BIOLOGICA NYSSANA
15 (2) December 2024: 97-104
DOI: 10.5281/zenodo.13842094

Evaluation of wing morphology changes in *Drosophila melanogaster* treated with *Pseudotsuga menziesii* (Pinaceae) essential oil

Original Article

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Abstract:
The assessment of the toxicity of essential oils (EOs) from conifer species in pest insects has recently gained importance. Fumigant, deterrent, repellent and larvicidal activities of EOs are the most evaluated in insects while studies on EOs-induced changes in wing morphology are rare. This study deals with evaluation of EOs derived from *Pseudotsuga menziesii* var. *menziesii* (PMEO) influence on wing morphology changes in the insect model *Drosophila melanogaster*. After treatment at the larval stage with 3% of PMEO, and finishing the development, wings were analyzed using geometric morphometrics. The results suggest that PMEO induced significant changes in wing morphology, particularly with moderate effects in wing shape. The correlation of the obtained results with available literature on moderate influence on developmental time and weak larvicidal activity in *Drosophila,* as well as chemical composition of PMEO was discussed. We believe that this finding would significantly contribute to overall knowledge of PMEO bioactivity.

Key words:
Pseudotsuga menziesii, *Drosophila*, essential oils, wing morphology, geometric morphometrics, toxicology

Apstrakt:
Procena promene morfologije krila kod *Drosophila melanogaster* tretirane etarskim uljem *Pseudotsuga menziesii* (Pinaceae)
Ispitivanja toksičnosti etarskih ulja (EU) na insektima štetočinama su od nedavno dobila na značaju. Fumigantna, odvraćajuća, obijajuća i larvicidna aktivnosti EU su najčešće ispitivane aktivnosti, dok su promene morfologije krila uzrokovane EU veoma retko istraživane na insektima. Ovo istraživanje se bavi procenom uticaja EU četinara vrste *Pseudotsuga menziesii* var. *menziesii* (PMEU) na promenu morfologije krila na modelu insekta *Drosophila melanogaster*. Nakon tretmana larvi sa 3% PMEU i završetka razvića, krila su analizirana pomoću metode geometrijske morfometrije. Rezultati ukazuju da PMEU indukuje značajne promene u morfologiji krila izazivajući umereni efekat na oblik krila. Diskutovana je korelisanost dobijenih rezultata sa prethodno publikovanim rezultatima o slaboj larvicidnoj aktivnosti, umerenom uticaju na dužinu vremena razvića i hemijskom sastavu PMEU. Verujemo da će ovaj nalaz značajno doprineti sveukupnom znanju o bioaktivnostima PMEU.

Ključne reči:
Pseudotsuga menziesii, *Drosophila*, etarska ulja, morfologija krila, geometrijska morfometrija, toksikologija

Received: June 30, 2024
Revised: September 10, 2024
Accepted: September 11, 2024

Introduction

Pseudotsuga menziesii (Mirb.) Franco (Douglas fir) is a representative of the Pinaceae family indigenous to western North America with two varieties: coastal Douglas fir (var. *menziesii*) and inland Douglas fir (var. *glauca* (Beissn.) Franco) (Da Ronch et al., 2016). It was introduced to Europe in the 19th century as an ornamental tree, and today it is the most common non-native coniferous species in European forest plantations (Schmid et al., 2014). The analyses of chemical composition of *P. menziesii* var. *menziesii* EO (PMEO) are frequently performed (Buchbauer et al., 1994; Gambliel & Cates, 1995; Jirovetz et al., 2000) while studies on biological activities are rare (Han, 2017; Mitić et al., 2021), especially on insect models. Recently, anti-proliferative activity of PMEO in a human dermal fibroblast model was shown (Han, 2017), fumigant toxicity against store product pest insect *Sitophilus oryzae* and contact toxicity against aphid *Phyllaphis fagi* (Yazdgerdian et al., 2015), toxicity against brine shrimp *A. salina* (Mitić et al., 2021) and antimicrobial properties (Tešević et al., 2009; Mitić et al., 2021).

Plant essential oils (EOs) were recognized as potential eco-friendly pesticides against arthropods (Park & Tak, 2016). Pest insects are surely the most abundant in this group and consequently most of the published results are related to larvicidal (Mitić et al., 2018), fumigant (Negahban et al., 2007), antifeedant (Nikolić et al., 2022), repellent (Nerio et al., 2010) effect of the EOs against pest insects. Surely, targeted toxicological studies against real threats, certain pest insect species, are the most valuable. However, screening of the bioactivities of the EOs on some universal insect models such as *Drosophila melanogaster* Meigen, 1830 (Drosophilidae) is also of great importance. This species has been proved as an effective model insect in different studies that involve EOs (Mihajilov-Krstev et al., 2014; Ickovski et al., 2024).

