Biochemical and insecticidal effects of plant essential oils on insecticide resistant and susceptible populations of *Musca domestica* L. point to a potential cross-resistance risk

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Abstract

Essential oils (EOs) can provide important alternatives to chemical insecticides in the control of pests. In this study, 12 EOs of native plant species from Iran were evaluated for their adulticidal activity against the house fly. In addition, we examined the insecticidal activity of *Zataria multiflora* and *Rosmarinus officinalis* EOs on adult female house flies from pyrethroid and organophosphate resistant and susceptible populations, using both fumigant and topical bioassays. The involvement of detoxification enzymes in susceptibility was investigated with synergism experiments *in vivo*, while the inhibitory effects of *R. officinalis* and *Zataria multiflora* EOs on detoxification enzyme activities were determined by enzymatic inhibition assays *in vitro*. The EOs of *Z. multiflora*, *Mentha pulegium*, *R. officinalis* and *Thymus vulgaris* were the most effective against adults in contact topical assays, while oils extracted from *Eucalyptus cinerea*, *Z. multiflora*, *Citrus sinensis*, *R. officinalis*, *Pinus eldarica* and *Lavandula angustifolia* where the most effective in fumigant assays. *Rosmarinus officinalis* and *Z. multiflora* EOs were selected for further investigation and showed higher toxicity against a susceptible population, compared to two insecticide-resistant populations. Correlation analysis suggested cross-resistance between these EOs and pyrethroids in the resistant populations. The toxicity of both EOs on the resistant populations was synergized by three detoxification enzyme inhibitors. Further, *in vitro* inhibition studies showed that *R. officinalis* and *Z. multiflora* EOs more effectively inhibited the activities of the detoxification enzymes from flies of the susceptible population compared to those of the pyrethroid resistant populations. Synergistic and enzymatic assays further revealed that increased activities of P450s, GSTs, and CarEs are possibly involved in the cross-resistance between EOs and pyrethroids. Investigating the molecular mechanisms of P450s, GSTs, and CarEs in the resistance to EOs should be subject to further studies.

Keywords: essential oils, detoxifying enzymes, enzyme inhibition, *Musca domestica* L., cross-resistance, botanical insecticides

1. **Introduction**

The house fly, *Musca domestica* (Linnaeus) (Diptera: Muscidae), is one of the most important medical and veterinary pests (Scott et al., 2014). Different groups of insecticides such as pyrethroid and organophosphate insecticides (OPs) have been used extensively to control *M. domestica* (Freeman et al., 2019; Scott, 2017). Pyrethroids modulate voltage-gated sodium channels and act on the central nervous system of target organisms, while OPs bind to acetylcholinesterase, also disrupting nervous functions (Sparks and Nauen, 2015). The frequent use of these insecticides in controlling house flies led to the development of resistance (Kristensen et al., 2006; Scott, 2017), in addition to causing environmental contamination problems, human health risks and adverse effects on non-target organisms (Kumar et al., 2014). In our previous study, two house fly populations (Isfahan and Mobarake) were found resistant against both pyrethroid and OP insecticides via enhanced detoxification and target site modification (Ahmadi and Khajehali, 2020; Ahmadi et al., 2020).

A number of botanical extracts with often non-specified mode of action have recently been included in the IRAC MoA classification scheme as part of the 'biologics' group (Sparks et al., 2020). Botanicals have gained considerable attention in agriculture, addressing the need for inexpensive, easily sourced, and biodegradable or environment friendly alternatives to classical pesticides (Isman, 2006; Bajda et al. 2021; Pavela and Benelli, 2016). These alternative tactics, when incorporated into integrated pest management programs, can be used as an effective management tool to delay the development of resistance to conventional insecticides (Khater, 2012). Insecticidal properties of EOs against house flies have been documented in several studies (Ahsaei et al., 2020; Benelli et al., 2018a; Rossi and Palacios, 2015). Various EOs have chemical components that have insecticidal effects on the house fly, such as monoterpenoids, including limonene, myrcene, terpineol, linalool, and pulegone (Coats et al., 1991). Thus, EOs could potentially replace chemical insecticides in the control of arthropods of medical and agricultural importance (Campos et al., 2019), such as *M. domestica*, however, their effectiveness may be reduced due to resistance development (Lee et al., 2000).

The development of resistance to EOs may occur more slowly than toward synthetic insecticides, because of the mixture of active ingredients with multiple mode of action (Park and Tak, 2016). It has been reported that adult females of the bean weevil, *Acanthoscelides obtectus*, developed 8.6-fold resistance to lavender EO vapor after eight generations of selection (Papachristos and Stamopoulos, 2003). When increased tolerance or development of resistance toward EOs is observed, it may also be the result of synthetic insecticide cross-resistance. For example, a chlorpyrifos-methyl resistant strain of the saw-toothed grain beetle, *Oryzaephilus surinamensis*, showed cross-resistance to some EOs (Lee et al., 2000; Lee., 2002). The role of cytochrome P450 dependent monooxygenases (P450s), carboxylesterases (CarEs) and glutathione *S*-transferases (GSTs) has been confirmed in insecticide resistance and in the biosynthesis of many endogenous compounds in arthropods (Dermauw et al., 2013; Francis et al., 2005; Li et al., 2007; Scott, 2017; Wybouw et al., 2015). When enhanced detoxification activity of P450s, GSTs and CarEs provides resistance to synthetic insecticides (Scott, 2017; Van Leeuwen and Dermauw, 2016; Vontas et al., 2005), these metabolizing enzymes can potentially also detoxify EOs (Francis et al., 2005; Li et al., 2007; Rossi et al., 2012; Rossi and Palacios, 2013, 2015). The lower toxicity of some EOs for some pest species was therefore suggested to be partly caused by higher levels of detoxification enzymes that effectively metabolize the toxic EO components (Norris et al., 2015; Li et al., 2007) via shared degradation mechanisms, such as higher activities of P450s, GSTs and CarEs in the insecticide resistant strains. Numerous studies have indicated that EOs may have neurotoxic activities and inhibitory effects on acetylcholinesterase (Coats et al., 1991; Huang et al., 2020; Kumrungsee et al., 2014; Lee et al., 2000; Liao et al., 2016). In addition, EOs have the ability to induce or inhibit metabolizing enzymes in insects, including P450s, GSTs, and CarEs (Benelli et al., 2018b; Czerniewicz et al., 2018; Huang et al., 2020; Lee et al., 2000; Rossi and Palacios, 2015; Tak et al., 2017).

