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Biopesticide formulation based on essential oils in *Drosophila suzukii* management as a future of pest control

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Abstract: *Drosophila suzukii* poses a significant threat to berry fruits with its uncontrolled spread. Essential oils (EOs) have emerged as potential bioinsecticides due to their natural origin, mode of action, and biodegradability. Although EOs show potential for use in agriculture due to ecotoxicologically favourable characteristics, additional research is required to enhance their effectiveness, stability, and application for practical implementation in pest management. The primary objective of this research was the development of a bioinsecticide formulation based on a combination of three EOs – *Pelargonium graveolens*, *Anethum graveolens*, and *Pinus sylvestris* followed by the assessment of formulated bioinsecticide physicochemical properties. Using a two-choice bioassay, this study aimed to evaluate the effects of formulated bioinsecticides on *D. suzukii*, regarding their insecticidal properties through oviposition deterrence. The developed formulation exhibited favourable physicochemical properties and demonstrated a decrease in the number of larvae in fruits. Bioinsecticides present an environmentally friendly approach to pest control. However, further research and development are imperative to fully exploit their potential for effective crop protection in the field, followed by comprehensive research to evaluate the potential side effects on natural enemies, ensuring that their implementation doesn't harm beneficial organisms and maintain ecological equilibrium.

Keywords: bioinsecticide; eco-friendly; oviposition; biocontrol; sustainable

Drosophila suzukii Matsumura [spotted wing drosophila (SWD)] was initially documented by Matsumura in 1931. This pest belongs to the order Diptera, family Drosophilidae Kanzawa (1939). The majority of species within this genus are not considered pests as they typically deposit eggs in rotten or damaged fruits. However, SWD lays eggs in undamaged, healthy fruits Crava et al. (2019). This species has a wide distribution across moderate-climate regions Rota-Stabelli et al. (2013).

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The first report of *D. suzukii* in Serbia was documented in 2014 by Toševski et al. (2014). The rapid and uncontrolled spread was contributed by neglecting the point of entry through the global trade of fresh fruit, followed by the cryptic nature of eggs and larvae inside the fruit, high reproductive rate, short generation time, and lack of specific natural enemies (Rota-Stabelli et al. 2013; Cini et al. 2014). *Drosophila suzukii* females have a large serrated ovipositor to lay eggs inside the fruit.

Females primarily attack soft-skinned fruits, such as berries, cherries, grapes, and other similar fruits. When infested by *D. suzukii*, the fruit will display visible marks, such as small holes in its skin. These marks and larval feeding inside the fruit are followed by a softening of the fruit, which outcomes in spoilage, deterioration, and a decrease in marketable quality Walsh et al. (2011). The focus of protection against *D. suzukii* primarily revolves around the use of synthetic insecticides, which poses a specific challenge due to concerns regarding pesticide residues. Currently, in Europe, insecticides belonging to the chemical classes of pyrethroids, organophosphates, spinosyns, and neonicotinoids are predominantly used for managing SWD infestations Shaw et al. (2019). Repeated exposure to synthetic pesticides in combination with the specific biology of the species can lead to the development of genetic traits that make them resistant, which reduces the overall effectiveness. Natural or organic pesticides serve as alternatives to synthetic pesticides, aiming to diminish dependence on artificial chemicals and foster more sustainable and environmentally friendly pest control methods. Botanical insecticides are natural chemicals extracted or obtained from plants or plants. They can affect insects by disrupting the nervous system, interfering with their feeding or reproductive processes, or causing paralysis or death (Isman 2004; Šunjka & Mechora 2022). Plant EOs are among the highly promising plant metabolites with valuable insecticidal activity. Composed of a complex mixture of natural compounds, EOs offer potential as effective botanical insecticides Turek and Stintzing (2013). Despite the numerous benefits, it is important to highlight certain limitations of the EOs application. They exhibit a limited range of effectiveness, and their action may be slower than synthetic insecticides. The efficacy relies on environmental conditions like photodegradation and evaporation (Pavela 2014; Isman 2020). A significant drawback is their tendency to degrade shortly after application. While this rapid degradation can be advantageous in terms of environmental impact compared to synthetic pesticides, it poses a considerable challenge during the formulation of these products. One way to address this issue is through biotechnology, which allows controlled release of the active ingredient. This application enhances the efficiency and stability of the bioinsecticide, particularly in field conditions Šunjka and Mechora (2022). The inactive ingredients used in formulating botanical insecticides are generally not considered to have significant toxicological effects. Therefore, when assessing the impact of a formulated bioinsecticide, the focus is primarily on the active substance.