Studies on alternative, ecofriendly pesticides are very important and urgent due to growing risk to environment and human health regarding the use of chemical pesticides (Rattan, 2010) including developing of the resistance to chemical pesticides (Pavela et al., 2016). The EOs derived from conifers are known as potential biocides against insects (Mitić et al., 2019, 2022; Cvetković et al., 2023, 2024). Considering that in most cases, high concentrations of the EOs are needed for the biocide effect, consequently possible side effects against non-targeted organisms are therefore certain. Thus, the evaluation of the effects of the lower, non-lethal doses of the EOs is crucial in further studies of the EOs. The detrimental effect of the test agents at low doses could also be reflected in the morphology of the body parts in the model organism. For example, the toxicity of the TiO_2 nanoparticles reflected in the wing morphology of the *D. melanogaster* in lower doses through 20 generations (Cvetković et al., 2020) even though it was not lethal for treated flies. Thus, it might be assumed that lower doses of an EOs intended for pest insect controlling also have potential to disrupt morphology of the insects' body parts, including wings what may potentially reduce insects' pest activity as it was suggested recently (Cvetković et al., 2024). Hence, the potential of PMEO to disrupt wing morphology in model insect *D. melanogaster* was evaluated in this study. The geometrical morphometrics is reliable method that enables the visualization and evaluation of potential changes in wing morphology resulting from the application of conifers' EOs (Cvetković et al., 2024).

Materials and Methods

Plant material, EO isolation and characterization

In this study, twigs with needles of *Pseudotsuga menziesii* var. *menziesii* were used. Details about collection, location and deposition of the material as well as isolation and chemical characterization of the EO, were described in Mitić et al. (2021). In brief, hydrodistillation using the Clevenger-type apparatus was performed, and for qualitative and quantitative analyses were conducted using Gas Chromatography-Mass Spectrometry (GC-MS), and Gas Chromatography-Flame Ionization Detector (GC-FID) were used, respectively.

Model insect cultivation – fruit fly Drosophila melanogaster

A laboratory strain (Oregon) of the fruit fly *Drosophila melanogaster* was used. Fruit flies were reared under standard laboratory conditions for this species, which included a temperature of 25 °C, relative humidity of 60%, and a 12-hour light-dark cycle. The flies were fed on a standard cornmeal-based feeding medium that contained agar, sucrose, water, yeast, and fungicide (4 methyl hydroxybenzoate).

Dietary treatment and collection of the fruit flies

The adult fruit flies used in this study were treated and collected as described in a recent study by Mitić et al. (2021). Briefly, three-days-old *D. melanogaster* larvae were treated with 3% PMEO through their diet (cornmeal feeding medium) in triplicate. The control larvae were fed on media without PMEO. A total of sixty larvae were set up in three replicates, with twenty larvae per replicate. After completing their life cycle, the adults were collected, separated by sex, and preserved in 70% ethanol prior to wing dissection. Since PMEO was shown to induce detrimental and significant effects in *D. melanogaster*, mostly at 3%, (Mitić et al., 2021) we considered the 3% concentration as the most relevant for the testing of its influence on wing morphology changes. Also, the 3% was the only concentration used in the study with similar methodology (Cvetković et al., 2024).

Wing preparation and geometrics morphometrics method

Wing preparation and analysis were conducted following the detailed methodology described by Cvetković et al. (2020). In summary, 15 male and 15 female fruit flies were randomly selected from both the treatment and control groups. Using entomological needles and forceps, wings were detached from the flies, washed in distilled water and mounted on microscopic slides. Prepared slides were photographed and processed using a Leica binocular microscope DFC 320 (M2 16 A) (Leica Microsystems, Weltzar, Germany) at 40 × magnification. Wing shape was depicted using 15 landmarks as described by Gidaszewski et al. (2009) and the nomenclature of the wing cells was set according to Morgan et al. (1919). Information on wing shape as Procrustes coordinates (Rohlf & Slice, 1990; Dryden & Mardia, 1998) was obtained by performing the Generalized Procrustes analysis (GPA) in the geomorph software package (Adams et al., 2023) in RStudio (RStudio team, 2020).