In our previous studies, the Isfahan and Mobarake house fly populations were found resistant to pyrethroid and OP insecticides while the Koohrang population was relatively susceptible (Ahmadi and Khajehali, 2020; Ahmadi et al., 2020). In the current study, we examined the fumigant and contact toxicity of twelve EOs from

plants native to Iran on the susceptible Koohrang population. Then, to investigate possible cross-resistance between these synthetic insecticides and EOs, the insecticidal activity of *Rosmarinus officinalis* and *Zataria multiflora* EOs was tested against the susceptible and insecticide-resistant populations. Furthermore, the *in vitro* inhibitory activities of EOs on P450s, GSTs, and CarEs were investigated and *in vivo* synergism assays were performed to elucidate possible mechanisms conferring cross-resistance between EOs and OPs or pyrethroids.

2. Material and methods

2.1. *Chemicals and EOs*

Chemical substances including 2,4-dinitrochlorobenzene (CDNB), α-naphthyl acetate (α-NA), α-naphthol, reduced glutathione (GSH), Coomassie Brilliant Blue G-250, bovine serum albumin (BSA), 3,3′,5,5′-tetramethylbenzidine (TMBZ), cytochrome C from the equine heart (95%), fast blue RR salt, acetylthiocholine iodide (ATChI), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), and triphenyl phosphate (TPP) were purchased from Sigma-Aldrich (Darmstadt, Germany). Piperonyl butoxide (PBO) and diethyl maleate (DEM) were purchased from Sigma-Aldrich (Bornem, Belgium). The EOs as listed in Table 1 were obtained from Barij Essence Company, Kashan, Iran (http://www.barijessence.com) and were stored at 4 °C.

2.2. GC–MS analysis

Chemical analysis of *Z. multiflora* and *R. officinalis* EOs was performed on a GC–MS 6890–5975 system (Agilent Technologies, Palo Alto, CA, USA) with HP-5MS (5% diphenyl) dimethylpolysiloxane capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). For GC–MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium was used as the carrier gas at a flow rate of 1 mL/min. The EOs were first diluted in hexane, then one μL was injected. The GC conditions were as follows: injector and detector temperatures were set at 220 °C and 290 °C, respectively. The temperature was maintained at 40 °C for 3 min initially and then raised at the rate of 3 °C/min to 280 °C. The oil components were identified by comparison of

their retention indices (RI) and mass spectra with the National Institute of Standards and Technology (NIST), and also with data previously reported in the literature.

2.3. House fly populations

As previously described, two insecticide-resistant populations of house flies were collected from industrial cattle farms located in Isfahan (32.6546° N, 51.6680° E) and Mobarake (32.3347° N, 51.5571° E), and a relatively susceptible population was collected from Koohrang (32.3297° N, 50.1112° E) (Ahmadi and Khajehali, 2020; Ahmadi et al., 2020). The populations were kept in separate cages in a rearing room at 25 ± 1 ˚C, 60 ± 5% relative humidity, and a photoperiod of 16:8 h (L: D). Adults were fed on a diet containing sesame meal and wheat bran (1:3) in a plastic container, supplemented with a mixture of water and sugar (10%). Hatched larvae were transferred to plastic buckets containing 20 g of sesame meal and wheat bran (1:3), 1.5 g milk powder, 1.5 g honey mixed with 8 mL of water.

2.4. Topical bioassay

The topical bioassays were performed according to the method of Benelli et al. (2018a). First, EOs were dissolved in acetone, and five to six different concentrations of EOs (from 2 to 250 μg/fly) were used in each bioassay. Then one μL of the EO solution was applied on the pronotum of CO_2-anesthetized flies (3- to 5-day old females) using a repetitive syringe dispenser (Nichiryo Model 8100, Tokyo, Japan) and acetone was used as the control treatment. In all bioassays, groups of 15–20 flies were treated with the EO acetone solution. Treated flies were kept in the plastic containers (50 mL) and were fed with 10% sugar water. Each test was replicated at least three times. After 24 h, mortality was recorded. All topical bioassays were performed at 25 ± 2 ˚C.

2.5. Fumigant bioassay

The fumigant bioassay was conducted based on the method of Rossi and Palacios (2015) with slight modifications. Briefly, twenty flies (3- to 5-day old females) were placed in a glass jar (650 mL) fitted with a screw cap. Different concentrations of the EOs (dissolved in 20 μL acetone) were applied on a cotton pad inside

a Petri dish, and the dish was placed inside the jar. The dishes were covered with a fine mesh to prevent the flies from coming into contact with the cotton pad. Each test was replicated at least three times. The glass jars were sealed tightly and kept at 25 ± 2 ˚C for 30 min. Acetone was used as the control. After 30 min, mortality was recorded. Based on both topical and fumigant assays, the most active oils, *R. officinalis* and *Z. multiflora* EOs, were selected for further investigations including bioassays on two insecticide resistant populations, synergism tests and biochemical assays.

2.6. Inhibitory effect of EOs on the activities of detoxifying enzymes

For the determination of the GST activity, whole bodies of three adult female house flies (3- to 5-day old after pupal eclosion) from each population (in each replication) were homogenized in 1 mL of sodium phosphate buffer (0.1 M, pH 7) in ice-cold conditions (0–4 ˚C). After centrifugation (12,000 g at 4 °C for 15 min), the supernatant was used for the enzyme assay. Similar procedures were used for the measurement of the CarE and P450 activities, but adults were homogenized in sodium phosphate buffer, (0.1 M, pH 7.5), containing 0.1% (w/v) Triton X-100 and potassium phosphate buffer (0.1 M, pH 7.5), respectively.

The GST activity was assayed using the method of Habig et al. (1974), with slight modifications. Briefly, to determine *in vitro* inhibition of GST activity by EOs and to calculate the median inhibitory concentrations (IC_{50}), 150 µL enzyme source was incubated for 10 min at 25 ± 2 ˚C with 150 µL of increasing concentrations of EOs (varying between 0.01 and 1000 mg/L) in sodium phosphate buffer (0.1 M, pH 7, stock solutions of 100 g/L oils were prepared in acetone). The reactions were started with adding 200 µL GSH (10 mM in sodium phosphate buffer, 0.1 M, pH 7) and 200 µL CDNB (1.2 mM, dissolved in methanol) to 30 µL mixture of the EO and enzyme source. The absorbance was measured using a spectrophotometer (Unico, Model UV-2100, USA) at 340 nm at 30s intervals for 10 min at 25 ± 2 ˚C. The activity without EOs was also determined. The GST activity was calculated using the CDNB extinction coefficient of 9.6 mM^{-1} cm^{-1} and was expressed as nmol min^{-1} mg of $protein^{-1}$.

The *in vitro* inhibitory effects of two EOs on house fly CarE activity were determined based on the method of Van Leeuwen et al., (2005) and Wang et al., (2016) with slight changes. Briefly, the enzyme source (250 µL) was incubated for 10 min with serial concentrations of EOs (250 µL, 0.01 to 1000 mg/L) in sodium phosphate buffer 0.1 M, pH 7.5. The reactions were started by adding 100 µL substrate (α-NA, 4 mM), 300 µL Fast Blue RR 0.8% and 50 µL sodium phosphate buffer (0.2 M, pH 6) to 50 µL of reaction mixtures of the selected EO and enzyme extract. Absorbance was measured at 450 nm at 30 s intervals for 10 min at 25 ± 2 ˚C. The CarE activity without adding EOs was also determined.

