mixtures caused the appearance of numerous empty spaces within the ovarioles. The oils also disturbed arrangement and distribution of oocytes/ova with two or more ova merging and fusing to produce a lumpy mass inside the ovarioles (Jacob and Qamar, 2013). The treatment of *T. confusum* with a diterpene, forskolin, isolated from the roots of *Coleus forskohlii* reduced ovarian development. The ovaries showed variation in the size and length of the ovarioles, oocyte degeneration, resorption and inability of the mature oocytes to oviposit (Lingampally *et al.,* 2012). Finally, thymol, a monoterpene, was reported to interfere with the development of oocytes of *Rhipicephalus sanguineus* and produce degeneration signs (Matos *et al.,* 2014).

In summary, the tested monoterpenes, (1*R*, 2*S*, 5*R*)-menthol, (*R*)-carvone and (*R*)-camphor showed remarkable biological activities on *B. zonata* adults, including toxicity, ovarian damage and sterilizing effects. These results demonstrated that the tested monoterpenes cause their effects on this insect through multiple targets. Thus, the tested monoterpenes might be suitable components in *B. zonata* control programs.

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اثرات سمّی و بافت‌شناسی مونوترپن‌ها روی تخمدان مگس میوه هلو، *Bactrocera zonata* (Diptera: Tephritidae)

سمیر آ. ام. عبدالغاله[1*]، محمد آ. الریان[2]، عبدالعزیز ام. المنشوی[2]، گدهاک جی. گدهاک[2] و رفیع آ. رباب[2]

۱- گروه شیمی آفت‌کش‌ها و فنآوری، دانشکده کشاورزی، دانشگاه اسکندریه، مصر.
۲- گروه حشره‌شناسی کاربردی، دانشکده کشاورزی، دانشگاه اسکندریه، مصر.
پست الکترونیکی نویسنده مسئول مکاتبه: samirabdelgaleil@gmail.com
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چکیده: در مطالعه حاضر، سمّیت سه مونوترپن اکسیژنه، شامل کاروون، کامفور و منتول روی حشرات کامل مگس میوه هلو، *Bactrocera zonata* (Saunders) بررسی شد. اثرات این مونوترپن‌ها بر تخم‌گذاری، طول عمر حشرات کامل و رشد تخمدان‌ها نیز مورد بررسی قرار گرفت. ترکیبات آزمایش شده اثرات سمّی قوی روی حشرات کامل نشان دادند. با این وجود خاصیت حشره‌کشی کاروون بیش‌تر از منتول و کامفور بود. علاوه بر این، مونوترپن‌های آزمایش شده باعث کاهش معنی‌دار طول عمر حشرات کامل در غلظت ۲۰ و ۵۰ میلی‌گرم در کیلوگرم در مقایسه با شاهد شد، به‌طوری که کاروون مؤثرتر از بقیه بود. به‌علاوه، تخم‌گذاری در غلظت ۲۰ میلی‌گرم در کیلوگرم کاروون و ۵۰ میلی‌گرم در کیلوگرم کامفور و منتول متوقف شد. مطالعات بافت‌شناسی نشان داد که مونوترپن‌ها موجب تأخیر در رشد تخمدان‌های حشرات ماده تیمار شده در رژیم غذایی مصنوعی حاوی ۲۰ و ۵۰ میلی‌گرم در کیلوگرم شدند. هم‌چنین تغییراتی مانند فضاهای خالی تخم، ژرماریوم‌های ضعیف، فضای خالی بین تخمدان‌ها و اختلال در تشکیل سلول‌های تخم مشاهده شد.

واژگان کلیدی: *Bactrocera zonata*، مونوترپن‌ها، سمّیت، باروری، تکامل تخمدان

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