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Orijinal araştırma (Original article)

The toxic effects of *Perilla frutescens* essential oils in combination with CO_2-enriched modified atmospheres on the life stages of *Dermestes maculatus* Degeer (Coleoptera: Dermestidae)

CO_2 bakımından zengin değiştirilmiş atmosferler ile kombine edilmiş *Perilla frutescens* uçucu yağlarının *Dermestes maculatus* Degeer (Coleoptera: Dermestidae)' un biyolojik dönemleri üzerine toksik etkileri

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Summary

Fumigation with essential oils from plants is a popular, safe, and environment friendly alternative tool for pest control in stored products. To provide a range of choices and to reduce costs in a variety of contexts, more plant-derived pest management control agents from local plant species should be identified and more effective fumigant methods must be developed. This paper describes the toxic effects of the essential oils from *Perilla frutescens* (L.) Britt. (Lamiaceae) as a fumigant, either alone or in combination with CO_2-enriched modified atmospheres, for the control of *Dermestes maculatus* Degeer. The essential oils showed strong fumigant activities against this pest. After six hours of fumigation with the essential oils at a concentration of 0.10 µL/L, mortalities for each life stage was 73.0% (adult), 61.2% (larva), 39.6% (pupa) and 55.4% (egg). Furthermore, the effects were enhanced when CO_2 levels were increased. At an essential oil concentration of 0.08 µL/L in the treatment group with 60% CO_2, mortality rate for all life stages was 100%. We propose that CO_2 concentration plays a direct role during joint fumigation, as atmospheres with high CO_2 levels cause the permanent opening of insect spiracles, thereby increasing the uptake of plant essential oils.

Keywords: *Perilla frutescens*, *Dermestes maculatus*, pest control, fumigation, CO_2

Özet

Depolanmış ürün zararlılarının mücadelesinde bitkilerden elde edilen uçucu yağlar ile fumigasyon popüler, güvenli ve çevre dostu alternatif bir yöntemdir. Geniş bağlamda çeşitliliği arttırmak ve maliyetleri azaltmak için yerel bitki türlerinden bitki kökenli zararlı kontrolünde kullanılabilecek daha fazla maddeler tanımlanmalı ve daha etkili fumigasyon metotları geliştirilmelidir. Bu çalışma, *Perilla frutescens* (L.) Britt. (Lamiaceae)'dan elde edilen uçucu yağların, fumigant olarak, tek başına ya da CO_2 bakımından zengin değiştirilmiş atmosferler ile birlikte kullanımının, *Dermestes maculatus* Degeer. (Coleoptera: Dermestidae)'a karşı toksik etkisini açıklamaktadır. Uçucu yağlar bu zararlıya karşı kuvvetli fumigant etki göstermiştir. Uçucu yağın 0.10 µL/L konsantrasyonunda altı saatlik fumigasyondan sonra yaşam dönemlerinin yüzde ölüm oranları %73 (ergin), % 61.2 (larva), % 39.6 (pupa) ve %55.4 (yumurta) bulunmuştur. Ayrıca, CO_2 seviyesi arttığında fumigant etki de artmıştır. %60 CO_2 içeren ortamda uçucu yağın 0.08 µL/L konsantrasyon uygulaması tüm yaşam dönemlerinde %100 ölüme neden olmuştur. Yüksek CO_2 konsantrasyonu böcek stigmalarının sürekli açık kalmasına, dolayısıyla bitki uçucu yağlarının alımının artmasına neden olduğu için ortak fumigasyonda CO_2 konsantrasyonunun uçucu yağın fumigant etkisinde önemli bir rol aldığını düşünmekteyiz.