The method of Brogdon et al. (1997) was followed to measure P450 heme peroxidase activity and the ability of EOs to inhibit this activity, with slight modifications. Briefly, TMBZ (TMBZ solution was including 0.01 g of TMBZ in 5 mL methanol with 15 mL of 0.25 M sodium acetate buffer, pH 5.0) was used as the substrate, and 40 µL of the enzyme source was mixed with different concentrations of EOs (8 µL, 0.01 to 1000 mg/L) dissolved in acetone and then diluted in potassium phosphate buffer 0.1 M, pH 7. Then, 152 µL potassium phosphate buffer (0.1M, pH 7.5), 400 µL TMBZ solution and 50 µL hydrogen peroxide (3%) were added, and all reactions were incubated for two hours at 25 ± 2 ˚C. Absorbance was measured at 620 nm. As a control, the heme peroxidase activity was measured with acetone alone.

The protein concentration in the enzyme source was measured according to Bradford (1976) using BSA as a standard. For all enzymatic assays, three biological replicates were considered.

2.7. In vivo synergism studies with PBO, DEM and TPP

To investigate the metabolic resistance mechanism to EOs in the house fly populations, the enzyme inhibitors PBO, DEM, and TPP, diluted in acetone, were applied one hour before treatment with *Z. multiflora* and *R. officinalis* EOs. The synergists were used at their maximum sublethal concentrations (5, 7 and 10 µg/fly for PBO, DEM and TPP, respectively) through topical application on the notum of CO_2 anesthetized adults (3- to 5-day old females) using a repetitive syringe dispenser (Nichiryo Model 8100, Tokyo, Japan). All experiments were conducted at room temperature (25 ± 2 °C) (Ahmadi et al., 2020).

2.8. Statistical analysis

The LC_{50} or IC_{50} values were calculated by POLO-Plus software (Robertson et al., 2017). Data was only used when the probit model was accepted (chi-square goodness-of-fit test) and all the bioassay data were well described by the probit model (χ^2 test, $p > 0.05$). Pearson correlation analysis ($P < 0.05$) was used to determine the cross-resistance between EOs and insecticides (PROC CORR). The IC_{50} values were calculated by plotting the percentage enzyme inhibition versus concentration of EOs. Analysis of variance (ANOVA) followed by LSD mean separation was used to test the differences in the levels of IC_{50} values using SAS v. 9.4 software (Institute, 2017). The comparison charts were prepared using Origin software (OriginLab, 2018).

3. Results

3.1. Chemical composition of the isolated EOs

The results of GC–MS analysis of the *Z. multiflora* and *R. officinalis* EOs, obtained by hydrodistillation, are provided in Table S1. The *Z. multiflora* EO was comprised mainly of 1,8 cineole (14.1%), thymol (16.5%), and carvacrol (49.7%), while the major constituents of *R. officinalis* oil were α-pinene (14.2%), 1,8 cineole (33.2%) and camphor (25%).

3.2. Topical bioassay

The insecticidal activities of twelve EOs (Table 2 and Table 3) were assessed on an insecticide-susceptible population of the house fly, the Koohrang population (Ahmadi et al., 2020). The investigated EOs showed variable adulticidal topical activity and LC_{50} values ranged from 32.9 μg/fly (*Z. multiflora*) to 93.8 μg/fly (*Apium graveolens*) (Table 2). Subsequently, the EOs of *Z. multiflora and R. officinalis* were evaluated against two insecticide-resistant house fly populations (Mobarake and Isfahan populations). The LC_{50} value of the susceptible Koohrang population was estimated at 32.9 and 46.1 μg/fly for *Z. multiflora* and *R. officinalis*, respectively, while the resistant populations had LC_{50} values 1.9- to 2-fold higher compared to the susceptible population (Table 4).

3.3. Fumigant bioassay

All tested EOs showed variable fumigant toxicities against adult house flies of the Koohrang population. The lowest LC_{50} value was 10.3 μL/L (*Eucalyptus cinerea*) and the highest was 27.1 μL/L (*Ferula gummosa*) (Table 3). Then, EOs of *Z. multiflora* and *R. officinalis* were tested on populations with insecticide-resistance (Mobarake and Isfahan). According to the obtained fumigant LC_{50} values, the resistant populations showed significantly higher tolerance against both EOs (1.39- to 2.25-fold) than the susceptible Koohrang population (Table 5).

3.4. Inhibitory effect of EOs on the activities of detoxifying enzymes

The results of the *in vitro* inhibitory effect of selected EOs on the P450 peroxidase activity of female house flies of the different populations are given in Fig. 1A. Both the EOs from *Z. multiflora* and *R. officinalis* significantly inhibited P450 peroxidase activity in the different populations. *Zataria multiflora* and *R. officinalis* showed a statistically much higher inhibitory potential on the P450s of the Koohrang population (IC_{50} value of 0.68 and 0.61 mg/L, respectively) compared to that of Mobarake population (IC_{50} value of 3.43 and 3.1 mg/L, respectively) and Isfahan population (IC_{50} value of 3.16 and 2.2 mg/L, respectively). These IC_{50} values were from 3.6-fold to 6.3-fold higher compared to the Koohrang population.

The results of the *in vitro* inhibition assays of the two selected EOs on the GST activity of female house flies of different populations are shown in Fig. 1B. The highest GST inhibition of *Z. multiflora* and *R. officinalis* EOs was observed in the Koohrang population, with IC_{50} values of 0.79 and 1.15 mg/L, respectively. These values were much higher for GSTs of the Mobarake and Isfahan populations (4.3- to 6.8- fold, relative to the Koohrang population).

Figure 1C shows the inhibitory effect of EOs on the house fly CarE activity. The IC_{50} values of *Z. multiflora* and *R. officinalis* EOs on CarE activity of female house flies of the Koohrang population were estimated as 1.04 and 2.17 mg/L, respectively. That is 2.4- to 4.6-fold lower than the IC_{50} values of the Mobarake and Isfahan populations.

3.5. Synergism studies with PBO, DEM and TPP

In vivo synergism bioassays were performed with PBO, DEM, and TPP in the topical bioassay with *Z. multiflora* and *R. officinalis* EOs against the three house fly populations. The enzyme inhibitors did not increase the toxicity of *Z. multiflora* and *R. officinalis* EOs in the susceptible Koohrang population (Table 6). However, in the resistant populations, the EO toxicities were significantly synergized in the presence of PBO and DEM (1.6- to 1.9- fold), suggesting that P450s and GSTs were at least partially involved in the decreased susceptibility of flies to Z. *multiflora* and *R. officinalis* EOs. Also, TPP significantly increased the toxicity of both EOs (1.5- to 1.7- fold) in the Isfahan population, indicating that CarEs may also contributed to EO tolerance.

