

## Dietary exposure to essential oils of selected *Pinus* and *Abies* species leads to morphological changes in *Drosophila melanogaster* wings

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**Abstract:** Essential oils (EOs) from conifer species are a potential source of biopesticides due to their various bioactive effects against pest insects. Since the harmful activity of pest insects often relies on their ability to fly, the disruption of wing morphology due to the use of essential oils (EOs) could be significant. This study investigated changes in wing shape and size in the model insect *Drosophila melanogaster* after exposure to EOs derived from selected conifers species. *Drosophila* larvae were treated with a diet containing 3% EOs from 6 *Pinus* and 3 *Abies* species. After completing their life cycle, the wings of adults were dissected and analyzed using the geometric morphometrics method. Changes in *Drosophila* wing morphology were observed. The potential effect of the major compounds of the tested EOs on wing morphology is discussed. We believe that this study lays the foundation for further evaluation of *Abies* and *Pinus* EOs in targeted toxicological studies against pest insects by focusing on the potential of EOs to disrupt pest activities that depend on wing morphology.

**Keywords:** *Abies*, *Drosophila melanogaster*, essential oils, *Pinus*, wing morphology

### INTRODUCTION

The pine family, Pinaceae, is a significant coniferous taxon comprising 11 genera [1]. Among these is the genus *Abies* Mill. (firs), encompassing 52 species and the genus *Pinus* L. (pines) containing 133 species, representing the most diverse groups [1]. In traditional medicine of different cultures, certain essential oils (EOs) derived from *Abies* and *Pinus* have been recognized for their beneficial effects in treating respiratory infections and for muscle relaxation [2,3]. Consequently, during the last decades, there has been an increased interest in studying the chemical profiles and bioactivities of fir and pine EOs. Many studies have shown that EOs contain an array of bioactive compounds characterized by antimicrobial [4-6], molluscicidal [7], insecticidal [4-6,8,9], antiradical [10,11], antiplatelet [12], and antitumor properties [13].

The fruit fly *Drosophila melanogaster* Meigen, 1830 is an important model organism [14,15] in biological research, widely recognized as a multicellular eukaryotic

insect model for numerous reasons. It is a low-cost animal model with a rapid life cycle and well-understood biology. Several assays that include *D. melanogaster* are frequently used in studies of the biological activity of plant-derived extracts [16], EOs [17-19], plant-derived oil [20], or fruit-derived compounds [21]. Furthermore, *D. melanogaster* is an effective model organism for investigating EOs as potential biopesticides for pest insect control, particularly closely related species such as *Drosophila suzukii* (Matsumura). Recent reports have highlighted the promising impact of volatile plant compounds against this invasive pest insect [22], demonstrating both insecticidal and deterrent effects [23-24].

However, recently published studies have demonstrated a significant impact of EOs extracted from conifers on the different aspects of *D. melanogaster* life cycle, such as survival rate, pupation rate, adult eclosion, and life cycle duration [4-6,25]. It was observed that adult fruit flies that survived the dietary treatment with EOs of *Abies* and *Pinus* species during the larval stage did not exhibit visible large-scale morphological

changes on the body or the wings of *D. melanogaster* [4-6]. The question has been raised about whether treatments using EOs extracted from six *Pinus* and three *Abies* species affect the micro-scale morphology of *D. melanogaster*, particularly their wings. Based on recently published papers [26,27], one approach is to use the geometric morphometric method. Cvetković et al. [26] found micro-scale changes in wing morphology in *D. melanogaster* treated with TiO<sub>2</sub> nanoparticles despite their normal appearance at a larger scale. Using this sensitive method, changes in insect wings caused by active substances such as insecticides were examined in store-product coleopteran pests, including the Khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae) [28], *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), *Prostephanus truncatus* (Horn), and *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) [29]. Furthermore, this method has demonstrated its suitability for distinguishing between groups subjected to specific treatments, as in Culicidae [30], which, like *D. melanogaster*, are dipterans. A recent investigation into the influence of EOs on the wing morphology of dipterans, particularly mosquitos, showed that *Croton tetradenius* EO influenced the wing morphometry of *Aedes aegypti* [27]. Changes in wing morphology can affect dipteran dispersion and flying capability [30]. Thus, it can be assumed that changes in wing morphology potentially induced by treatment with EOs could affect flying insect pests and reduce their impact on economically important crops. The aim of this study was to explore potential changes in wing shape and size in the insect model *D. melanogaster* following dietary exposure to EOs from selected *Abies* and *Pinus* species.

## MATERIALS AND METHODS

### Ethics statement

This study does not involve any live vertebrates or human subjects.

### Plant material processing

Needles of six *Pinus* species (*P. halepensis* Mill., *P. heldreichii* Christ, *P. mugo* Turra, *P. nigra* J.F. Arnold, *P. peuce* Griseb. and *P. sylvestris* L.), and twigs with needles of three *Abies* species (*A. alba* Mill., *A. × borisii-regis* Mattf., and *A. cephalonica* Loudon) used in this

study were two years old and collected from their native populations in the Balkan Peninsula. Vernacular names for the species whose EOs were tested are *P. halepensis* – Aleppo pine, *P. heldreichii* – Bosnian pine, *P. mugo* – Mountain pine, *P. nigra* – European black pine, *P. peuce* – Macedonian pine, *P. sylvestris* – Scots pine, *A. alba* – Silver fir, *A. × borisii-regis* – King Boris fir and *A. cephalonica* – Greek fir. Details regarding the collection of plant material, as well as the locations and ecological conditions of the populations of studied species of *Abies* and *Pinus* have already been reported [4-6]. Voucher specimens of each taxon were deposited in the “Herbarium Moesiacum Niš” (HMN) of the Faculty of Sciences and Mathematics, Department of Biology and Ecology, University of Niš under acquisition numbers 13548, 13885, 12810, 6920, 12812, 6938, 13944, 13943, and 13999, respectively.

### Isolation and chemical characterization of EOs

The isolation and determination of the chemical composition of the three *Abies* and six *Pinus* species EOs used in this study were performed and published in previous studies [4-6]. Briefly, plant material was subjected to hydrodistillation using a Clevenger apparatus. The qualitative analysis of the EOs was conducted through gas chromatography-mass spectrometry (GC-MS), while quantitative analysis involved gas chromatography-flame ionization detector (GC-FID) analysis. Both analyses were carried out under the previously presented operating parameters [31].

### Bioassay: insects and their husbandry

Specimens of *D. melanogaster* were used as a model system in this experiment (Oregon stock, Bloomington Indiana University, USA). Flies in stock were reared *en masse* on a standard cornmeal-based feeding medium (agar, sucrose, yeast, water, corn meal) with the addition of methyl 4-hydroxybenzoate as a fungicide. The laboratory conditions for maintenance of the flies were standard: 25°C, 60% relative humidity, and a light regime of 12h of day and 12h of night.