Anahtar sözcükler: *Perilla frutescens*, *Dermes tesmaculatus*, zararlı yönetimi, fumigasyon, CO_2

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Introduction

Dermestes maculatus Degeer (Coleoptera: Dermestidae) is a major cosmopolitan insect pest of dried fish and other products of animal origin, and it frequently causes severe damage (Lambkin & Khatoon, 1990). This insect attacks not only dried animal flesh, bones, hides, horns, and feathers, but also destroys other stored products, including dried blood, leather and cheese (Hiton, 1945; Macquillan & Shipp, 1976; Osuji, 1975; Islam et al., 2009). In addition to damaging stored products, tunneling larvae seeking to construct pupation chambers can severely damage wood, cork and other materials, including packaging (Khatoon & Heather, 1990). For these reasons, in surveys on pest control, *D. maculatus* has also been identified as a serious museum pest (Linnie, 1994, 1999). *D. maculatus* is a favorite experimental insect pest, as large numbers for each life stage can be readily reared in laboratory cultures (Linnie & Keatinge, 2000).

Conventional control methods for *D. maculatus* populations around the world are primarily based on the continued application of chemicals such as sodium arsenate, chlorinated hydrocarbons, and organophosphate insecticides (Wheatley, 1971; Macquillan & Shipp, 1976; Linnie & Keatinge, 2000). However, repeated use of these chemicals over decades has led to resistance in the target insects, undesirable effects on non-target organisms, and environmental and human health concerns (Kim et al., 2003). Increased concern over the adverse effects of pesticides highlights the need to develop alternative strategies for the control of *D. maculatus*, including the development of chemical substitutes, exploitation of controlled atmospheres, and integration of physical methods (Rajendran & Sriranjini, 2008).

Plant materials represent an attractive alternative to current insect control agents, as they are a rich source of bioactive chemicals. Plant materials are relatively inexpensive, widely available, generally safe, broad-spectrum in application, biodegradable, and environment friendly (Egwunyenga et al., 1998). In particular, some plant extracts and essential oils display effective insecticidal activities against the different life stages of *D. maculatus* (Rajendran & Sriranjini, 2008; Islam et al., 2009). For example, Fasakin & Abererjo (2002) showed that the pulverized powder of *Piper guineense* Schumach & Thonn and *Afromomum melegueta* K. Schum significantly inhibited egg hatching and adult emergence in *D. maculatus* when applied to smoked catfish. However, to provide a range of choices and to reduce costs in different contexts, more plant-derived pest management control agents from local plant species should be developed.

Perilla frutescens (L.) Britt is an annual herb of the Lamiaceae family that is traditionally grown in China, India, Japan, and other Asian countries. This herb has been in common use as a traditional Chinese medicine for over 1,000 years. Edible fresh leaves are typically used as vegetables, commonly used for seasoning pickles, or as a garnish for raw fish dishes in Japan. This plant is also a popular leafy vegetable in Korea that is generally consumed with a pickle or used for wrapping roasted meats (You et al., 2014). *P. frutescens* has significant anti-allergic, anti-inflammatory, and antitumor activities (Banno et al., 2004). Moreover, the essential oils of *P. frutescens* exhibit strong insecticidal and repellent activities against several pests of stored products, including *Lasioderma serricorne* (F.) and *Liposcelis bostrychophila* Badonnel (Hori, 2003, 2004; Zhao et al., 2012; You et al., 2014). Considering these previous findings, we decided to test whether the essential oils of *P. frutescens* also display toxicity against *D. maculatus*.

The aim of this study was to evaluate the effectiveness of the essential oils from the leaves of *P. frutescens* as a fumigant for the control of *D. maculatus*, either alone or in combination with CO_2-enriched modified atmospheres.

Materials and Methods

Extraction of the essential oils

Extraction of the *P. frutescens* essential oils was performed according to the method of Zhao et al. (2012). Fresh leaves of *P. frutescens* were obtained from the South China Botanical Garden,

Chinese Academy of Sciences. Fresh leaves were used to avoid volatilization. The leaves were subjected to hydro distillation using a modified Clevenger-type apparatus for 6 h and extracted with *n*-hexane. Anhydrous sodium sulfate was used to remove water following extraction. The essential oils were stored in airtight containers in a refrigerator at 4°C for subsequent experiments.