3.6. Cross-resistance between Z. multiflora and R. officinalis EOs and insecticides

A correlation analysis was performed between topical LC_{50} values of two EOs (*Z. multiflora* and *R. officinalis*) and four insecticides (permethrin, cypermethrin, deltamethrin and dichlorvos) obtained in our previous studies (Ahmadi and Khajehali, 2020; Ahmadi et al., 2020). The analysis revealed a significant correlation between the $\log_{10}$ LC_{50} values of *Z. multiflora* EOs and four tested insecticides ($r = 0.99$, $P < 0.04$). However, for *R. officinalis* EOs, this correlation was significant only for dichlorvos ($r = 0.99$, $P < 0.02$) (Table 7).

4. Discussion

Essential oils have shown insecticidal potential in controlling *M. domestica* and other insect pests in many parts of the world (Benelli et al., 2020; Benelli et al., 2018a; Huang et al., 2020; Koul et al., 2008; Kumrungsee et al., 2014; Liao et al., 2016). Here, we report on the toxicity of 12 EOs from Iranian aromatic plants against house fly adults, both in topical and fumigant applications. *Zataria multiflora*, *E. cinerea*, and *R. officinalis* demonstrated higher fumigant toxicities compared to the other tested oils. EOs of *Z. multiflora*, *M. pulegium*, *R. officinalis* and *T. vulgaris* were the most potent in the topical assay. Based on both topical and fumigant toxicity tests, *Z. multiflora* and *R. officinalis* exhibited the highest insecticidal activity on the female house flies. Other studies have also documented insecticidal properties of *Z. multiflora* and *R. officinalis* EOs (Ahsaei et al., 2020; Benelli et al., 2020; Tak et al., 2016). The insecticidal activity of EOs is the result of their major constituents or

a synergy between the major and some minor compounds. The differences in the growth stage, harvesting time, physicochemical variables, environmental parameters and genetic background, may affect the chemical composition of the EOs (Masotti et al., 2003). Hence, the chemical composition of the main EOs used in present study was analyzed using GC–MS, and highlighted carvacrol (49.7%) and 1,8 cineole (33.2%), repectively, as the predominant components of *Z. multiflora* and *R. officinalis* EOs.

The development of resistance to EOs may be the result of direct selection (Papachristos and Stamopoulos, 2003) or cross-resistance with synthetic insecticides (Lee et al., 2000). Our previous studies have revealed that the Mobarake and Isfahan populations are resistant to pyrethroids and OPs, and that the Koohrang population is relatively susceptible (Ahmadi and Khajehali, 2020; Ahmadi et al., 2020). The present study demonstrated a link between insecticide resistance in *M. domestica* and insecticidal activity of *Z. multiflora* and *R. officinalis* EOs (Supplementary Table S1). Topical and fumigant toxicity assays of oils from both plants confirmed different activity of EOs against the resistant (Mobarake and Isfahan) and susceptible (Koohrang) house fly populations. According to the estimated topical and fumigant LC_{50} values (Table 4, Table 5), the two resistant populations showed higher tolerance to *Z. multiflora* and *R. officinalis* EOs relative to the susceptible population. Correa et al., (2015) have also reported that pyrethroid-susceptible and -resistant populations of the maize weevil *Sitophilus zeamais* exhibit different susceptibility to EOs of *Syzygium aromaticum* L., and *Cinnamomum zeylanicum* L. (Correa et al., 2015). It has also been demonstrated that EOs of *Lippia sidoides* have different toxicity (resistance ratio < 3.3) on five populations of *S. zeamais* (Oliveira et al., 2018). Our results, presented in Table 7, confirmed a correlation between topical and fumigant LC_{50} of *Z. multiflora* and *R. officinalis* EOs with pyrethroid (permethrin, cypermethrin, and deltamethrin) and OP (dichlorvos) LC_{50} values. However, the resistance ratios in Mobarake and Isfahan populations against *Z. multiflora* and *R. officinalis* EOs (RRs ≈ 2-fold) were much lower than those of pyrethroids (RRs > 124- fold) and OPs (RRs > 80- fold).

The insecticidal mode of action of EO compounds is not fully understood, but they elicit characteristic neurotoxic symptoms including agitation, hyperactivity, paralysis and knockdown and based on several studies,

EOs also have an inhibitory effect on detoxifying enzymes (P450s, GSTs and CarEs) in insects (Benelli et al., 2018b; Czerniewicz et al., 2018; Huang et al., 2020; Lee et al., 2000; Rossi and Palacios, 2015; Seo et al., 2015; Tak et al., 2017). In addition, several P450s, GSTs, and CarEs have been described and characterized for their role in insecticide resistance and biosynthesis of many endogenous compounds in arthropods (Dermauw et al., 2020; Van Leeuwen and Dermauw, 2016; Vandenhole et al., 2020; Vontas et al., 2005). On the other hand, the induction of insect detoxification enzymes by plant allelochemicals reported in several phytophagous insects suggests that detoxification enzymes may also be involved in detoxification of allelochemicals (Vandenhole et al., 2020; Li et al., 2007). Previous studies have indicated that detoxifying enzymes contribute to the biodegradation of EO or monoterpenes in *M. domestica* (Rossi et al., 2012; Rossi and Palacios, 2013). Heterologous expression of the house fly *CYP6A1*, involved in insecticide resistance, demonstrated that this cytochrome P450 can metabolize terpenoid compounds (Andersen et al., 1997). Also, CarEs play a role in the decomposition of plant-derived insecticides (Yang et al., 2005) and it was shown that GSTs participate in the detoxification of xenobiotics such as plant allelochemicals and insecticides (Francis et al., 2005). Most arthropod species have highly efficient and diverse detoxifying enzyme genes, including CarEs, GSTs and P450s (Dermauw et al., 2018; Dermauw et al., 2020). To determine the role of detoxification enzymes in insecticide resistance, synergism assays can be used with PBO, DEM, and TPP as inhibitors of P450, GST and CarE activity, respectively (Snoeck et al., 2017). In the present study, treating populations with these three synergists revealed that they did not enhance toxicity of *R. officinalis* and *Z. multiflora* EOs in the susceptible population. The lack of synergism by three known enzymatic inhibitors showed that for these two tested EOs in an insecticide susceptible strain of house fly, inhibition of detoxification enzyme activities is unlikely to play a key role in the EO detoxification, but also in mode of action. Alternatively, it is also possible that the tested synergists do not inhibit specific detoxification isozymes involved in EO metabolism, and the lack of synergism is never a conclusive evidence that metabolism is not involved in detoxification. However, pretreatment of the resistant populations (Mobarake and Isfahan) with PBO, DEM, and TPP synergized the toxicity of *R. officinalis* and *Z. multiflora* EOs. From Table 6, 1.31- to 1.89- fold enhanced toxicity was observed in Mobarake and Isfahan