Statistics

The Principal component analysis (PCA) was conducted to test the differences in wing morphology caused by the exposure of fruit fly larvae to the EO of *Pseudotsuga menziesii* var. *menziesii*, compared to the wings of the control group. Statistical testing of changes in wing shape was performed using multivariate analysis of variance (MANOVA) in the geomorph package. Afterward, the Linear discriminant analysis (LDA) was conducted using the MASS package (Venables & Ripley, 2002) incorporated in RStudio.

Results

The morphospace displayed by PCA shows that the first principal component (PC1) separates the treatment groups from the control groups, positioning the treatments in the positive part of PC1 and the controls in the negative part (**Fig. 1**). The male control group shows a more distinct separation compared to the female control group. The wings of the treated groups of *Drosophila* tend to be narrower. Along with the second principal component (PC2), there is minimal discrimination. Sexual dimorphism is observable in the control groups, while such differences are less evident in the treatment groups. Cumulatively, the first two principal components (PC1+PC2) account for 51.15% of the total variation in shape and size of the tested wings, indicating a moderate level of discrimination.

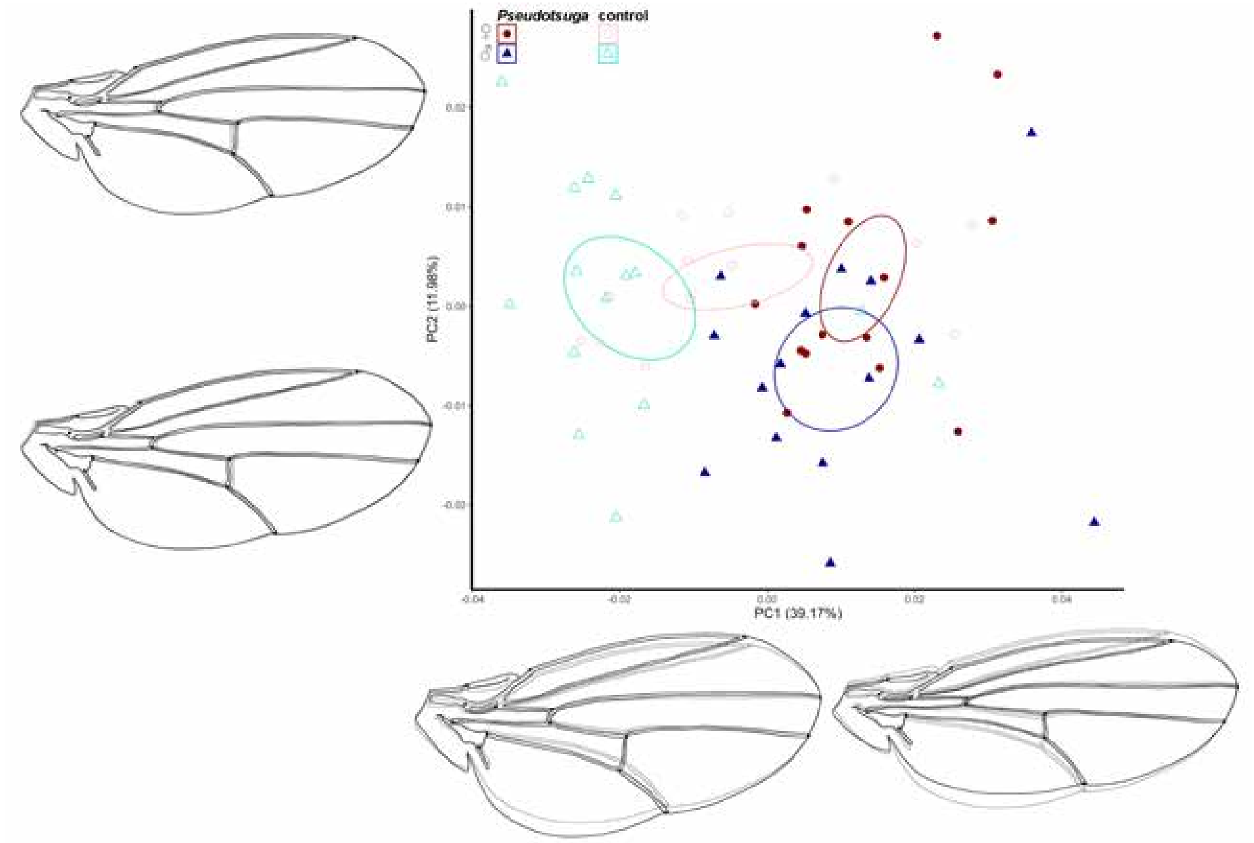

Fig. 1. Results of wing morphology changes visualized in PCA morphospace defined by the first two PC axes. The ellipses represent the 90% mean confident intervals for each group. The changes in wing morphology are depicted by outline graphs; the black line for maximum/minimum values of each axis, whereas the grey line for mean wing shape

Statistical analyses revealed highly significant differences in wing shape, both between treatment groups ($F_{(1\text{-}55)}$=12.433, P=0.001) and between sexes ($F_{(1\text{-}55)}$=4.948, P=0.001). Significant differences in wing shape were also observed in the interaction of treatment and sex ($F_{(1\text{-}55)}$=1.961, P=0.042). Pairwise test of MANOVA reveals significant differences in wing shape between control and treatment group, as well as between sexes (due to sexual dimorphism) (P<0.001).