Gas chromatography and mass spectrometry analysis

Components of the essential oil were identified by gas chromatography-flame ionization detection (GC–FID) and gas chromatography-mass spectrometry (GC–MS) using an Agilent 7890A gas chromatograph hooked to an Agilent 5975C mass selective detector. The same column and analysis conditions were used for both GC–FID and GC–MS. They were equipped with a HP-5MS (50 m × 0.32 mm × 0.52 µm) capillary column. The oven temperature was programmed to increase from 50 ^{o}C to 250 ^{o}C at a rate of 5 ^{o}C/min and finally held for 10 min. The injector and detector temperatures were maintained at 250 ^{o}C. Helium was used as the carrier gas at a flow rate of 1.0 mL/min with a split ratio equal to 1/20. 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 a homologous series of *n*-alkanes (C_5–C_{36}) under the same operating conditions. Further identification was made by comparison of their mass spectra with those stored in NIST 10 and Wiley 275 libraries or with mass spectra from the literature (Adams, 2001). Relative percentages of the individual components of the essential oil were obtained by averaging the GC–FID peak area% reports.

Insect cultures

Unsexed *D. maculatus* adults were collected from naturally infested museum specimen of *Ctenopharyngodon idellus* Cuvier & Valenciennes at the Shenzhen Museum. The beetles were transferred into Kilner jars containing disinfested dried fish from *C. idellus* to initiate new colonies and to create a parent stock for experimental use. The culture jars were maintained at ambient conditions (28-32 °C and 60–65% RH). The Kilner jars and culture media were disinfested in a laboratory drying cabinet (Model LCON 53 CL) at 70 °C for 1 hour and then allowed to cool to room temperature prior to the introduction of the insects and culture media.

Preparation of gas treatment

The controlled atmosphere apparatus used in this experiment was modified as described by Hashem et al. (2014). Treatment with the gas mixtures was performed inside sealed, gastight wide-mouth bottles (1 L). Each bottle was tightly plugged with a customed glass stopper equipped with two lateral valves (inlet and outlet) leading into two vertical glass tubes. One of these tubes was long and reached near to the bottom of the bottle and acted as a gas inlet. The other tube was shorter and reached approximately one quarter of the way down the bottle and acted as a gas outlet. At the beginning of the treatment, the valves were opened until the desired gas concentration was reached, as indicated by an oxygen analyzer. Then, various doses of the essential oils were injected through the lid onto filter paper placed at the bottom of the bottle to reach the desired concentrations.

Fumigant activity

Different life stages (egg, larva, pupa, and adult) of *D. maculatus* were exposed to one normal atmosphere as a control (AIR) as well as two modified atmospheres, CA1 (25% CO_2, 15% O_2, and 60% N_2) and CA2 (60% CO_2, 8% O_2, and 32% N_2).

Five pairs (F_3 generation) of adults, ten pupae, ten third-instar larvae, or ten eggs were placed in small glass tubes (25–50 mm) with culture media, and the open ends were covered with muslin cloth. The tubes were hung in the geometric center of the bottles.

Fumigations were conducted at various concentrations (0.0, 0.02, 0.04, 0.06, 0.08, and 0.10 µL/L) for 6 hours with five replicates for the control and the treatments. After exposure, the insects were transferred to clean vials with the culture media, placed in incubators maintained at 30 ± 2 °C, and monitored daily for different numbers of days depending on the life stage. Survival of the adults and

larvae were determined by counts after two days of monitoring. Egg mortality was indirectly assessed by counting the hatching rate after seven days of monitoring. Pupal survival was measured by counting the number of F_1 adults that successfully emerged after 20 days of monitoring.

Differences in percentages of mortality for different life stages exposed to each of the experimental conditions were analyzed by analysis of variance using the SPSS software program (Anonymous, 1999). The percentage data were arcsine-transformed to meet the assumption of homogeneity and normality for analysis of variance. Tukey's HSD test was used to compare the means.