populations. Previously, our studies revealed that resistance to OPs and pyrethroids in the resistant populations (Mobarake and Isfahan) was at least partly associated with enhanced activities of P450s, GSTs, and CarEs (Ahmadi and Khajehali, 2020; Ahmadi et al., 2020). Therefore, based on the results of synergism assays, it is very likely that cross-tolerance between *R. officinalis* and *Z. multiflora* EOs in OPs and pyrethroids resistant populations was at least partially caused by enhanced metabolic detoxification. Thymol, the second major component of *Z. multiflora* (16.5%), was shown to be metabolized by GSTs in *Trichoplusia ni* larvae (Tak et al., 2017) and was shown to be detoxified through glycosylation in the cabbage looper (Passreiter et al., 2004). It has also been reported that the toxicity of camphor, the second main constituent of *R. officinalis* (25.01%) was synergized when mixed with GST and P450 inhibitors (Tak et al., 2017). In addition, 1,8-cineole, a major compound of both tested EOs, has been demonstrated to be oxidized to (+)-2β- hydroxycineole in *Paropsisterna tigrina* adults and larvae (Southwell et al., 1995). Moreover, transcriptional surveys showed that exposure to terpinen-4-ol can alter the expression levels of GSTs, esterases, and especially P450s in *S. zeamais* (Huang et al., 2018).

Previous studies have confirmed that EOs are likely capable of inhibiting insect P450s, GSTs, and CarEs (Huang et al., 2020; Kumrungsee et al., 2014; Liao et al., 2016) and that these enzymes can be considered as target sites for EOs in insects (Huang et al., 2020; Koul et al., 2008; Kumrungsee et al., 2014; Lee et al., 2000; Liao et al., 2016). However, the exact mechanisms of enzyme inhibition by EOs are unknown (O'Neal et al., 2019). The results of *in vitro* enzyme inhibition assays show that the IC_{50} values of *R. officinalis*, and *Z. multiflora* EOs on P450, GST, and CarE activity were found to be 2.41- to 6.77-fold higher in the resistant populations compared to those of the susceptible Koohrang population. As it was shown by synergism assays, enhanced detoxification enzyme activities in the resistant strains may be associated with higher tolerance and higher IC_{50} values of EOs. Elucidating inhibition mechanisms of these enzymes by tested EOs would be subject to future study. Although the target site of DEET, a mosquito repellent, has been reported to be olfactory receptor neurons, a significant correlation was found between P450-inhibition and repellency of several EOs, which highlighted the

potential of multiple target sites for insect repellents (Ramirez et al., 2012). It has been also reported that the EO of *Piper sarmentosum* and its major component, myristicine, exhibited inhibitory activity on the CarE and GSTs of *Brontispa longissima* larvae (Qin et al., 2010). Although we have demonstrated an association between detoxification enzyme activities and EO insecticidal activities, the involved mechanism was not elucidated. The present study demonstrated that the tested EOs are potent *in vitro* inhibitors of insect P450s, GSTs, and CarEs but their potential as synergists needs to be investigated. However, as observed in *in vivo* synergism assays, EOs may be metabolized by detoxification enzymes, thus reducing their inhibitory effect assessed by the *in vitro* assays.

5. Conclusions

The tested EOs in this study displayed insecticidal activity against house fly populations. The EOs of *R. officinalis* and *Z. multiflora* showed the most promising insecticidal activities against the susceptible population (Koohrang), however, two insecticide-resistant populations (Mobarake and Isfahan) were less susceptible. The results revealed a correlation between the toxicity of EOs with OPs and pyrethroid resistance. Synergism studies demonstrated that the decreased toxicity is most likely caused by enhanced metabolism. Finally, *in vitro* enzyme inhibition assays revealed that tested EOs have the capability to inhibit P450s, GSTs and CarEs, but are less potent in the resistant populations.

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Table 1. Specification of EOs extracted from twelve Iranian plant species.

Plant name	Family	Common name	Place of collection	Plant part used	Oil yield (%) (v/w)	Oil color
Apium graveolens	Apiaceae	Celery	Isfahan	Seed	1.50	Pale yellow
Citrus sinensis	Rutaceae	Sweet oranges	Northern Iran	Fruit peel	0.30	Yellow
Matricaria chamomilla	Asteraceae	Chamomile	Kashan - Alliance Farm	Flower	0.10	Dark green
Ferula gummosa	Apiaceae	Galbanum	Firuzkouh Highlands	Resin	22.00	Colorless
Eucalyptus cinerea	Myrtaceae	Argyle apple	Southern Iran	Leaf	0.60	Colorless
Pinus eldarica	Pinaceae	Brutia pine	Kashan	Leaf	0.20	Colorless
Lavandula angustifolia	Lamiaceae	Lavender	Kashan - Barij Essen Co.	Flower and stem	0.60	Colorless
Mentha pulegium	Lamiaceae	Pennyrile	Kerman - Lalehzar Farm	Flower and stem	0.40	Yellow
Thymus vulgaris	Lamiaceae	Garden thyme	Tehran	Flower and stem	0.80	Yellow
Zataria multiflora	Lamiaceae	Avishan-e-Shiraz	Bandar Abbas	Flower and leaf	1.40	Yellow
Rosmarinus officinalis	Lamiaceae	Rosemary	Dezful	Stem	0.40	Colorless
Pelargonium graveolens	Geraniaceae	Pelargonium	Kashan	Stem	0.10	Pale yellow

Table 2. LC_{50} values and 95% confidence limits determined by topical application of 12 EOs on female house flies of the Koohrang population.

Essential oils	n[a]	LC_{50} (µg/fly) (95% CI)[b]	LC_{95} (µg/fly) (95% CI)	Slope ± SE	χ^2 (df)[c]
A. graveolens	250	93.83 (76.81-112.89)	614.95 (436.52–1015.04)	2.01 ± 0.21	0.57 (3)
C. sinensis	300	80.76 (65.84-96.98)	503.84 (363.19–813.56)	2.06 ± 0.22	0.76 (3)
M. chamomilla	300	76.98 (62.23-92.86)	503.45 (359.97–825.85)	2.01 ± 0.22	0.69 (3)
F. gummosa	300	67.40 (52.40-82.99)	535.01 (370.25–937.03)	1.82 ± 0.21	2.05 (3)
E. cinerea	376	53.01 (41.88-65.28)	641.29 (408.04-1279.52)	1.51 ± 0.17	1.83 (3)
P. eldarica	300	55.04 (45.13-66.74)	394.67 (270.27–692.10)	1.92 ± 0.21	0.88 (3)
L. angustifolia	250	62.20 (49.2-78.7)	557.68 (343.41–1213.63)	1.72 ± 0.22	1.54 (3)
M. pulegium	280	39.45 (28.84-50.46)	472.44 (287.92–1086.11)	1.52 ± 0.21	1.43 (3)
T. vulgaris	250	47.21 (37.86-57.94)	316.82 (216.23–568.85)	1.99 ± 0.23	1.97 (3)
Z. multiflora	300	32.93 (25.75-40.38)	246.01 (172.51–420.81)	1.88 ± 0.22	0.80 (3)
R. officinalis	296	46.12 (31.80–64.08)	1604.64 (744.17–5939.16)	1.06 ± 0.15	0.91 (3)
P. graveolens	250	89.93 (72.64-109.37)	539.67 (380.15–915.10)	2.11 ± 0.24	0.81 (3)

[a] number of flies used in bioassays.