Adult male and female flies used in this analysis originated from previous studies where the treatment protocol was explained in detail [4-6]. Briefly, the treatment protocol included the following steps: initially, 3-day-old larvae were obtained from young

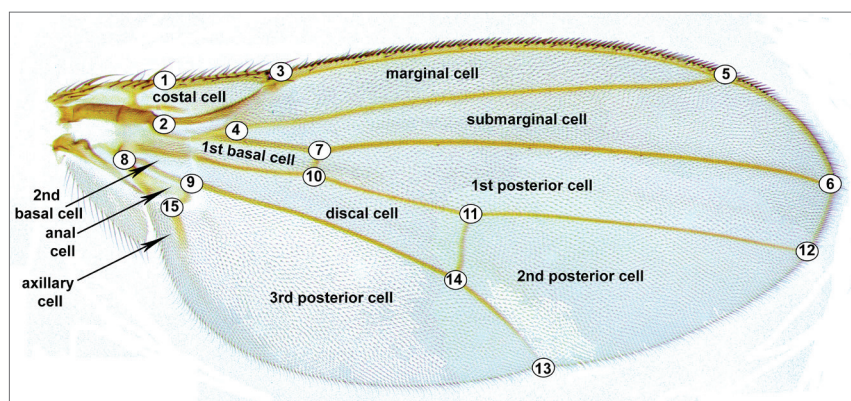
adults of the same age that were being mass-cultured. Subsequently, treatments were prepared by adding 100% pure EOs from one of the tested *Abies* or *Pinus* species into fresh cornmeal-based feeding media, achieving a final concentration of 3% essential oil within the media. The 3% concentration of the EOs was selected based on prior findings, which exhibited a positive correlation with significant larvicidal effects and delayed developmental time in *D. melanogaster* [4-6]. Therefore, it is presumed that the 3% EO concentration has the potential to induce certain morphological changes in the wings, in contrast to lower concentrations of 1.5%, 0.75%, 0.38%, and 0.19% that were used in previous studies. For the control group, a feeding medium without essential oils was used. Then, twenty larvae per replicate were transferred onto each treatment as well as the control feeding medium. Experimental groups were incubated under standard laboratory conditions, as explained in the previous paragraph of this section. At the end of the fruit flies' life cycle, i.e., after the eclosion of adults (imago), all successfully emerged males and females were collected and preserved in 70% ethanol prior to further analysis.

### Wing dissection and slide preparation

Wing dissection from the fruit fly's body was conducted using fine forceps and entomological needles, conducted in small plastic trays filled with distilled water. Detached wings were mounted on microscopic slides using Berlese medium. Details of the process are given in [26]. In each group exposed EOs and in control groups alike, 15 specimens were randomly chosen, and their wings were carefully detached using the method described earlier. Wings were photographed with a Leica Flexicam C3 on a Leica M165 stereomicroscope (Leica Microsystems, Wetzlar, Germany) with 40× magnification.

### Geometric morphometrics – positioning of landmarks

The assessment of possible variations in *D. melanogaster* wing shape resulting from different EO treatments was based on geometric morphometrics [32]. To depict



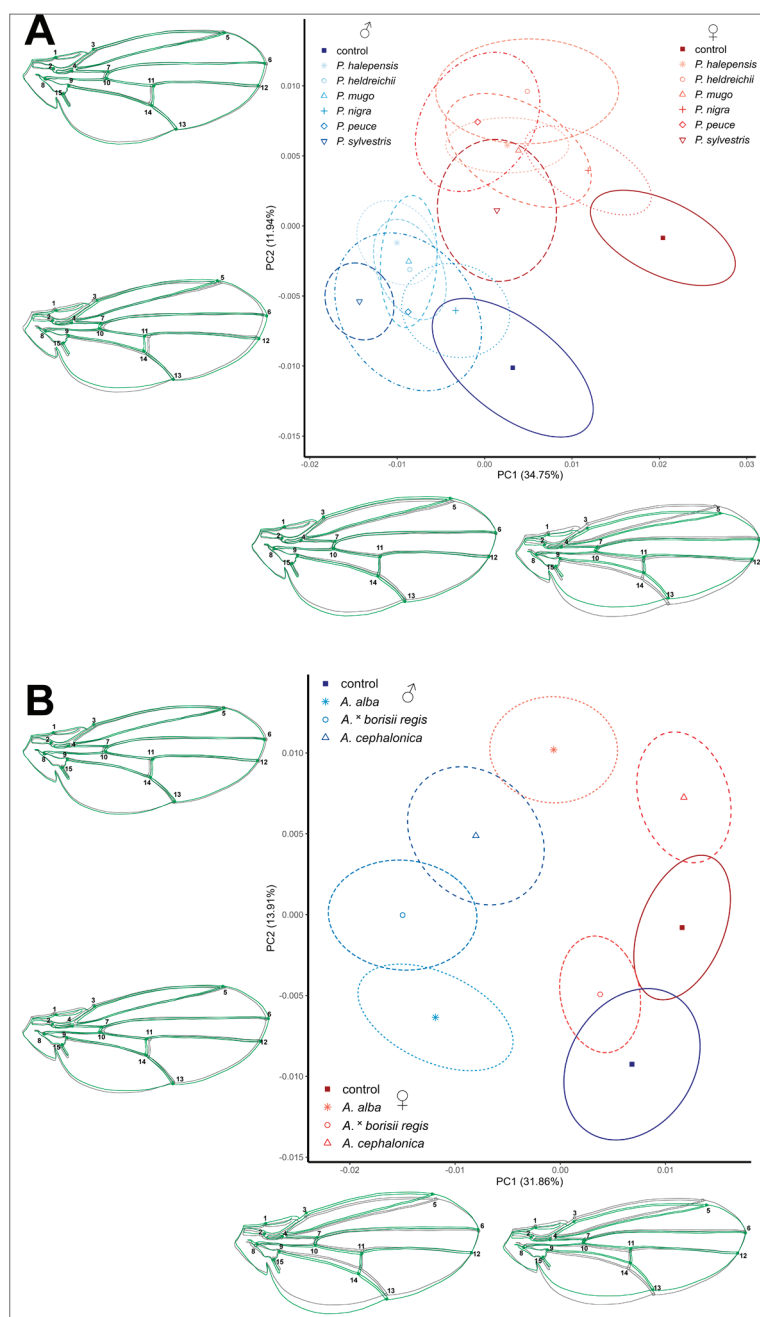
**Fig. 1.** The landmark position on the female wing from the control group. Fifteen landmarks (LM) depict the shape of the following wing cells: LM 1-3 – costal cell, LM 2-5 – marginal cell, LM 4-6 – submarginal cell, LM 2, 4, 7, 8, and 10 first basal cell, LM 6-7 and LM 10-12 – first posterior cell, LM 8-9 – length of second basal cell, LM 9-11 and LM 14 – discal cell, LM 11-14 – second posterior cell, LM 8-9 and LM 15 – anal cell, LM 9 and LM 13-15 – third posterior cell. Wing cell nomenclature is given according to [35].

the wing shape, 15 landmarks were positioned in the tpsDig2 software [33] following Gidaszewski et al. [34]. All landmarks are placed at the intersection of veins (Fig. 1). Wing cell nomenclature follows Morgan et al. [35]. To eliminate discrepancies in position, rotation, and translation, a generalized Procrustes analysis (GPA) was performed as outlined by Dryden and Mardia [36] and Rohlf and Slice [37]. The shape and size were obtained as Procrustes coordinates and centroid size (CS), respectively. GPA was performed in MorphoJ software [38].