Results and Discussion

The essential oil was yellow with a yield of 0.07% (v/w) and density of 0.92 g/mL at 20 ℃. A total of 26 components of the essential oil were identified, account for 98.09% of the total oil (Table 1). The main compounds in the essential oil were perilla aldehyde (53.97%), caryophyllene (11.85%), limonene (9.13%) and trans-shisool (4.39%).

Table 1. Chemical composition of the essential oil from fresh leaves of *Perilla frutescens*

Compound	RI (min)	Relative content (%)
(Z)-3-Hexen-1-ol	12.122	0.10
Benzaldehyde	15.911	0.27
1-Octen-3-ol	16.178	1.18
(1S)-(1)-β-Pinene	16.579	0.15
3-Octanol	16.733	0.18
Limonene	18.226	9.13
Eucalyptol	18.395	0.11
β-Linalool	20.360	2.17
α-Terpineol	23.684	0.30
trans-Citral	25.981	0.19
trans-Shisool	26.233	4.39
Perillaldehyde	26.557	53.97
Perillol	27.039	2.92
Eugenol	28.697	0.20
α-Copaene	29.576	0.26
β-Elemene	29.898	0.42
Caryophyllene	31.021	11.85
β-Farnesene	31.221	0.35
α-Humulene	31.955	1.44
(Z,E)-α-Farnesene	32.203	2.41
β-Cubebene	32.646	3.33
δ-Cadinene	33.511	0.29
(Z)-β-Farnesene	34.135	0.33
Elemicin	34.329	0.22
Caryophyllene oxide	35.511	0.42
Asarone	36.970	1.51
Total		98.09

Mortality was significantly affected in each life stage by the CO_2 concentration (Figure 1). In the control group (AIR), in which the atmosphere was not modified, no deaths were observed when the essential oils were not added. However, CO_2 alone had some effect, especially on the adult stage. The highest mortality (37.4%) was observed for the adult stage in the CA2 treatment group with a CO_2 concentration of 60%.

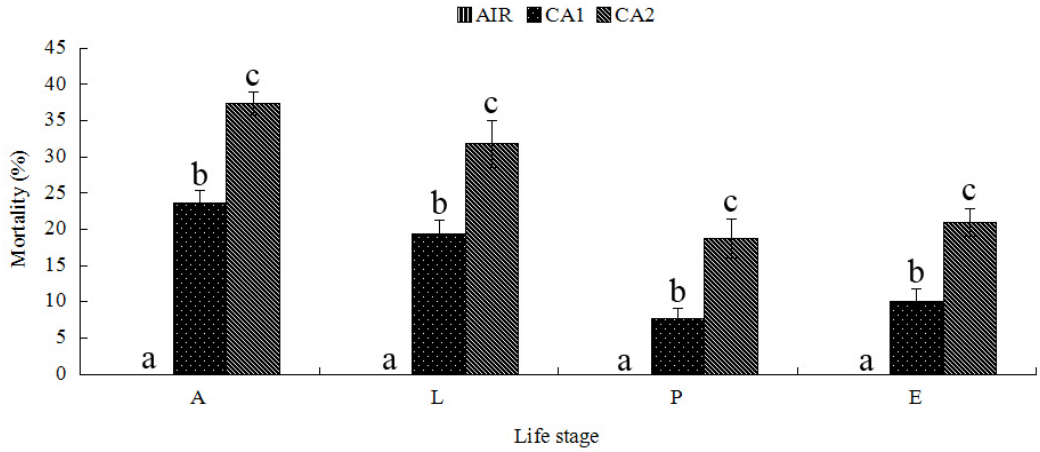

Figure 1. Total mortality (Mean ± SE) of adults (A), larvae (L), pupae (P), and eggs (E) of *Dermestes maculatus* exposed to different CO_2 concentrations (AIR, normal atmosphere; CA1, 25% CO_2; CA2, 60% CO_2) at 28–32 °C and 60–65% RH for 6 hours. Means indicated with the same letters were not significantly different ($P < 0.05$).