The aim of this study was to formulate a bioinsecticide containing three EOs and assess the physicochemical properties of the formulated bioinsecticides. This research aimed to evaluate the formulated bioinsecticide's effects on *D. suzukii* oviposition deterrence by two-choice bioassay.

MATERIAL AND METHODS

Insect colony. A laboratory colony of *D. suzukii* was initiated by using the flies that emerged from blackberries (*Rubus fruticosus* L.) collected in Vojvodina Province, Serbia. The colony has been maintained in a climatic chamber under an air temperature of 23 °C ± 1 °C, relative humidity (RH) of 65% ± 5%, and photoperiod of 12:12 h (light: dark). Adults were transferred to a new jar with fresh medium and water supply weekly Bošković et al. (2023) to rear the colony. An artificial diet was provided and placed in a Petri dish with *ad libitum* access Schlesener et al. (2017). The genetics of the colony were enriched once a year using wild-caught flies from infested fruits to prevent the likelihood of multiple breeding. Adults aged between 4 and 7 days were used for the bioassay.

Formulation of bioinsecticide. The composition of the formulation includes EOs as an active ingredient, a solvent and an emulsifier as an inactive ingredients. Before assessing the impact of the EOs mixture in formulation, as a part of the control treatment to evaluate the potential impact of the solvent (rapeseed, sunflower, or MCT oil), berries were treated with a formulation consisting solely of these oils individually along with an emulsifier. A blend of three EOs was combined: geranium

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Table 1. Ingredients and their weight for formulation of bioinsecticide

Ingredients	MIX 1 (g)	MIX 2 (g)	MIX 3 (g)
Geranium	1.4523	1.4728	1.4777
Dill	1.5964	1.5783	1.5852
Scots pine	1.5637	1.5560	1.5707
Feronol	32.3357	32.2952	32.9889
Rapeseed oil	13.4702	–	–
Sunflower oil	–	13.0656	–
MCT oil	–	–	14.1905

MCT – medium chain triglycerides

(*Pelargonium graveolens* L'Hér.), dill (*Anethum graveolens* L.), and Scots pine (*Pinus sylvestris* L.), sourced from Avena Lab – Farmadria© (Vršac, Serbia). Three vegetable oils were used as the solvent: rapeseed oil (Granum Food; Suncokret doo, Hajdukovo, Serbia), sunflower oil (Dijamant AD, Zrenjanin, Serbia), and MCT oil – medium chain triglycerides (OstroVit, Zambrow, Poland). The geronol FF/6 (Solvay, Milan, Italia) was used as the emulsifier. The proportions of these components were maintained at a ratio of 5:15:30. Weight (g) of ingredients is given in Table 1. The EOs mixture was stirred at 800 rpm using magnetic stirring for 15 min. Afterward, solvent was added to the EOs and stirred for 20 min at 1 200 rpm using Ulturatux. Then, the emulsifier was added and stirred again for 20 min at a higher speed.

Physicochemical properties. The physicochemical properties of the spray liquid were evaluated according to FAO specifications FAO (2022). The following physical properties were evaluated: *(i)* appearance; *(ii)* pH range (MT 75.3); *(iii)* foam persistence (MT 47.3); *(iv)* emulsion stability and re-emulsification (MT 36.3); *(v)* specific weight-γ (MT 3.2) (CIPAC K 2003; CIPAC F 2007). The particle size distribution of the formulated bioinsecticide was determined by the Mastersizer 2 000 laser diffraction particle size analyser (Malvern Instruments, Malvern, UK). Particle size distributions (PSD) were characterised using the volume mean diameter *d* (4.3 μm) and parameters such as *d* (0.1 μm), *d* (0.5 μm), and *d* (0.9 μm), which denote the sizes where 10%, 50%, or 90% of the total particle volume comprises particles smaller than the specified size, respectively. The Mastersizer 2 000 software was used to quantify the results as the volume-based particle size distribution Lončarević et al. (2021).