Linear discriminant analysis (LDA) was used to highlight the real differences in wing morphology that were already indicated by PCA (**Fig. 2**). Cumulatively, LD1+LD2 account for 89.15% of the total variability in wing shape. LD1 effectively separates the control groups from the treatment groups, with the wings of the treatment groups being slightly narrower than those of the controls, while LD2 reveals that sexual dimorphism is preserved in both the control and treatment groups.

Discussion

There are very few studies on the effects of EOs on insect wings. A recent study reported that larvicidal doses of *Croton tetradenius* EO induced changes in the wings of mosquito *Ae. aegypti* (Silva et al., 2023). But this EO was not from conifer species and the chemical composition was significantly different from the tested PMEO, so it is difficult to compare with the obtained results from this study. Very recently one more study on three *Abies* and six *Pinus* species EOs influence on wing morphology in *Drosophila melanogaster* was published (Cvetković et al., 2024). Also, a few studies on the bioactivity of PMEO have been published. For example, anti-proliferative activity in a human dermal fibroblast model was shown (Han, 2017), but the data on PMEO bioactivity in insects are limited. In a *Drosophila* model, PMEO did not show larvicidal activity, but only did cause a slight delay in developmental time, especially at the highest applied concentration of 3% (Mitić et al., 2021). Regarding these findings, PMEO showed certain but weak detrimental effects against insects. In the current study, wing morphology in *Drosophila* was affected to some extent under the treatment of 3% of the PMEO.

Due to sexual dimorphism, significant differences between female and male flies were obvious. This indicates that differences in the wing morphology between females and males were maintained and did