### Statistical analysis

The previous experiments, from which the flies used in this study originated, were categorized according to genus affiliation (*Pinus* and *Abies*) and conducted at different times. Differences in the composition of the EOs between these two genera led us to treat them as distinct entities throughout the manuscript and perform separate analyses within the *Pinus* and *Abies* groups. Also, considering that we analyzed EOs from nine species, for practical reasons and readability, we decided to separate the analysis according to genus affiliation. The positioning of males and females in the morphospace suggests that any changes in wing morphology resulting from exposure to EOs were smaller compared to the inherent differences in wing





**Fig. 2** Principal component analysis (PCA) in the wings of females and males exposed to the essential oils of 6 *Pinus* species (A) and 3 *Abies* species (B) and their corresponding control groups. The morphospace was constructed using the first two PC axes. EO treatments are shown as ellipses with dashed lines and assigned symbols; controls are in a solid line. Females are represented by shades of red, and males in shades of blue. The ellipses represent a 90% confidence interval for group means. Changes in wing morphology along the PC axes are shown via contour graphs; grey lines show the mean shape of the wing, and green lines describe the changes in the maximum or minimum value for both axes.

shape and size between males and females in *D. melanogaster*. Therefore, separate analyses were conducted within the male and female groups for both *Abies* and *Pinus* species' EOs.

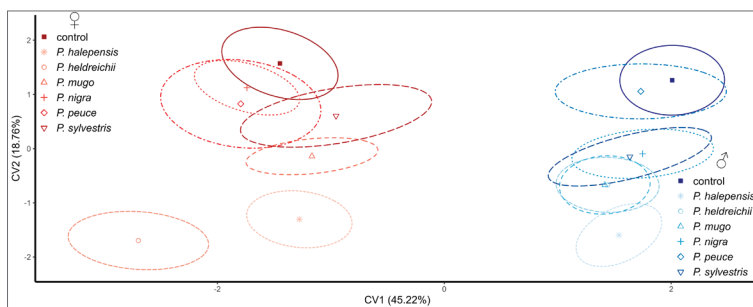
To assess the differences in wing size, analysis of variance (ANOVA) was conducted using the logarithmic value of centroid size (logCS) as the dependent variable. The factors considered were sex, treatment, and their interaction. The specific tests included differences in wing size between sexes (logCS ~ sex), differences in wing size among treatments (logCS ~ treatment), and interaction effects of sex and treatment on wing size (logCS ~ sex \* treatment). For analyzing wing shape, multivariate analysis of variance (MANOVA) was applied to Procrustes coordinates, focusing on the following factors: shape differences between sexes (shape ~ sex), shape differences among treatments (shape ~ treatment), interaction effects on shape between sex and treatment (shape ~ sex \* treatment). All statistical analyses were performed using the geomorph package version 4.0.5. [39] in R Studio software [40]. Significant differences were considered as  $P < 0.05$ . Spearman's rank correlation coefficient (Spearman's Rho) was used to analyze correlations between the selected compounds, constituents of the tested essential oils (EOs) (Supplementary Table S1), and the centroid size value (which describes wing size). These analyses were performed separately within *Pinus* and *Abies* species, as well as separately for females and males. The correlation analysis was done in software SPSS version 15.0 (SPSS Inc, Chicago, IL, USA). Principal component analysis (PCA) and canonical variate analyses (CVA) were used for testing wing divergence. Both PCA and CVA were performed in the MorphoJ software [38]. The results of PCA and CVA were extracted and used for generating scatterplot graphs in R Studio software [40] via the ggplot2 software package [41]. Additionally, within the CVA, the Procrustes distances were calculated as a value of deviations in the wing shape of treated flies from the control group.

## RESULTS

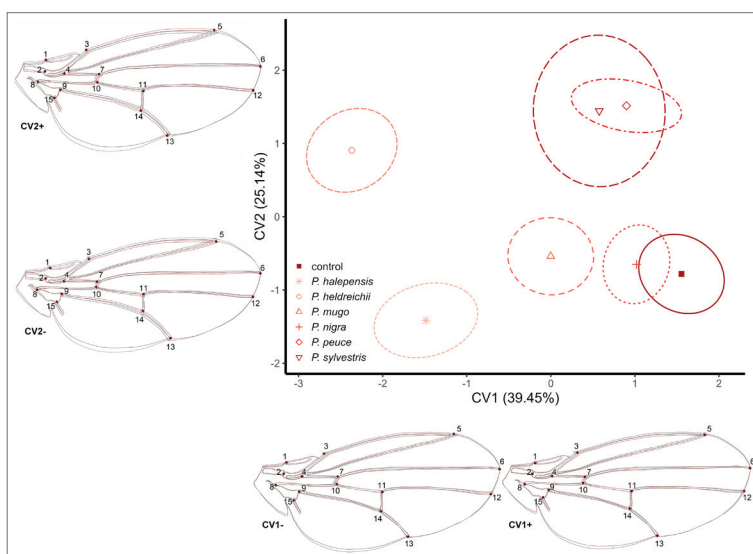
Principal component analysis (PCA) revealed distinct patterns of wing shape variation between the control and treated groups, as well as between sexes. In both females and males, discrimination between control groups was evident along the first principal component (PC1) axis, whereas differentiation between sexes was observed along the second principal component (PC2) axis (Fig. 2A). The discrepancy between the control group and the specimens exposed to essential oils mainly pertained to the width of the wings. Female controls exhibited the narrowest wings, whereas wings in the treated groups and male controls were similar in shape. Notably, the widest wings were found in the male groups exposed to EOs, with the wings of those exposed to the *P. sylvestris* EO being the widest compared to the male control group. Among female groups, the greatest difference was between wings exposed to the EO of *P. peuce* and the control group (Fig. 2A). Small differences along the PC2 axis (PC2 = 11.94%) explained sex-related differences, with females exhibiting slightly wider and shorter wings with shorter first and second posterior cells. In contrast, males showed elongated and narrower wings with longer first and second posterior cells (Fig. 2A).