The essential oils showed strong fumigant activity against each of the life stages in the AIR control group (Figure 2). Furthermore, the toxicity of the fumigant progressively increased with increasing concentrations of the oil. At a concentration of 0.10 µL/L, mortality for each life stage was 73.0% (adult), 61.2% (larva), 39.6% (pupa), and 55.4% (egg).

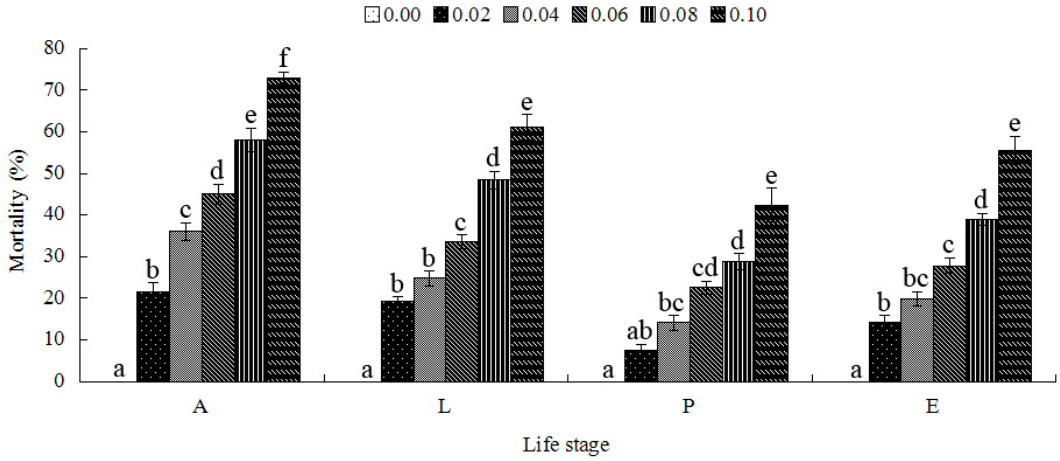

Figure 2. Total mortality (Mean ± SE) of adults (A), larvae (L), pupae (P), and eggs (E) of *Dermestes maculatus* exposed to different concentrations of essential oils from *Perilla frutescens* (0.0, 0.02, 0.04, 0.06, 0.08, and 0.10 µL/L) under a normal atmosphere at 28–32 °C and 60–65% RH for 6 hours. Means indicated with the same letters were not significantly different ($P < 0.05$).

The effects of the essential oils on *D. maculatus* also increased when the CO_2 concentration of the atmosphere was modified (Figure 3). Higher mortality was observed for each life stage in the CA1 and CA2 treatments compared with the AIR control group. Additionally, pest mortality in the CA2 treatment group with 60% CO_2 was higher than in the CA1 treatment group with 25% CO_2. The mortality of all life stages reached 100% in the CA2 treatment group at an essential oil concentration of 0.08 µL/L.