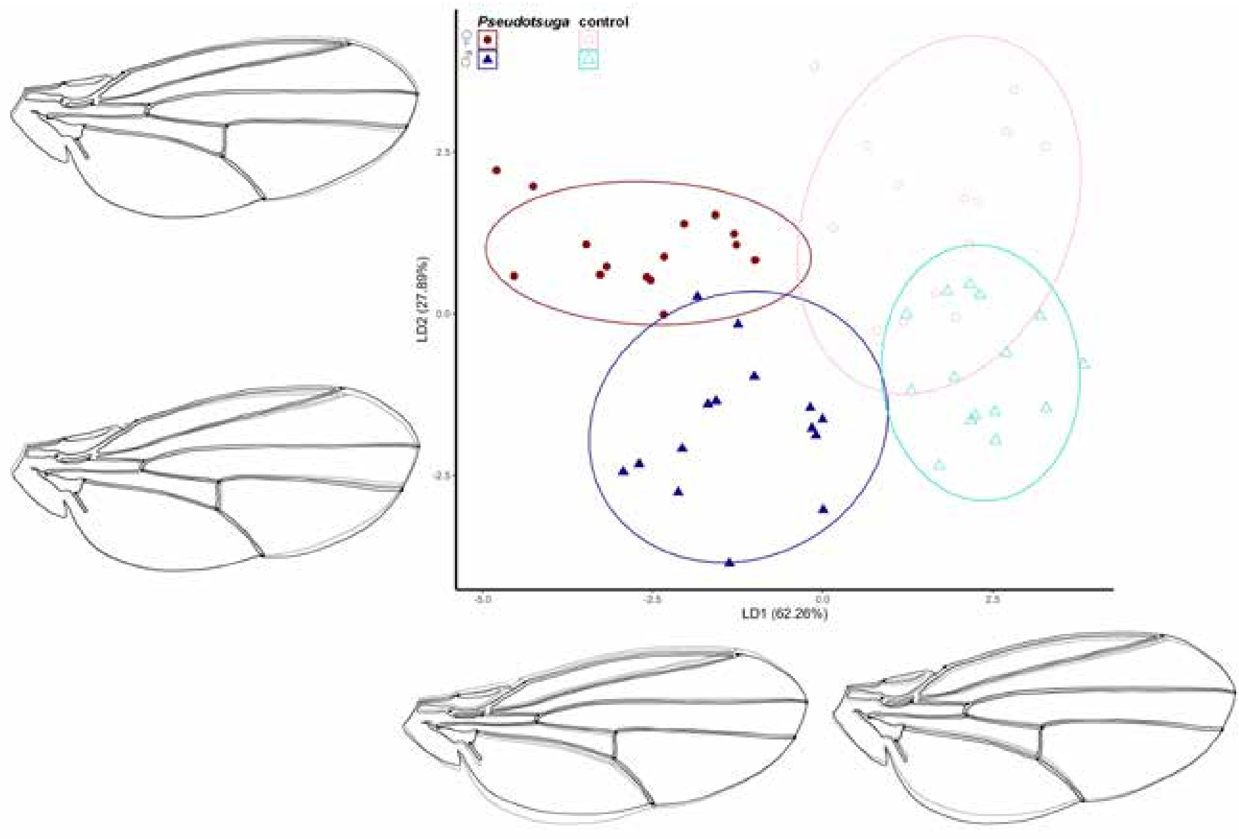

Fig. 2. Wing morphology analysis visualized in LDA morphospace defined by the first two LD axes. The ellipses represent the 90% mean confident intervals for each group. Outline graphs show wing shape changes; the black line for a maximum/minimum value of each axis, the grey line for mean wing shape

not change after PMEO treatment. Additionally, the results indicate that the differences and significance in wing shape between the sexes are greater than the differences between the groups treated with PMEO and the corresponding controls, i.e. separately within males and within female group. Therefore, it was reasonable to observe the effects on wing morphology induced by PMEO treatment separately within male and female groups. The wings of the males treated with PMEO were more affected than those of female when compared with the corresponding control, the untreated flies. Generally, the wings of the treatment groups were slightly narrower than those of the controls.

Despite the observed significant changes in wing morphology, the general structure of the wings in all flies treated with PMEO was maintained and all were able to fly. Additionally, the observed changes in the wing shape suggests that the changes were at moderate level due to overlapping of the results from the compared groups in presented morphospace in PCA and LDA analyses (**Figs. 1 and 2**). This is consistent with mild effect on developmental time and weak, non-significant larvicidal effect of PMEO applied at 3% concentration in *Drosophila* (Mitić et al., 2021). Considering that some *Abies* and *Pinus* EOs with larvicidal effects, also significantly disturb wing morphology (Cvetković et al., 2024) some conclusions could be drawn from the results of the present study. EOs from certain *Abies* and *Pinus* species have demonstrated stronger larvicidal activity in *Drosophila* (Mitić et al., 2018, 2019, 2022) than PMEO (Mitić et al., 2021), and also induced more pronounced and significant wing morphology alterations (Cvetković et al., 2024) than PMEO in the present study. Hence, larvicidal activity and probably other toxic effects in insects (fumigant effect, repellency, antifeedant effect, deterrent effect) of the EOs might be correlated with the proportional level of changes in wing morphology. As it was concluded in the recent study by Cvetković et al. (2024) the results from this study are important because of the potential of EOs to induce morphological changes in insects' wings might be used as agent for controlling the insect pest activities and behavior that mostly depends on wing morphology such as flight in mosquitos or serious pest *Drosophila suzukii,* Asian fruit fly. Certainly, this should be evaluated in specific pest insect species.

The chemical composition of the same batch of PMEO used in the presented study was presented in detail in a study by Mitić et al. (2021). The dominant components in this EO are sabinene (17.9%), β-pinene (15.2%) and α-terpinolene (22.7%). These components exhibited various biological activities, such as shown in recently published studies (da Silva Rivas et al., 2012; Matias et al., 2016; Liu et al., 2019), but very few studies have focused on their effects in insects. Some activities of these components, that are constituents of the PMEO, in insects have been recently published. In the red flour beetle *Tribolium castaneum*, repellent activity of β-pinene was demonstrated (Pajaro-Castro et al., 2017). In the rice weevils *Sitophilus oryzae* (L.), EO of the *Bacopa caroliniana* with the most abundant component α-terpinolene exhibited insecticidal activity (Liu et al., 2019). Sabinene was also shown as toxic for this species (Sharma & Tiwari, 2021). This study did not test the influence of the major components of PMEO, what might be potential limitation of the present study. Therefore, while PMEO had a moderate effect in the wings of *Drosophila*, its separate components such as sabinene, β-pinene and α-terpinolene could have more significant influence on wing morphology. Understandably, this assumption should be verified in future studies. Afterall, we believe that the results of this study are of great importance, as they provide insights into the relationship between the toxicity of the PMEO and its influence on the wing shape in *D. melanogaster* as potential ecofriendly agent against flying pest insects.