[b] CI = confidence interval.

[c] degrees of freedom.

Table 3. LC_{50} values and 95% confidence limits determined by fumigation of 12 EOs on female house flies of the Koohrang population.

Essential oils	n[a]	LC_{50} (µL/L) (95% CI)[b]	LC_{95} (µL/L) (95% CI)	Slope ± SE	χ^2 (df)[c]
A. graveolens	360	17.02 (12.64-22.40)	440.85 (230.72-1235.76)	1.16 ± 0.14	0.20 (4)
C. sinensis	300	11.09 (8.63-13.88)	118.35 (74.84-241.980)	1.60 ± 0.19	1.69 (3)
M. chamomilla	360	24.85 (19.29-31.05)	299.96 (196.93-554.12)	1.52 ± 0.16	1.09 (4)
F. gummosa	300	27.06 (21.82-33.26)	232.34 (151.98-443.85)	1.76 ± 0.20	0.14 (3)
E. cinerea	360	10.28 (8.31-12.69)	106.66 (70.01-194.67)	1.61 ± 0.16	0.68 (4)
P. eldarica	300	12.22 (9.83-14.99)	102.36 (67.93-190.89)	1.78 ± 0.20	0.80 (3)
L. angustifolia	300	13.71(10.43-16.99)	103.95 (73.28-177.43)	1.87 ± 0.22	0.12 (3)
M. pulegium	350	22.06 (16.74-27.83)	291.47 (191.04-541.08)	1.46 ± 0.15	0.28 (4)
T. vulgaris	300	20.12 (16.08-24.52)	152.05 (105.32-263.43)	1.87 ± 0.21	0.44 (3)
Z. multiflora	360	10.47 (8.28-13.20)	145.91 (89.02-302.78)	1.43 ± 0.15	1.15 (4)
R. officinalis	300	11.42 (9.35-13.76)	74.99 (53.26-123.33)	2.01 ± 0.21	0.46 (3)
P. graveolens	300	18.66 (14.36-23.26)	183.24 (119.75-355.86)	1.65 ± 0.20	0.24 (3)

[a]number of flies used in bioassays.

[b] CI = confidence interval.

[c] degrees of freedom.

Table 4. Topical toxicity of *Zataria multiflora*, and *Rosmarinus officinalis* EOs on house flies of the Koohrang, Mobarake and Isfahan populations.

Essential oils	Population	n[a]	LC_{50} (µg/fly) (95% CI)[b]	LC_{95} (µg/fly) (95% CI)	Slope ± SE	χ^2 (df)[c]	RR_{50}[d](95% CI)
Z. multiflora	Koohrang	300	32.93 (25.75-40.38)	246.01 (172.51–420.81)	1.88 ±0.22	0.80 (3)	-
	Mobarake	325	66.31 (52.65-84.09)	849.36 (489.78-2050.24)	1.48 ± 0.18	1.69 (3)	2.01 (1.46–2.77)
	Isfahan	300	65.69 (53.66-80.76)	527.48 (344.95–1002.74)	1.81 ± 0.20	0.98 (3)	1.99 (1.47–2.69)
R. officinalis	Koohrang	296	46.12 (31.80–64.08)	1604.64 (744.17–5939.16)	1.06 ± 0.15	1.91 (3)	-
	Mobarake	300	86.00 (59.6-128.7)	3165.80 (1091.86-31755.29)	1.05 ± 0.20	0.62 (3)	1.86 (1.32–3.07)
	Isfahan	300	92.07 (72.76-121.15)	1136.35 (620.86–3059.36)	1.50 ± 0.19	1.16(3)	1.99 (1.30-3.05)

[a] number of flies used in bioassays.

[b] CI = confidence interval.

[c] degrees of freedom.

[d] resistance ratio (RR): LC_{50} of Mobarake or Isfahan / LC_{50} of Koohrang.

Table 5. Fumigant toxicity of *Zataria multiflora*, and *Rosmarinus officinalis* EOs on house flies of the Koohrang, Mobarake and Isfahan populations.

Essential oils	Population	n[a]	LC_{50} (µL/L) (95% CI)[b]	LC_{95} (µL/L) (95% CI)	Slope ± SE	χ^2 (df)[c]	RR_{50}[d] (95% CI)
Z. multiflora	Koohrang	360	10.47 (8.28-13.20)	145.91 (89.02-302.78)	1.43 ± 0.15	1.15 (4)	-
	Mobarake	360	14.57 (11.54-18.71)	214.53 (124.06-487.70)	1.40 ± 0.15	0.79 (4)	1.39 (0.99–1.94)
	Isfahan	360	14.86 (11.86-18.96)	198.15 (117.55-429.82)	1.46 ± 0.15	0.41 (4)	1.42 (1.02–1.97)
R. officinalis	Koohrang	300	11.42 (9.35-13.76)	74.99 (53.26-123.33)	2.01 ± 0.21	0.46 (3)	-
	Mobarake	300	21.02 (17.21-26.18)	183.51 (115.81-368.23)	1.74 ± 0.19	0.14 (3)	1.84 (1.38–2.44)
	Isfahan	300	25.80 (20.84-33.18)	245.15 (149.92–578.13)	1.65 ± 0.19	0.51 (3)	2.25 (1.67–3.04)

[a] number of flies used in bioassays.

[b] CI = confidence interval.

[c] degrees of freedom.

[d] resistance ratio (RR): LC_{50} of Mobarake or Isfahan / LC_{50} of Koohrang.

Table 6. Synergistic effect of enzyme inhibitors on the topical toxicity of *Zataria multiflora*, and *Rosmarinus officinalis* EOs against house fly populations.