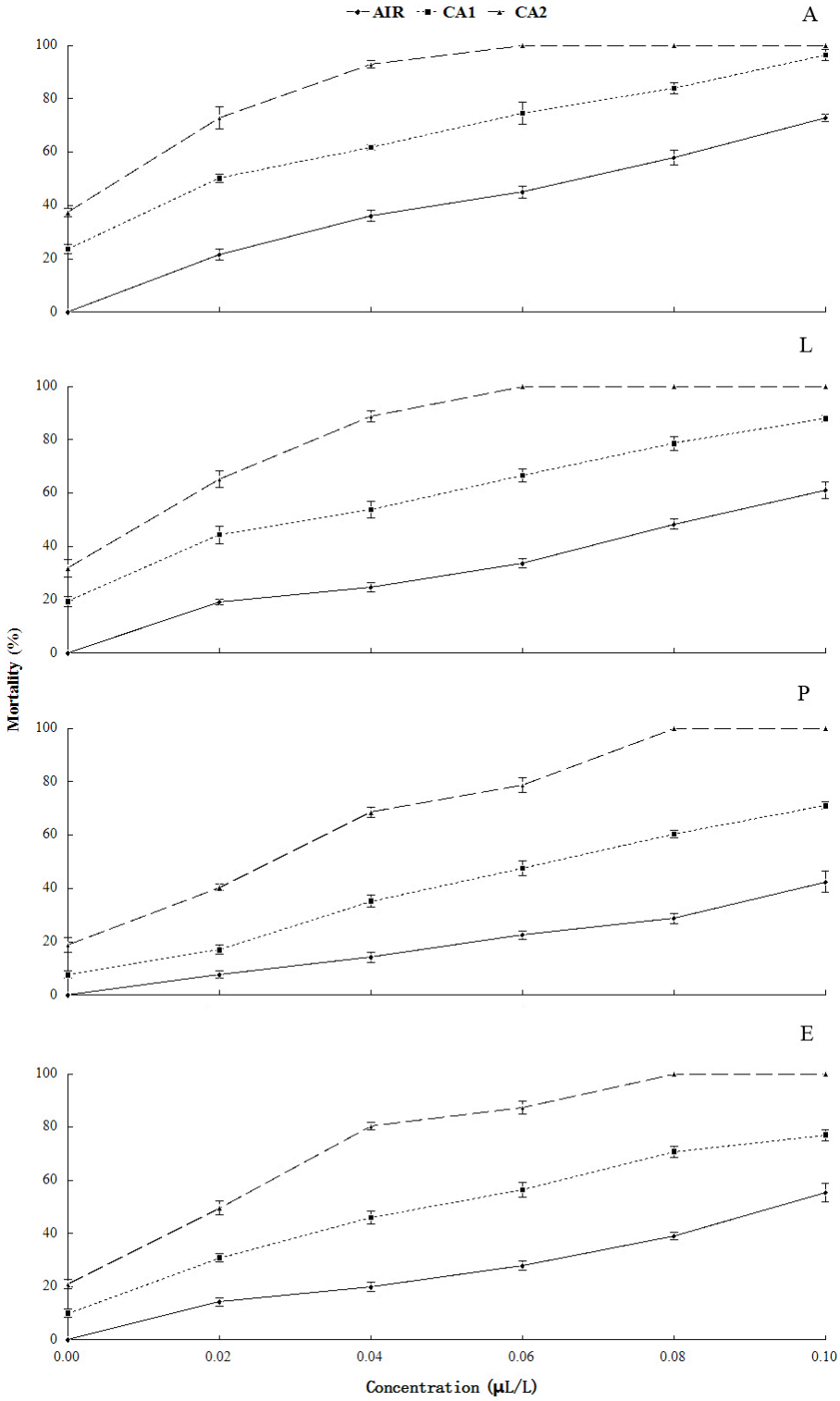

Figure 3. Total mortality (Mean ± SE) of adults (A), larvae (L), pupae (P), and eggs (E) of *Dermestes maculatus* exposed to different CO_2 concentrations (AIR, normal atmosphere; CA1, 25% CO_2; CA2, 60% CO_2) in combination with different concentrations of essential oils from *Perilla frutescens* (0.0, 0.02, 0.04, 0.06, 0.08, and 0.10 µL/L) at 28–32 °C and 60–65% RH for 6 hours.

The different life stages showed different degrees of susceptibility to the essential oils alone (AIR) or in combination with modified atmospheres (CA1 and CA2). Probit analyses showed that adults (LC_{50} = 0.06 µL/L) were the most susceptible, followed by larvae (LC_{50} = 0.09 µL/L), eggs (LC_{50} = 0.11 µL/L) and pupae (LC_{50} = 0.16 µL/L) in the AIR control groups (Table 2). Similar susceptibility trends were observed for the different life stages in the CA1 and CA2 treatments (Table 2).