Conclusion

The results from the presented study suggest that the wing morphology of fruit flies treated with PMEO was significantly changed, particularly wing shape. However, the observed changes in the wing shape were moderate, but did not impair the flies' ability to fly. The level of changes was proportional to previously published larvicidal effects (Mitić et al., 2021), which were weak and moderately delayed the developmental time. In our opinion and based on the observed effects on wing morphology of *D. melanogaster*, PMEO could be candidate for the potential treatment against pest insects or arthropods. Especially its major components such as sabinene, β-pinene and α-terpinolene could have more significant impact on wing morphology that can impact pest activities of the treated flying insects. Studies on the effect of EOs on insect's body morphology, including wings, are very rare. Therefore, we believe that this finding significantly contributes to the overall knowledge of the conifer EOs effect in insect models. Further research is warranted to explore these correlations in depth and to clarify the mechanisms by which EOs affect both the structural and functional aspects of insect wing morphology.

Acknowledgements. This work was funded by the Ministry of Science, Technological Development and Innovations of the Republic of Serbia [Grant number: 451-03-65/2024-03/200124 and 451-03-66/2024-03/200124].

References

Adams, D., Collyer, M., Kaliontzopoulou, A., & Baken, E. (2023). Geomorph: software for geometric morphometric analyses. R package version 4.0.5. https://cran.r-project.org/package=geomorph. Accessed 28 August 2023

Buchbauer, G., Jirovetz, L., Wasicky, M., & Nikiforovt, A. (1994). Comparative investigation of Douglas fir headspace samples, essential oils, and extracts (needles and twigs) using GC-FID and GC-FT-IR-MS. *Journal of Agricultural and Food Chemistry*, *42*, 2852–2854. https://doi.org/10.1021/jf00048a037

Cvetković, V.J., Jovanović, B., Lazarević, M., Jovanović, N., Savić-Zdravković, D., Mitrović, T., & Žikić, V. (2020). Changes in the wing shape and size in *Drosophila melanogaster* treated with food grade titanium dioxide nanoparticles (E171)–A multigenerational study. *Chemosphere*, *261*, 127787. https://doi.org/10.1016/j.chemosphere.2020.127787

Cvetković, V.J., Lazarević, M., Mitić, Z.S., Zlatković, B., Stojković Piperac, M., Jevtović, S., Stojanović, G., & Žikić, V. (2024). Dietary exposure to essential oils of selected *Pinus* and *Abies* species leads to morphological changes in *Drosophila melanogaster* wings. *Archives of Biological Sciences*. https://doi.org/10.2298/ABS240527019C

Cvetković, V.J., Mitić, Z.S., Stojanović-Radić, Z., Matić, S.L., Nikolić, B.M., Rakonjac, L., Ickovski, J., & Stojanović, G. (2023). Biological Activities of *Chamaecyparis lawsoniana* (A. Murray bis) Parl. and *Thuja plicata* Donn ex D. Don Essential Oils: Toxicity, Genotoxicity, Antigenotoxicity, and Antimicrobial Activity. *Forests*, *15*(1), 69. https://doi.org/10.3390/f15010069

Da Ronch, F., Caudullo, G., & de Rigo, D. (2016). In: San-Miguel-Ayanz, J., de Rigo, D., Caudullo, G., Houston Durrant, T., Mauri A. (Eds.), European Atlas of Forest Tree Species (pp. e01a4f5+). Luxembourg: Publ. Off. EU.

da Silva Rivas, A.C., Lopes, P.M., de Azevedo Barros, M.M., Costa Machado, D.C., Alviano, C.S., & Alviano, D.S. (2012). Biological activities of α-pinene and β-pinene enantiomers. *Molecules*, *17*(6), 6305-6316. https://doi.org/10.3390/molecules17066305

Dryden, I.L. & Mardia, K.V. (1998). *Statistical shape analysis: Wiley series in probability and statistics.* Chichester: John Wiley & Sons

Gambliel, H.A. & Cates RG. (1995). Terpene changes due to maturation and canopy level in Douglas-fir (*Pseudotsuga menziesii*) flush needle oil. *Biochemical systematics and ecology*, *23*(5), 469-476. https://doi.org/10.1016/0305-1978(95)00033-Q

Gidaszewski, N.A., Baylac, M., & Klingenberg, C.P. (2009). Evolution of sexual dimorphism of wing shape in the *Drosophila melanogaster* subgroup. *BMC Evolutionary Biology*, *9*, 110. https://doi.org/10.1186/1471-2148-9-110