Population	Essential oils	n[a]	LC_{50} (µg/fly) (95% CI)[b]	LC_{95}(µg/fly) (95% CI)	Slope ± SE	χ^2 (df)[c]	RR_{50}[d] (95% CI)	RR_{95}[e] (95% CI)	SR[f] (95% CI)
Koohrang	*R. officinalis*	296	46.12 (31.80–64.08)	1604.64 (744.17–5939.16)	1.06 ± 0.15	1.91 (3)	-	-	-
	+PBO	300	45.45 (34.04-59.34)	733.35 (428.94-1664.15)	1.36 ± 0.12	0.11 (3)	-	-	1.01 (0.65 - 1.57)
	+DEM	330	45.10 (34.81-57.21)	580.60 (368.69-1126.47)	1.48 ± 0.17	0.60 (3)	-	-	1.02 (0.67 - 1.56)
	+TPP	350	46.08 (34.79-60.03)	940.23 (533.37-2213.34)	1.25 ± 0.14	2.26 (3)	-	-	1.00 (0.64 - 1.55)
	Z. multiflora	300	32.93 (25.75-40.38)	246.01 (172.51–420.81)	1.88 ±0.22	0.80 (3)	-	-	-
	+PBO	312	31.02 (24.47-37.71)	203.60 (148.47-323.83)	2.01 ± 0.22	0.23 (3)	-	-	1.06 (0.78 - 1.44)
	+DEM	332	30.09 (23.67-36.67)	220.23 (158.69-356.00)	1.90 ± 0.19	1.03 (3)	-	-	1.09 (0.80 - 1.49)
	+TPP	344	31.89 (25.62-38.35)	216.33 (157.78-342.30)	1.97 ± 0.21	0.36 (3)	-	-	1.03 (0.76 - 1.39)
Mobarake	*R. officinalis*	300	86.00 (59.6-128.7)	3165.80 (1091.86-31755.29)	1.05 ± 0.20	0.62 (3)	1.86 (1.10-3.03)	2.13 (0.28-15.8)	-
	+PBO	300	50.49 (40.35-62.22)	452.07 (294.98-870.01)	1.72 ± 0.20	0.33 (3)	1.71 (1.12-2.62)	8.71 (1.48-51.21)	1.70 (1.11-2.59)
	+DEM	330	53.58 (44.22-64.41)	391.21 (271.41-667.54)	1.90 ± 0.18	0.86 (3)	1.60 (1.06-2.42)	10.29 (1.8-58.62)	1.60 (1.06-2.41)
	+TPP	355	62.73 (51.60-76.33)	562.50 (371.03-1038.21)	1.72 ± 0.18	0.48 (3)	1.37 (0.90-2.08)	6.95 (1.19-40.43)	1.37 (0.90-2.07)
	Z. multiflora	325	66.31 (52.65-84.09)	849.36 (489.78-2050.24)	1.48 ± 0.18	1.69 (3)	2.01 (1.46-2.77)	3.89 (1.53-9.86)	-
	+PBO	341	35.81 (29.01-42.89)	234.59 (171.94-367.72)	2.01 ± 0.21	1.79 (3)	1.87 (1.38-2.53)	4.19 (1.72-10.23)	1.85 (1.36-2.50)
	+DEM	355	38.74 (30.55-47.32)	356.07 (244.27-624.88)	1.70 ± 0.19	0.58 (3)	1.73 (1.26-2.39)	2.60 (1.04-6.69)	1.71 (1.24-2.35)
	+TPP	300	50.27 (41.24-60.62)	339.85 (237.47-576.55)	1.98 ± 0.21	1.85 (3)	1.32 (0.97-1.80)	2.73 (1.08-6.91)	1.31 (0.97-1.78)
Isfahan	*R. officinalis*	300	92.07(72.76-121.15)	1136.35(620.86–3059.36)	1.50 ± 0.19	1.16 (3)	1.96 (1.28-3.03)	0.64 (0.15-2.60)	-
	+PBO	340	48.48 (39.95-57.99)	343.60 (243.84-564.12)	1.93 ± 0.20	0.86 (3)	1.92 (1.40-2.63)	3.60 (1.36-9.49)	1.89 (1.39-2.59)
	+DEM	332	49.96 (41.27-59.81)	345.95 (245.25-568.16)	1.95 ± 0.21	1.91 (3)	1.86 (1.34-2.55)	3.54 (1.34-9.33)	1.84 (1.34-2.51)
	+TPP	325	53.92 (43.84-65.68)	460.50 (306.53-847.56)	1.76 ± 0.19	1.49 (3)	1.72 (1.24-2.38)	2.56 (0.92-7.09)	1.70 (1.23-2.35)
	Z. multiflora	300	65.69 (53.66-80.76)	527.48 (344.95–1002.74)	1.81 ± 0.20	0.98 (3)	1.99 (1.47-2.69)	2.24 (1.03-4.88)	-
	+PBO	300	35.86 (29.09-43.04)	214.50 (156.59-339.98)	2.11 ± 0.23	1.57 (3)	1.85 (1.39-2.46)	2.62 (1.25-5.48)	1.83 (1.38-2.42)
	+DEM	325	39.02 (32.46-46.09)	212.43 (158.95-320.90)	2.23 ± 0.22	1.26 (3)	1.70 (1.29-2.23)	2.65 (1.30-5.41)	1.68 (1.28-2.20)
	+TPP	300	44.54 (37.89-51.92)	187.34 (145.16-267.19)	2.63 ± 0.25	0.44 (3)	1.49(1.15-6.32)	3.19 (1.61-6.32)	1.47 (1.14-1.90)

[a] number of flies used in bioassays.

[b] CI = confidence interval.

[c] degrees of freedom.

[d] resistance ratio (RR_{50}): LC_{50} of Mobarake or Isfahan / LC_{50} of Koohrang.

[e] resistance ratio (RR_{95}): LC_{95} of Mobarake or Isfahan / LC_{95} of Koohrang.

[f] SR, synergism ratio.

Table 7. Pairwise correlation between log LC_{50}s of the two selected EOs and synthetic insecticides.

	Essential oils	
	Zataria multiflora	*Rosmarinus officinalis*
Fixed effect: permethrin		
Intercept	1.42	1.58
Slope	0.14	0.13
P^*	0.002	0.06
r	0.99	0.99
Fixed effect: cypermethrin		
Intercept	1.46	1.61
Slope	0.13	0.12
P^*	0.007	0.07
r	0.99	0.99
Fixed effect: deltamethrin		
Intercept	1.51	1.65
Slope	0.13	0.13
P^*	0.01	0.07
r	0.99	0.99
Fixed effect: dichlorvos		
Intercept	1.41	1.56
Slope	0.15	0.145
P^*	0.04	0.02
r	0.99	0.99

*, *P* values were estimated by the slope values of the fixed effect.