ANOVA indicated statistically significant differences in wing size ( $Z = 5.899$ ,  $P = 0.001$ ) and in the interaction between sex and treatment ( $Z = 2.111$ ,  $P = 0.018$ ). However, differences in wing size between sexes and between treatments alone were not statistically significant. The multivariate analysis of variance (MANOVA) revealed statistically significant differences in wing shape between sexes ( $Z = 12.940$ ,  $P = 0.001$ ), among treatments ( $Z = 6.343$ ,  $P = 0.001$ ), and in the interaction between sex and treatment ( $Z = 1.703$ ,  $P = 0.047$ ).

Due to the statistically significant results of ANOVA and MANOVA, CVA was applied. The sexual dimorphism in wing morphology was sustained even after exposure of *D. melanogaster* larvae to the essential oils (Fig. 3). Notably, all female groups, including the control, clustered in the negative part of the CV1 axis, while all males, along with their respective control, clustered in the positive part of CV1. Despite



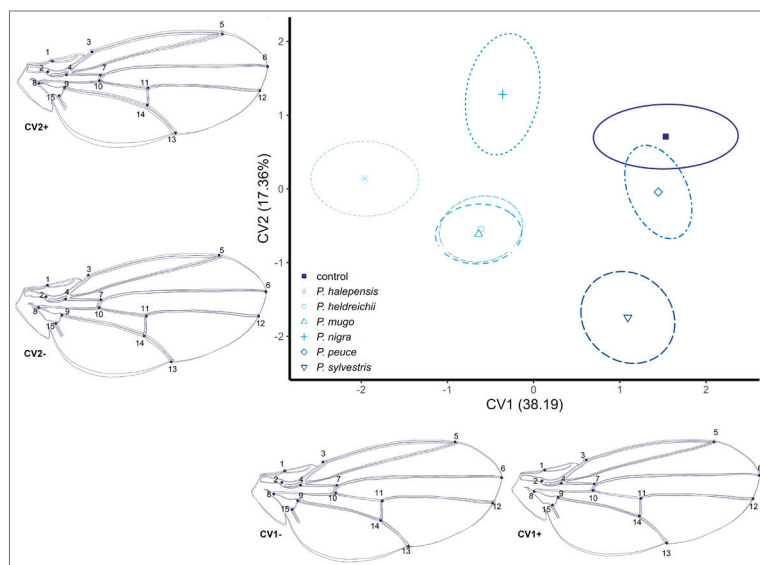
**Fig. 3.** Comparative analysis of changes in the wings of females and males exposed to the essential oils of six *Pinus* species and their control groups. The morphospace was constructed using the first two CV axes. EO treatments are shown as ellipses with dashed lines and assigned symbols; controls are in a solid line. Females are represented by shades of red and males in shades of blue. The ellipses represent a 90% confidence interval for group means.



**Fig. 4.** Variation of females' wings exposed to essential oils of six *Pinus* species and control group in CV1  $\times$  CV2 morphospace. EO treatments are shown as ellipses with dashed lines and assigned symbols; controls are in a solid line. Changes in wing morphology along the CV axes are shown via contour graphs; grey lines show the mean shape of the wing, and red lines describe the changes in the maximum or minimum value for both axes. The ellipses represent a 90% confidence interval for group means.

the influence of the *Pinus* EOs on wing morphology, sexual dimorphism is evident. This fact eliminates any concerns about mixing groups of different sexes, which is essential for the subsequent interpretation of the results when analyzing the wing changes separately in females and males (Figs. 4 and 5.)

The total variability of wing morphology summarizing CV1+CV2 was 64.59%. The most noticeable separation along the CV1 axis is demonstrated



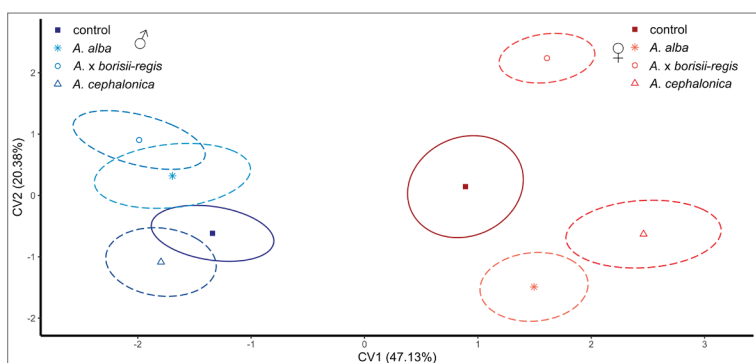
**Fig. 5.** Variation of male wings exposed to essential oils of six *Pinus* species and control group in CV1 × CV2 morphospace. EO treatments are shown as ellipses with dashed lines and assigned symbols; controls are in a solid line. The changes in wing morphology along the CV axes are presented in contour graphs. Grey lines show the mean shape of the wing, and blue lines describe the changes in the maximum or minimum value for both axes. The ellipses represent a 90% confidence interval for groups' means.

by the *P. halepensis* and *P. heldreichii* groups (Fig. 4). CV1 explains 39.45% of the overall variability in wing shape. The control group of females is positioned opposite the *P. halepensis* and *P. heldreichii* treatments and is located at the extreme range of the positive part of the CV1 axis. Changes in wing morphology, as observed on the deformation grid, were attributed to alterations in the following wing cells: the third posterior cell, first basal cell, and discal cell. Parallel elongation of the wings results from the shortening of the first and second posterior cells. Along the CV2, which accounts for about 25% of the total wing variability, distinct morphological differences are evident in the treatments involving *P. heldreichii*, *P. peuce* and *P. sylvestris* compared to the control group and the other three treatments: *P. halepensis*, *P. nigra*, and *P. mugo*. Changes in wing shape along the CV2 axis are similar to those observed along the CV1. Wings of specimens exposed to the EOs of *P. heldreichii*, *P. peuce*, and *P. sylvestris* are generally wider, with wider submarginal and second posterior cells (Fig. 4). The *P. nigra* treatment overlaps significantly with the control group, although the effect of the essential oils of this pine species on wing shape is minimal.