Han, X. (2017). *In vitro* biological activities of Douglas fir essential oil in a human skin disease model. *Cogent Biology*, *3*(1), 1336886. https://doi.org/10.1080/23312025.2017.1336886

Ickovski, J.D., Cvetković, V.J., Jovanović, N.M., Mitrović, T.L., & Stojanović, G.S. (2024). Serbian *Artemisia* species–chemical composition, acute toxicity and larvicidal activity of the essential oils. *Natural Product Research*, 1-12. https://doi.org/10.1080/14786419.2024.2334312

Jirovetz, L., Puschmann, C., Stojanova, A., Metodiev, S., & Buchbauer, G. (2000). Analysis of the essential oil volatiles of Douglas fir (*Pseudotsuga menziesii*) from Bulgaria. *Flavour and fragrance journal*, *15*(6), 434-437. https://doi.org/10.1002/1099-1026(200011/12)15:6<434::AID-FFJ935>3.0.CO;2-0

Liu, T.T., Chao, L.K., Hong, K.S., Huang, Y.J., & Yang, T.S. (2019). Composition and insecticidal activity of essential oil of *Bacopa caroliniana* and interactive effects of individual compounds on the activity. *Insects*, *11*(1), 23. https://doi.org/10.3390/insects11010023

Matias, E.F., Alves, E.F., Silva, M.K., Carvalho, V.R., Figueredo, F.G., Ferreira, J.V., Coutinho, H.D., Silva, J.M., Ribeiro-Filho, J., & Costa, J.G. (2016). Seasonal variation, chemical composition and biological activity of the essential oil of *Cordia verbenacea* DC (Boraginaceae) and the sabinene. *Industrial Crops and Products*, *87*, 45-53. https://doi.org/10.1016/j.indcrop.2016.04.028

Mihajilov-Krstev, T., Jovanović, B., Jović, J., Ilić, B., Miladinović, D., Matejić, J., Rajković, J., Đorđević, L., Cvetković, V., & Zlatković, B. (2014). Antimicrobial, antioxidative, and insect repellent effects of *Artemisia absinthium* essential oil. *Planta medica*, *80*(18), 1698-1705. https://doi.org/10.1055/s-0034-1383182

Mitić, Z.S., Jovanović, B., Jovanović, S.Č., Mihajilov-Krstev, T., Stojanović-Radić, Z.Z., Cvetković, V.J., Mitrović, T.Lj., Marin, P.D., Zlatković, B.K., & Stojanović, G.S. (2018). Comparative study of the essential oils of four *Pinus* species: Chemical composition, antimicrobial and insect larvicidal activity. *Industrial Crops and Products*, *111*, 55-62. https://doi.org/10.1016/j.indcrop.2017.10.004

Mitić, Z.S., Jovanović, B., Jovanović, S.Č., Stojanović-Radić, Z.Z., Mihajilov-Krstev, T., Jovanović, N.M., Nikolić, B.M., Marin, P.D., Zlatković, B.K., & Stojanović, G.S. (2019). Essential oils of *Pinus halepensis* and *P. heldreichii*: Chemical composition, antimicrobial and insect larvicidal activity. *Industrial crops and products*, *140*, 111702. https://doi.org/10.1016/j.indcrop.2019.111702

Mitić, Z.S., Stojanović-Radić, Z., Cvetković, V.J., Jovanović, S.Č., Dimitrijević, M., Ickovski, J.D., Jovanović, N., Mihajilov-Krstev, T., & Stojanović, G.S. (2021). *Pseudotsuga menziesii* (Pinaceae): Volatile profiles, antimicrobial activity and toxicological evaluation of its essential oil. *Chemistry & Biodiversity*, *18*(9), e2100424. https://doi.org/10.1002/cbdv.202100424

Mitić, Z.S., Stojanović-Radić, Z.Z., Jovanović, S.Č., Cvetković, V.J., Nikolić, J.S., Ickovski, J.D., Mitrović, T.L., Nikolić, B.M., Zlatković, B.K., & Stojanović, G.S. (2022). Essential oils of three Balkan *Abies* species: Chemical profiles, antimicrobial activity and toxicity toward *Artemia salina* and *Drosophila melanogaster*. *Chemistry & Biodiversity*, *19*(6), e202200235. https://doi.org/10.1002/cbdv.202200235

Morgan, T.H., Bridges, C.B., & Sturtevant, A.H. (1919). *Contributions to the Genetics of Drosophila Melanogaster*. (No. 278). Carnegie Institution of Washington.