Table 2. Fumigant toxicity of the essential oils from *Perilla frutescens* on the eggs (E), larvae (L), pupae (P), and adults (A) of *Dermestes maculatus*

Treatment	Life stages	LC_{50}	Slope ± SE	Chi-square (X^2)
AIR	A	0.06 (0.05–0.07)	1.89 ± 0.35	4.08
	L	0.09 (0.07–0.13)	1.65 ± 0.36	4.86
	P	0.16 (0.11–0.43)	1.68 ± 0.42	2.86
	E	0.11 (0.08–0.19)	1.70 ± 0.38	5.11
CA1	A	0.02 (0.02–0.03)	2.00 ± 0.36	8.91
	L	0.03 (0.02–0.03)	1.75 ± 0.34	6.06
	P	0.06 (0.05–0.07)	2.12 ± 0.36	2.25
	E	0.04 (0.03–0.05)	1.77 ± 0.34	3.03
CA2	A	0.01 (0.01–0.02)	3.79 ± 0.82	4.05
	L	0.02 (0.01–0.02)	3.88 ± 0.71	4.69
	P	0.03 (0.02–0.03)	3.33 ± 0.43	12.07
	E	0.02 (0.02–0.03)	3.29 ± 0.47	7.77

The chemical composition of the essential oil from *P. frutescens* in the present study was not same as that reported in previous studies. For example, carvone, perilla aldehyde, caryophyllene, and 2-furyl methyl ketone were the main volatile components of *P. frutescens* harvested from Beijing City, China (You et al., 2014). However, β-caryophyllene,2-hexanoylfuran, β-farnesene, 1,4,7-cycloundecatriene-1,5,9,9-tetramethyl-zzz, and 1-cyclohexane-1-carboxaldehyde were common constituents in *P. frutescens* collected from herb markets in China, and content and composition of the essential oil of *P. frutescens* were various in different parts of *P. frutescens* (Liu et al., 2013). These differences of chemical content and composition of the essential oils might have been due to harvest time and local, climatic and seasonal factors, storage duration of medicinal herbs as well as extraction method, and these differences may result in different biological activities (Huang et al., 2011; Liu et al., 2013; You et al., 2014).

As a stored-product insect pest, *D. maculatus* is an ideal laboratory animal for the testing of biocontrol agents due to its vitality, ease of culturing, and high reproductive rate. In this study, adequate numbers of all life stages were obtained easily from laboratory cultures, simply by providing suitable living conditions (Wong-Corral et al., 2013). The ease with which an insect can be cultured is a primary consideration for such experiments, as large numbers of each life stage will be required to test many chemicals under different exposure conditions (Linnie & Keatinge, 2000). Moreover, in this study, no deaths were observed in the control groups over the nearly a month-long research period, confirming that the rearing conditions for *D. maculatus* were satisfactory.

The essential oils showed significant fumigant activity against all developmental stages of *D. maculatus*, causing significant mortality at higher concentrations. The essential oils used in this study contained different kinds of bioactive components, such as aldehydes, alcohols, terpenes, and esters. These chemical components have insecticidal, nematicidal, fungistatic, antimicrobial, and insect repellent properties (Hori, 2003; Choi et al., 2007; Dimri et al., 2008; Zhao et al., 2012; Tian et al., 2014). Our findings are consistent with other studies that have demonstrated the toxicity of these essential oils and their associated components against a variety of stored-product pests (Dimri et al., 2008; Zhao et al., 2012; You et al., 2014). For example, You et al. (2014) demonstrated that the components of the essential oils, including carvone, perilla aldehyde, 2- furyl methyl ketone, and β-caryophyllene, are highly toxic to *L. serricorne*.