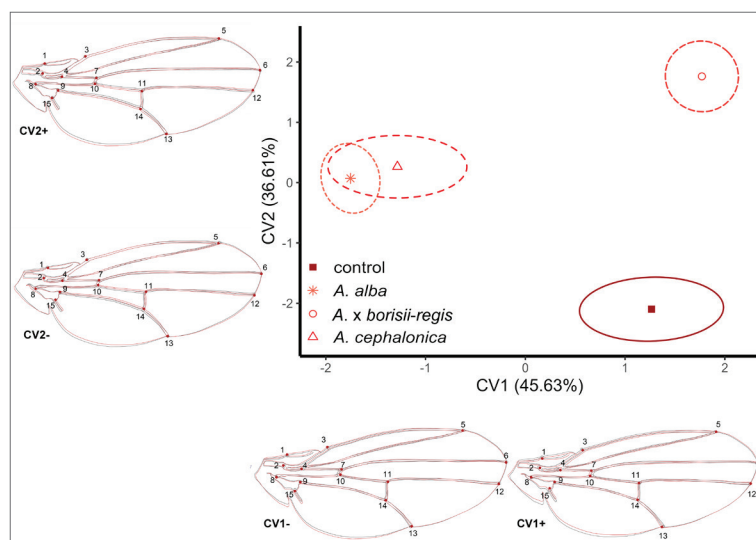
In the male groups (Fig. 5), within the two-dimensional morphospace defined by CV1 and CV2, which together account for 55.55% of the total wing variability, the control group was located in the CV1(+) × CV2(+) quadrant. The other treatments are scattered throughout the CVA morphospace, indicating that each treatment induces distinct changes in *D. melanogaster* wing shape, except for *P. mugo* and *P. heldreichii*, which overlap along the first canonical axis, which accounts for 38.19% of the total wing morphology variability, the treatments *P. peuce* and *P. sylvestris* are positioned in the positive part of CV1, while the other four treatments, *P. halepensis*, *P. heldreichii*, *P. nigra*, and *P. mugo*, are placed in the opposite part (Fig. 5). In these four treatments, changes can be observed in the form of an expansion of the entire wing, primarily the third posterior cell. Slight changes were observed, primarily as a mild lengthening of the first basal cell and a subtle shortening of the first posterior cell of the wing. In comparison to the other treatments, the wings of flies subjected to *P. peuce* EOs displayed the smallest changes in morphology, with considerable overlap with the control group. The second canonical axis, contributing only 17.36% to wing differentiation, polarizes the remaining two EO treatments, *P. nigra* in the CV2 (+) direction and *P. sylvestris* in the CV2 (-) direction. Male wings exposed to *P. sylvestris* EOs have wide second discal and third posterior cells (Fig. 5), in contrast to wings treated with *P. nigra* EOs.

Visualization of the results by PCA revealed clear distinctions in wing shape between the groups exposed to *Abies* EOs and the control groups. In males, the control group was clearly separated from those treated with EOs along the PC1 axis. For females, the control group differed significantly from the group exposed to *A. alba* EOs (Fig. 2B). These differences were primarily attributed to variations in wing width. Specifically, control males and females had narrower wings, while males exposed to EOs from the three *Abies* species had wider wings (Fig. 2B). Along the PC2 axis, the male control and *A. alba* groups were separated from the *A. cephalonica* group. In females, exposure to *A. × borisii-regis* EO resulted in narrower wings with slightly longer first and second posterior cells compared to the effects observed with *A. alba* (Fig. 2B).





**Fig. 6.** Comparative analysis of changes in the wings of females and males exposed to the essential oils of three *Abies* species and their control groups. The morphospace was constructed using the first two CV axes. EO treatments are shown as ellipses with dashed lines and assigned symbols; controls are in a solid line. Females are represented by shades of red, and males in shades of blue. The ellipses represent a 90% confidence interval for group means.



**Fig. 7.** Variation of females' wings of females exposed to essential oils of three *Abies* species and control group in CV1  $\times$  CV2 morphospace. EO treatments are shown as ellipses with dashed lines and assigned symbols; controls are in a solid line. The changes in wing morphology along the CV axes are presented in contour graphs. Grey lines show the mean shape of the wing, and red lines describe the changes in the maximum or minimum value for both axes. The ellipses represent a 90% confidence interval for group means.

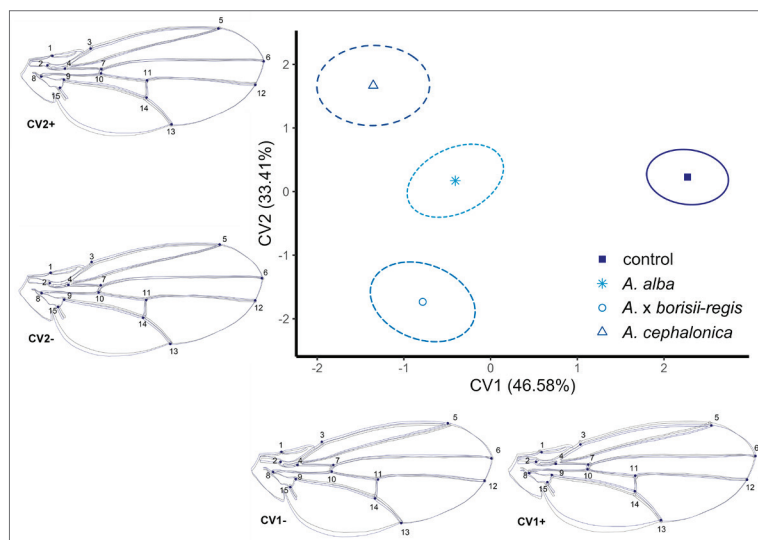
Results of ANOVA indicated statistically significant differences in wing size ( $Z=5.3116$ ,  $P=0.001$ ) and the interaction between wing size and treatment ( $Z=3.911$ ,  $P=0.001$ ). However, no significant differences were observed in wing size between the sexes or in the interaction between sex and treatment. MANOVA showed significant differences in wing shape between sexes ( $Z=8.170$ ,  $P=0.001$ ) across treatments ( $Z=3.743$ ,

$P=0.001$ ), and in the interaction between sex and treatment ( $Z=1.713$ ,  $P=0.039$ ).

Similar to the previous analysis of wing differentiation in *Pinus* treatments, Fig. 6 confirms the presence of sexual dimorphism in the *Abies* EO treatments as well. Along the CV1 axis, all female groups, including the control group, are positioned positively, whereas all male groups, including their respective controls, are positioned negatively. The analyses were conducted separately for male and female groups.

In female *Abies* specimens, both CV axes are crucial for distinguishing individuals based on wing shape and size. CV1, explaining 45.63% of the total variability, separates the control and *A. x borisii-regis* treatment from the *A. alba* and *A. cephalonica* treatments (Fig. 7). Morphological differences in wings between the *A. alba* and *A. cephalonica* treatments are minimal. Conversely, the control group is visibly segregated from the *A. x borisii-regis* treatment along the CV2 axis (describes 36.61% of the total wing variability). Changes in wing shape are reflected in the width and length of the first basal, discal, first, second, and third posterior cells. Wings of specimens exposed to EOs of *A. alba* and *A. cephalonica* have a wider third posterior cell, vaguely elongated first basal and discal cells, and marginally shorter first and second posterior cells (Fig. 7). EO of *A. x borisii-regis* affected the enlargement of the submarginal and second posterior cells, as well as the elongation of the second posterior cell and the reduction of discal cell length.