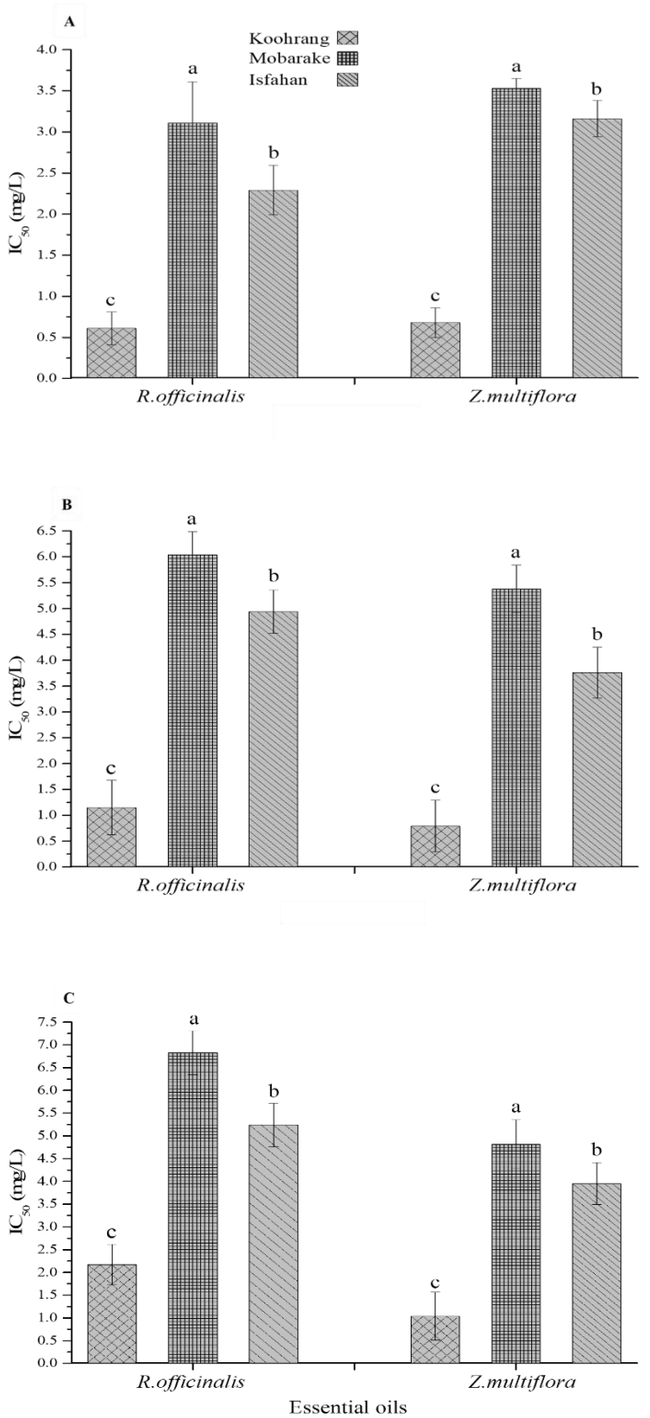

Fig 1. Inhibition rates of P450s, GSTs and CarEs from insecticide-resistant and susceptible populations of adult female house flies by *Zataria multiflora* and *Rosmarinus officinalis* EOs. Mean (±SE) values with different letters are significantly different from each other: **A** (P450); *R. officinalis*: $F_{2,8} = 1143.21$, $P < 0.001$; *Z. multiflora*: $F_{2,8} = 2085.49$, $P < 0.001$, LSD test at $P \leq 0.05$**; B (**GSTs)**;** *R. officinalis*: $F_{2,8} = 4.17$, $P < .0.01$; *Z. multiflora*: $F_{2,8} = 4882.56$, $P < 0.001$, LSD test at $P \leq 0.05$), **C (**CarE)**;** *R. officinalis*: $F_{2,8} = 427.35$, $P < 0.001$; *Z. multiflora*: $F_{2,8} = 236.72$, $P < 0.001$, LSD test at $P \leq 0.05$.

Supplementary Table S1. Relative concentrations (%) of the components of twelve EOs. RI, identification based on Retention indices.

		composition (%)											
Compound	RI	*A. graveolens*	*C. sinensis*	*M. chamomilla*	*F. gummosa*	*E. cinerea*	*P. eldarica*	*L. angustifolia*	*M. pulegium*	*T. vulgaris*	*Z. multiflora*	*R. officinalis*	*P. graveolens*
α- thujene	927			1.80	1.80								
α- pinene	934	8.47	1.20	1.30	9.09	3.61	15.18		1.90			14.22	
camphene	947	3.10	1.04		1.70		3.67					7.80	3.66
sabinene	969		1.40	4.00	3.10				1.60				
β- pinene	972	1.30		1.4	33.70		4.62		1.01			3.30	
beta myrcene	984		3.20	1.20		1.30	6.93			2.10	1.90	1.30	
α- phellandrene	997					2.90							
δ- 3 carne	1005	1.60	1.42		2.35		2.30						
α- terpinene	1011		1.40	10.38	1.30					1.20	1.20		
para-cymene	1019					10.45				23.54	5.90	1.50	
Limonene	1022	62.34	81.30		13.21		14.62		1.54				
1,8 cineole	1026			12.73		58.71		15.92	11.28	1.20	14.13	33.24	
cis-β-Ocimene	1037	6.50		5.40	2.90		4.28						
trans-β-Ocimene	1043			4.30			7.80						
γ- terpinene	1050	8.90	1.17	4.90	3.50	1.20				8.30	1.20	2.30	
cis linalool oxide	1072							3.12					
α- terpinolene	1081						6.80						
trans linalool oxide	1088							2.11					
linalool	1090		2.10	1.50	1.40			23.89	4.26	1.90	1.60	2.62	1.49
maltol	1108							6.74					
camphor	1114							12.28				25.01	
menthol	1116			1.20					9.75				
geraniol	1123												12.30
trans pinocarveole	1136					6.80							
menthone	1155			1.40					11.66				3.75
borneol	1168				2.10			8.30	6.90	2.60	1.30	2.20	
α- terpineol	1184				3.10	2.10	12.49	4.03	6.80			1.80	
citronellol	1229	1.20	1.14										30.95
pulegone	1231								12.60				
linalool acetate	1251							12.50					
thymol	1279									45.10	16.5		
citronellyl formate	1275												10.25
terpinen-4-ol	1178				2.10		2.83			1.50	1.30		
bornyl acetate	1285				5.20		3.42		2.11			1.40	
carvacrol	1300								4.12	6.57	49.66		
piperitenone	1338								12.40				
α-terpinyl acetate	1350						9.83						
caryophyllene	1420									1.40	2.70	2.30	
piperitenone oxide	1360								5.47				
beta gurjenene	1432												9.72
(e)-β-farnesene	1464			19.60									
Geranyl propionate	1472												4.25
α- humulene	1481	2.30							1.10				
bicyclogermacrene	1490			4.60	1.50	1.65							
geranyl isobutyrate	1516												3.20
caryophyllene oxide	1579			2.80				1.36	2.14	1.82	1.43		11.35
virdiflorol	1585					2.80							
β-eudesmol	1659				1.50	4.10							
α-eudesmol	1652					1.90							

α-bisabolol oxide b	1661			6.60									
α-bisabolol	1685			2.50	1.20			1.420					
α-bisabolol oxide a	1753			8.01									
caryophyllenol	1762				2.30								
	Total	95.71	95.37	95.61	93.05	97.52	94.71	91.67	96.64	97.10	98.79	98.99	90.92