Negahban, M., Moharramipour, S., & Sefidkon, F. (2007). Fumigant toxicity of essential oil from *Artemisia sieberi* Besser against three stored-product insects. *Journal of stored products research*, *43*(2), 123-128. https://doi.org/10.1016/j.jspr.2006.02.002

Nerio, L.S., Olivero-Verbel, J., & Stashenko, E. (2010). Repellent activity of essential oils: a review. *Bioresource technology*, *101*(1), 372-378. https://doi.org/10.1016/j.biortech.2009.07.048

Nikolić, B.M., Milanović, S.D., Milenković, I.L., Todosijević, M.M., Đorđević, I.Ž., Brkić, M.Z., Mitić, Z.S., Marin, P.D., & Tešević, V.V. (2022). Bioactivity of *Chamaecyparis lawsoniana* (A. Murray) Parl. and *Thuja plicata* Donn ex D. Don essential oils on *Lymantria dispar* (Linnaeus, 1758) (Lepidoptera: Erebidae) larvae and *Phytophthora* de Bary 1876 root pathogens. *Industrial Crops and Products*, *178*, 114550. https://doi.org/10.1016/j.indcrop.2022.114550

Pajaro-Castro, N., Caballero-Gallardo, K., & Olivero-Verbel, J. (2017). Neurotoxic effects of linalool and *β*-pinene on *Tribolium castaneum* Herbst. *Molecules*, *22*(12), 2052. https://doi.org/10.3390/molecules22122052

Park, Y.L. & Tak, J.H. (2016). Essential oils for arthropod pest management in agricultural production systems. In: Preedy, V.R., (Ed.) *Essential oils in food preservation, flavor and safety*. (Chapter 6, pp. 61-70). Academic Press. https://doi.org/10.1016/B978-0-12-416641-7.00006-7.

Pavela, R. & Benelli, G. (2016). Essential oils as ecofriendly biopesticides? Challenges and constraints. *Trends in Plant Sciences*, *21*, 1000-1007. http://dx.doi.org/10.1016/j.tplants.2016.10.005

Rattan, R.S. (2010). Mechanism of action of insecticidal secondary metabolites of plant origin. *Crop Protection*, *29*, 913–920. https://doi.org/10.1016/j.cropro.2010.05.008

Rohlf, F.J. & Slice, D. (1990). Extensions of the Procrustes method for the optimal superimposition of landmarks. *Systematic Biology*, *39*(1), 40-59. https://doi.org/10.2307/2992207

RStudio team. (2020). *Integrated Development for R*. Available online: http://www.rstudio.com/ (accessed on 20 April 2024).

Schmid, M., Pautasso, M., & Holdenrieder, O., (2014). Ecological consequences of Douglas fir (*Pseudotsuga menziesii*) cultivation in Europe'. *European Journal of Forest Research*, *133*(1), 13-29. https://doi.org/10.1007/s10342-013-0745-7

Sharma, J.H. & Tiwari, S.N. (2021). Fumigant toxicity of alpha-pinene, beta-pinene, eucalyptol, linalool and sabinene against rice weevil, *Sitophilus oryzae* (L.). *Pantnagar Journal of Research*, *19*(1), 50-55.

Silva, P.B., Santos, R.B., da Cruz, R.C., da Silva, D.C., & da Silva, P.S. (2023). Effect of *Croton tetradenius* essential oil on larval viability, pupal viability, and wing geometric morphometrics of *Aedes aegypti*. *Biocatalysis and Agricultural Biotechnology*, *51*, 102743. https://doi.org/10.1016/j.bcab.2023.102743

Tešević, V., Milosavljević, S., Vajs, V., Đorđević, I., Soković, M., Lavadinović, V., & Novaković, M. (2009). Chemical composition and antifungal activity of the essential oil of Douglas fir (*Pseudosuga menziesii* Mirb. Franco) from Serbia. *Journal of the Serbian Chemical Society*, *74*(10), 1035-1040.

Venables, W.N. & Ripley, B.D. (2002). *Modern Applied Statistics with S* (4th ed.). New York: Springer, ISBN 0-387-95457-0. https://www.stats.ox.ac.uk/pub/MASS4/

Yazdgerdian, A.R., Akhtar, Y., & Isman, M.B. (2015). Insecticidal effects of essential oils against woolly beech aphid, *Phyllaphis fagi* (Hemiptera: Aphididae) and rice weevil, *Sitophilus oryzae* (Coleoptera: Curculionidae). *Journal of entomology and zoology studies*, *3*(3), 265-271.