In males, there is significant discrimination along CV1 (46.58%) between the control group and those groups exposed to one of the three essential oils, as depicted in Fig. 8. The most distinctive wings, when compared to the wings from the control group, were those exposed to the *A. cephalonica* and *A. x borisii-regis* EOs. The most pronounced differences in wing



**Fig. 8.** Variation of males' wings exposed to essential oils of three *Abies* species and control group in CV1  $\times$  CV2 morphospace. EO treatments are shown as ellipses with dashed lines and assigned symbols; controls are in a solid line. The changes in wing morphology along the CV axes are presented in contour graphs. Grey lines show the mean shape of the wing, blue lines describe the changes of the maximum or minimum value for both axes. The ellipses represent a 90% confidence interval for group means.

morphology of *A. cephalonica* are characterized by a general broadening of the wings along the longitudinal axis. These wings have broader discal, second and third posterior, and submarginal cells, along with slightly elongated first basal cells (Fig. 8). On the second canonical axis, which accounts for 33.41% of the total variability, two groups can be discerned. The wings of *A. cephalonica* are positioned in the positive part, whereas the wings exposed to *A.  $\times$  borisii-regis* EO are grouped in the negative part of CV2. The main disparities between these two groups are seen in the width of the wing as well as in the width of the discal, marginal, second, and third posterior cells. The wings treated with *A.  $\times$  borisii-regis* EO are wider, with all the afore-mentioned cells being wider when compared to those treated with *A. cephalonica* EO (Fig. 8). The wings from the *A. alba* treatment are situated between the wings of the *A.  $\times$  borisii-regis* and *A. cephalonica* treatments.

We employed Procrustes distances as a numerical measure that indirectly indicates changes in wing shapes. This value quantifies the deviations in the wing shapes of treated fruit flies compared to the control (untreated flies). Procrustes distances were separately calculated for females and males, as well as individually

within the *Pinus* and *Abies* groups, all presented in Table 1. Procrustes distances were significant in all tested EOs except the EO of *P. nigra* which showed no significant influence on changes in wing shape in both genders, while the *P. peuce* EO did not exhibit significant influence in males. Additionally, in the *Abies* group among females, only the essential oil from *A. cephalonica* did not show a significant influence on wing shape.

Spearman's correlation analysis (Table 2) revealed that wing size is significantly negatively correlated with the concentrations of  $\alpha$ -humulene, (*E*)-caryophyllene, and thunbergol in both female and male individuals

treated with *Pinus* EOs. For  $\alpha$ -pinene, a negative correlation with wing size is only observed in males. In individuals treated with *Abies* EOs, wing size was significantly negatively correlated with the concentration of bornyl acetate and germacrene D in both females and males. Additionally, significant negative correlations were found between wing size and the concentration of  $\alpha$ -humulene and (*E*)-caryophyllene only in females and limonene+ $\beta$ -phellandrene and camphene only in males. However, considering that all obtained Rho values  $< 0.5$  (Table 2), these significant correlations can be classified as weak.

## DISCUSSION

Plant-derived essential oils have been acknowledged as botanical pesticides [42], showing significant potential for integration into organic crop production as alternatives to synthetic chemical pesticides, which may pose risks to both humans and the environment [43]. They have demonstrated insecticidal and repellent activities against mosquitoes [27,44] and insecticidal and deterrent effects against *D. sukukii*, which is an important invasive fruit pest [23,24]. In pursuit of this, frequently examined components found in EOs include thymol, thujone, carvacrol, and limonene, and there are even commercial products based on EOs [42].

At a concentration of 3%, *P. sylvestris* and *P. peuce* EOs induced significantly higher larvicidal activity and delayed developmental time in *D. melanogaster* compared to *P. nigra* and *P. mugo* EOs, respectively [4]. Additionally, the EO of *P. heldreichii* also exhibited



**Table 1.** Deviations of the wing shape of the treatment from the control (Procrustes distances) separately presented for females and males

Treatment		Pinus group						Abies group		
		<i>P. halepensis</i>	<i>P. heldreichii</i>	<i>P. mugo</i>	<i>P. nigra</i>	<i>P. peuce</i>	<i>P. sylvestris</i>	<i>A. alba</i>	<i>A. borisii</i> × <i>regis</i>	<i>A. cephalonica</i>
sex	females	0.0212***	0.0206**	0.0182**	0.0119	0.0238***	0.0207**	0.0189***	0.0139*	0.0114
	males	0.019***	0.0164**	0.0169**	0.0126	0.0147	0.021**	0.0204**	0.0258***	0.0214***

\*P&lt;0.05; \*\*P&lt;0.01; \*\*\*P&lt;0.001

**Table 2.** Correlation of the components from three *Abies* and six *Pinus* EOs with centroid size (describing wings size) separately for females and males

Compounds from the tested EO	Pinus EOs		Abies EOs	
	male	female	male	female
$\alpha$ -pinene	-0.204*	-0.122	-0.073	0.133
$\beta$ -pinene	-0.145	-0.075	-0.073	0.133
$\alpha$ -humulene	-0.317**	-0.410**	-0.240	-0.389**
limonene+ $\beta$ -phellandrene	0.065	0.093	-0.334*	-0.101
camphene	-0.103	-0.014	-0.334*	-0.101
bornyl acetate	-0.122	-0.037	-0.437**	-0.362**
germacrene D	-0.125	-0.120	-0.272*	-0.452**
$\beta$ -caryophyllene	-0.250*	-0.336**	-0.240	-0.389**
thunbergol	-0.221*	-0.270*	n/a	n/a
$\delta$ -3-Carene	-0.094	-0.101	-0.199	-0.166