In the present study, the modified atmospheres with high levels of CO_2 significantly reduced survival rate for the different life stages, and mortality observed following treatment with 60% CO_2 was significantly higher than with 25% CO_2. High levels of CO_2 are toxic to many stored-product insect species, such as *Sitophilus oryzae* (L.), *Sitotroga cerealella* (Olivier), *Stegobium paniceum* (L.) and *L. serricorne* (Annis & Morton, 1997, Gunasekaran and Rajendran, 2005, Hashem et al., 2014). Moreover, most stored-product insects eventually die under atmospheres containing more than 40% CO_2 (Navarro, 2006). The death of insects from high CO_2 concentrations is due to the combination of many effects. In insects, CO_2 poisoning directly affects the nervous, endocrine, respiratory and circulatory systems, as well as general metabolism (Wong-Corral et al., 2013). Furthermore, in many insects, high CO_2 induces permanent opening of the spiracles, leading to water loss and mortality (Wong-Corral et al., 2013). In addition, CO_2 can also have indirect effects on mortality, as Janmaat et al. (2001) demonstrated a strong narcotic and metabolic effects for high CO_2 concentrations, primarily due to changes in pH.

The mortality rate for all the life stages of *D. maculatus* fumigated with a combination of high CO_2 and essential oils was higher than in the groups where CO_2 or the essential oils were used alone. Therefore, the effects of the essential oils and CO_2 on mortality were synergistic when used in combination against *D. maculatus*. Similar synergistic toxic effects have been demonstrated for CO_2 in combination with other compounds. For example, adding CO_2 to fumigants such as sulfur dioxide, acrylonitrile, methyl bromide, phosphine, carbon disulfide, ethylene oxide, chloropicrin, methyl formate, and hydrogen cyanide increases their toxicity against insects and reduces the times required for treatment (Bond & Buckland, 1978; Riudavets et al., 2014). Moreover, the fumigant toxicity of essential oils from certain plants (*Allium sativum* L., *Citrus tangerina* Hort. ex Tanaka, *C. aurantium* L., *C. bergamia* R., *Pinus sylvestris* L., *Cupressus funebris* Endl, and *Eucalyptus citriodora* Hook) is also increased when combined with carbon dioxide for the control of stored-product insects (Wang et al., 2001; Isikber, 2010). In the present study, mortality was significantly higher in the treatment groups with higher CO_2 levels (60%) than in groups with lower CO_2 levels (25%). Therefore, it is likely that CO_2 concentration played a key role during the joint fumigations, as modified atmospheres with high CO_2 levels cause the permanent opening of insect spiracles, thereby increasing the uptake of plant essential oils (Nicolas & Sillans, 1989; Wang et al., 2001; Mitcham et al., 2006).

Our study revealed considerable variability in the susceptibility of the egg, larval, pupal, and adult stages to essential oil derived from *P. frutescens*. Notably, the susceptibility of each life stage was similar when exposed to CO_2, essential oils, or a combination of these factors. Generally, adults were the most sensitive, followed by larvae and eggs, whereas pupae were the least sensitive. Similar sensitivities to CO_2 during different life stages have been observed in other stored-product pest species, including *Callosobruchus maculatus* (F.), *Acanthoscelides obtectus* (Say), and *Zabrotes subfasciatus* (Boheman) (Wong-Corral et al., 2013). The effects of modified atmospheres with high CO_2 levels on pests could be related to differences in metabolic rates during different life stage. For example, pupae, which likely have the lowest oxygen demand, were most tolerant to the toxic effects of CO_2, whereas adults, with high metabolic rates, were the least tolerant (Mbata et al., 2000). Related to this, more essential oils are likely absorbed and accumulated in insects with higher metabolic rates, resulting in lower survival rates during fumigation with essential oils alone or in combination with CO_2.

The aim of this study was to determine the effectiveness of the essential oils from the leaves of *P. frutescens* as a fumigant for the control of *D. maculatus*, either alone or in combination with CO_2-enriched modified atmospheres. Results indicated that the essential oils showed strong fumigant activities against this pest under a normal atmosphere, with fumigant toxicity increasing with higher oil concentrations. Furthermore, the effects of the essential oils were enhanced as CO_2 levels were increased. At an essential oil concentration of 0.08 μL/L in the treatment group with 60% CO_2, the mortality rate for all life stages was 100%. It was suggested that the essential oils vapor and modified atmospheres could be used as an effective combined method to control the stored-product insects.

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