\* P&lt;0.05; \*\* P&lt;0.01; "n/a" – not applicable due to lack of the compound within the tested EO within genera

larvicidal effect at 3% against *D. melanogaster* [5]. However, changes in wing morphology do not exactly align with the reported larvicidal activity trend. For instance, in the female group, the impact on wing morphology was induced in the following order by EOs: *P. halepensis*, *P. heldreichii*, *P. peuce*, *P. sylvestris*, *P. mugo*, and *P. nigra*, from strongest to weakest, respectively. Interestingly, the EOs of *P. heldreichii*, *P. peuce*, and *P. sylvestris* had qualitatively different effects on wing morphology compared to treatments with *P. halepensis*, *P. nigra*, and *P. mugo*. These differences might be related to the previously reported larvicidal activity of the mentioned EOs against *D. melanogaster* [4,5]. Apart from *P. halepensis*,  $\alpha$ -pinene was one of the first two major compounds in all the studied *Pinus* EOs, and its content was positively correlated to the inhibition of developmental time and larvicidal activity [4,5]. In addition,  $\alpha$ -pinene is a well-known compound with bioactivity against insect pests, including fumigant toxicity against *Sitophilus oryzae* [45], *Plutella xylostella*, and *Megoura japonica* [46], while also affecting the reproduction of the green peach aphid, *Myzus persicae* [47]. However, a high relative

percentage content of pinenes in *Pinus* EOs did not correspond to the same intensity of changes in wing morphology changes. Notably, the EOs of *P. mugo* and *P. nigra* previously showed no toxicity or very weak toxicity against *D. melanogaster*, respectively [4]. Despite a high relative percentage content of pinenes, *P. nigra* EO had an insignificant impact on wing shape in both males and females, unlike the other tested *Pinus* EOs. The effect on female and male flies treated with *P. mugo* EO resulted in significant differences in wing shape (Table 1). Although  $\delta$ -3-carene, which was present in a higher percentage in *P. mugo*, has been reported to have synergistic activity with selected insecticides [48], it evidently may contributed to this observation. In contrast to females, *P. peuce* EO had an insignificant effect on changes in wing shape in males, while *P. sylvestris*, like its impact on females, had a moderate but significant effect on changes of wing shape. As in females, *P. sylvestris* EO had a qualitatively different impact on wing morphology in males compared to *P. halepensis*, *P. heldreichii*, *P. nigra*, and *P. mugo*. This observation may be attributed to the different composition of *P. sylvestris* EO compared to other *Pinus* EOs. In addition, a significant negative correlation between wing size and  $\alpha$ -pinene was found in the groups treated with *Pinus* EOs. Due to its activity against insect pests and vectors, the EO from *P. sylvestris* is already a candidate for potential use as an insecticide [49]. In both males and females, *P. heldreichii* EO exhibited a strong effect on changes in wing morphology, especially in shape. *Pinus heldreichii* EO apart from a high  $\alpha$ -pinene content, contained higher contents of germacrene D and limonene +  $\beta$ -Phellandrene than the EOs of *P. peuce*, *P. sylvestris*, and *P. mugo*. These components might affect only shape because they are not significantly correlated with wing size. A recent study has shown the toxic effects of germacrene D on third-instar larvae of insects [50]. Similarly, limonene has induced morphological and physiological deformities in *D. sukukii* [51], which aligns with the stronger impact of *P. heldreichii* EO

on wing shape in this study. The *P. halepensis* EO had the strongest effect on wing morphology, especially shape, in both sexes, despite its very low content of pinenes and the absence of larvicidal activity in *D. melanogaster* [5]. *P. halepensis* EO was distinct in its high relative percentage content of thunbergol and  $\beta$ -caryophyllene, unlike the other investigated pine essential oils where  $\alpha$ -pinene was consistently one of the two major compounds. While there are no specific toxicological studies on thunbergol for direct comparisons,  $\beta$ -caryophyllene showed potential as an anti-insecticidal agent, impacting the reproduction of *M. persicae* [47] and demonstrating fumigant toxicity against *Sitophilus oryzae* [45]. Furthermore,  $\beta$ -caryophyllene derived from *Cephalotaxus sinensis* showed the highest contact toxicity against *M. japonica* and *P. xylostella* [46]. This suggests that pinenes, known for their larvicidal and toxic effects on pest insects, may not be the primary drivers of wing morphology changes in *D. melanogaster*. Hence, EOs with a high relative percentage content of other components, such as  $\beta$ -caryophyllene and thunbergol or limonene combined with  $\beta$ -phellandrene and germacrene D, could play a more significant role. Consistent with this conclusion, a statistically significant negative correlation between wing size and the concentration of  $\beta$ -caryophyllene and thunbergol was found in both female and male groups. A noteworthy finding is that the variation of minor component, particularly  $\alpha$ -humulene, was also found to be significantly negatively correlated with wing size in both females and males treated with *Pinus* EOs.

The literature data regarding the effects of *Abies* EOs on insects is limited [6,48]. Recent studies revealed that *Abies balsamea* EO exhibited potential as a potent synergist for select insecticides, suggesting its use as an additive for insecticidal formulations [48]. Significant differences in wing morphology were observed in both female and male *D. melanogaster* flies treated with *Abies* EOs. Male flies exhibited more pronounced and significant changes in wing shape changes when treated with *A. cephalonica* and *A.  $\times$  borisii-regis* EOs. *Abies alba* had a milder effect on wing morphology but still showed a significant difference in wing shape compared to the control. This trend aligns with the toxicity of *Abies* EOs in *D. melanogaster* at a 3% concentration, where *A.  $\times$  borisii-regis* and *A. cephalonica* exhibited higher activity than *A. alba* EO [6]. The major volatiles in all three EOs were  $\beta$ -pinene and  $\alpha$ -pinene, with higher

levels in *A. cephalonica* than in the other two species, *A. alba* and *A.  $\times$  borisii-regis*. These components are known for their detrimental effect on insects, which might influence the wing morphology in *D. melanogaster*. For instance,  $\beta$ -pinene has repellent activity and affects neurotransmission-related genes in the red flour beetle *Tribolium castaneum* [52]. EO isolated from *Haplophyllum dauricum*, rich in  $\alpha$ -pinene and  $\beta$ -pinene, displayed contact and fumigant toxicity, as well as repellent activity against two pest insects [53]. In addition,  $\alpha$ -pinene exhibited an array of toxic activities against pest insects [45-47]. *Abies  $\times$  borisii-regis* was the richest in  $\beta$ -caryophyllene compared to the other two *Abies* species. The bioactivity of  $\beta$ -caryophyllene in insects was discussed in the preceding paragraph. Nevertheless, it is noteworthy that *A. cephalonica* and *A.  $\times$  borisii-regis* EOs induced distinct qualitative changes in male wing shape, which could be attributed to significant differences in the relative percentage content of  $\alpha$ -pinene and  $\beta$ -pinene. In addition, values of limonene +  $\beta$ -Phellandrene, bornyl acetate and germacrene D are significantly negatively correlated with wing size in males.

In the female group, *A. alba* EO induced the greatest changes in wing shape, followed by *A.  $\times$  borisii-regis*, while *A. cephalonica* induced minor differences in wing shape compared to the control and the other two species. Camphene and limonene +  $\beta$ -phellandrene were the third and fourth most abundant components in all three *Abies* EOs, with a higher percentage in *A. alba*. As previously mentioned, limonene is considered a promising bioinsecticide that causes morphological and physiological deformities in the well-known pest insect *D. suzukii* larvae [51], which is concurrent with the results of this study. In addition, (R)-(+)-limonene acts as a repellent against *D. melanogaster* and *D. suzukii* [54], while (-)-limonene exhibits strong contact and/or fumigant toxicities against *S. oryzae* and *T. castaneum* [55]. Additionally, *A. alba* essential oil exhibited the highest content of bornyl acetate and camphene compared to the other two *Abies* species, indicating that these components may play a significant role in inducing changes in wing morphology, either in conjunction with or independently from pinenes. Furthermore, a significant negative correlation was found between wing size and these two compounds within *Abies* essential oils. Camphene and bornyl acetate have demonstrated fumigant and contact toxicity against storage pest insects

[56]. Camphene has also shown insecticidal activity [57], including fumigant and contact toxicity against pests like the palm weevil *Rhynchophorus ferrugineus* [58], as well as larvicidal and insecticidal activities. [59]. In females, the induced wing morphological changes were small between *A. alba* and *A. cephalonica* but qualitatively different in terms of width and length compared to *A. × borisii-regis*. This could be due to the significant difference in relative percentage content of the major constituents pinenes, limonene +  $\beta$ -phellandrene, and bornyl acetate. The concentrations of  $\beta$ -caryophyllene,  $\alpha$ -humulene, and germacrene D within *Abies* EOs are significantly negatively correlated with wing size. There are limited studies on  $\alpha$ -humulene insecticidal activity [57] and none for its influence on wing morphology in insects. The observed differences in the response to the same treatment between males and females seen in this study have also been recorded in previous studies [26], and are likely due to physiological differences between the two sexes influencing their responses to the same treatment. Likewise, in treatments involving *Pinus* EOs, components such as limonene +  $\beta$ -phellandrene, camphene, bornyl acetate, and  $\beta$ -caryophyllene may play an important role in inducing wing morphology changes, either in conjunction with or separately from pinenes. Additionally, it is possible that the combined action of minor components, such as  $\alpha$ -humulene and germacrene D, with other components within the *Abies* EOs contributes to the observed changes in *D. melanogaster* wing morphology.

## CONCLUSIONS

The geometric morphometrics method demonstrates its potential for screening minute morphological changes in *D. melanogaster* wings induced by EO treatments. These changes can be correlated with EO treatment toxicity and the specific chemical composition of each tested EO. At the applied concentration of 3%, the *Abies* and *Pinus* EOs did not cause changes in wing morphology that would impair the wing function of flies. However, almost all recorded changes were significant compared to the control group, indicating a potential to cause morphological changes in wing structure. In both male and female flies, it was observed that EOs derived from *P. halepensis* showed the most pronounced influence on wing morphology, while *P. nigra* EO had the lowest impact, particularly

insignificant for wing shape. EOs from *P. heldreichii* and *P. sylvestris* exhibited moderate influence, followed by a moderate to lower impact by the *P. mugo* EO. In males, *P. peuce* EOs had none to lower impact and *vice versa* in females. The *A. × borisii-regis* EO exhibited the strongest influence in the male group, *A. alba* EO in the female group, while the *A. cephalonica* EO exhibited moderate but significant impact on wing shape in males but not in females. Pinenes may not be the primary and necessary factors driving changes in wing morphology in *D. melanogaster*. Other components, such as  $\beta$ -caryophyllene, thunbergol, limonene +  $\beta$ -phellandrene, camphene, bornyl acetate, or minor ones such as  $\alpha$ -humulene and germacrene D, could play a more significant role in inducing changes in *D. melanogaster* wing morphology. Considering that the dietary treatments caused pronounced morphological changes in *D. melanogaster* wings, it could be assumed that EOs from the six *Pinus* and three *Abies* species might also have the potential to cause changes in other body parts of the insects. We believe that this study lays the foundation for further investigations into the potential of selected *Abies* and *Pinus* EOs as biopesticides for insect pest control, with a focus on their capacity to disrupt certain activities that depend on the morphology of the wings, potentially incapacitating or significantly reducing pest insect activities and behaviors. Naturally, these suggestions should be verified in targeted toxicological investigations against pest insects.

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**Data availability:** Data underlying the reported findings have been provided as a raw dataset available here: [https://www.serbiosoc.org.rs/NewUploads/Uploads/Cvetkovic%20et%20al\\_9901\\_Raw%20Dataset.docx](https://www.serbiosoc.org.rs/NewUploads/Uploads/Cvetkovic%20et%20al_9901_Raw%20Dataset.docx)



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## SUPPLEMENTARY MATERIAL

**Supplementary Table S1.** Ten dominant compounds contained in the EOs of *Abies* and *Pinus* species.

Species	Relative percentage (%) content of dominant compounds from the corresponding EOs										Reference
	$\alpha$ -Pinene	Camphene	$\beta$ -Pinene	$\delta$ -3-Carene	Limonene+ $\beta$ -Phellandrene	Bornyl acetate	(E)-Caryophyllene	$\alpha$ -Humulene	Germacrene D	Thunbergol	
<i>P. halepensis</i>	3.4	tr	0.3	1.1	0.4	0.1	<b>32.2</b>	5.9	1.0	<b>29.2</b>	[5]
<i>P. heldreichii</i>	<b>23.8</b>	0.7	<b>8.8</b>	-	<b>34.4</b>	tr	11.1	1.1	<b>17.3</b>	-	
<i>P. mugo</i>	<b>18.0</b>	3.0	4.1	<b>21.3</b>	<b>7.6</b>	5.1	5.0	0.8	5.6	-	[4]
<i>P. nigra</i>	<b>35.4</b>	0.9	<b>10.0</b>	0.1	2.8	0.6	<b>7.4</b>	1.3	<b>28.1</b>	-	
<i>P. peuce</i>	<b>49.3</b>	<b>8.1</b>	<b>13.0</b>	0.1	5.5	<b>7.7</b>	1.8	0.2	6.5	-	
<i>P. sylvestris</i>	<b>41.9</b>	4.7	3.2	3.6	2.0	1.9	6.0	1.2	3.0	-	[6]
<i>A. alba</i>	<b>12.4</b>	<b>10.9</b>	<b>22.3</b>	tr	<b>17.0</b>	<b>8.8</b>	5.7	2.6	0.9	-	
<i>A. x borisii-regis</i>	<b>11.6</b>	7.0	<b>21.1</b>	tr	<b>12.1</b>	5.7	<b>8.0</b>	4.5	2.0	-	
<i>A. cephalonica</i>	<b>29.2</b>	<b>9.8</b>	<b>35.5</b>	tr	<b>12.4</b>	2.4	2.0	0.8	-	-	

tr – trace<0.1%; not detected compounds are marked as (-); compounds that are >7% are in bold