Two-choice bioassay. This research was conducted at the Department of Plant and Environmental Protection, Faculty of Agriculture, University of Novi Sad, Serbia. The two-choice test represents a modification of the multiple-choice test Bošković et al. (2023). The test was conducted in cages (35 cm × 35 cm × 40 cm) lined with transparent Plexiglas on the sides, fine mash on the top, and plywood on the bottom.

Organic blueberries were used for this experiment**.** Berries were placed in a smaller Petri dish (55 mm × 15), while a moistened paper wipe was placed in a larger one (90 mm × 15 mm). The experiment was conducted in four replicates. The spray liquid was made by dissolving 1 mL of formulated bioinsecticide in 99 mL of water until a water-milky appearance of the working solution was obtained. On the first day, all blueberries needed for the experiment were submerged in a bioinsecticide solution for two seconds and left to dry completely. Untreated berries were used as a control. After completely drying, one blueberry was placed separately in a small Petri dish. The remaining berries, which would be used in the following days, were placed in a plastic container, covered with fine mesh, and kept in the same condition as the experimental cages. The aim was to treat all berries only once (on the first day) to assess the post-treatment effect and degradation of the formulated product based on the mixture of EOs after 24, 48, 72, and 96 h.

After berries were placed in the cages, 10 flies per cage (5 males and 5 females) were aspirated and introduced inside the cage. Cages were kept in a climate chamber under controlled conditions (23 °C ± 1 °C, 65% ± 5% RH; 12 h:12 h light: dark). After 24 h, flies were removed from the cages. Each berry was removed from the Petri dish and placed in a separate small plastic cup, which was then covered with mesh. Then, the berries were placed in the same chamber under the same conditions to enable larvae in the berries to hatch.

Previously treated berries, which were kept in the plastic container, were exposed in the same way to demonstrate the efficacy of the EOs in the duration of the four above-described days. When new berries were added to the cages, newly emerged (non-used) flies were introduced in cages.

Counting larvae started after the fifth day of setting the berries in the cage because older larvae are easier to count. Before larval counting, a saline

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Table 2. Physicochemical properties of bioinsecticide formulation

Evaluated parameters	MIX 1	MIX 2	MIX 3
Appearance	thick orange liquid	thick light orange liquid	thick orange liquid
PH	7.36	7.43	7.41
Foam persistence	10 s – 42 mL 1 min – 38 mL 3 min – 34 mL 12 min – 26 mL	10 s – 56 mL 1 min – 45 mL 3 min – 42 mL 12 min – 38 mL	10 s – 46 mL 1 min – 42 mL 3 min – 35 mL 12 min – 30 mL
Emulsion stability and re-emulsification (RE)	½ h – / 2 h – / 24 h – 1 mL RE ½ h – 1 mL	½ h – / 2 h – 1 mL 24 h – 1 mL RE ½ h – 1 mL	½ h – / 2 h – / 24 h – 1 mL RE ½ h – 1 mL
Specific weight	0.9611	0.9984	0.9867

water solution was poured into cups with berries to force larvae to leave the fruits. Data analyses were conducted using Statistica software (version 14.0.0.15). The analysis of collected data initially employed the method of descriptive statistics. Subsequently, to test the difference between the control and treatment, the two-sample *t*-test was applied ($P < 0.05$) Winter (2019).

RESULTS

The physicochemical properties are shown in Table 2. All mixtures were thick orange liquids. The hue ranged in the orange spectrum, with MIX 2 featuring a lighter shade. In the concentration of 1%, all mixes had a pH level of around 7 at 21 C, in distilled water (pH 7.39). The amount of foam (assessed at the highest recommended rate) after 10 s was 42 mL for MIX 1, 58 mL for MIX 2, and 46 mL for MIX 3. The foam dropped after one minute, per the FAO specifications (less than 60 mL after 1 min). Regarding the emulsion stability, all mixtures were stable after half an hour. After two hours, a ring of cream (1 mL) was observed for MIX 2, which is in accordance with the CIPAC method (less than 5 mL). After 24 h, all formulations had a ring of cream on the surface (1 mL). The same ring appeared after re-emulsification.

In the formulated bioinsecticide, 10% of the particles have a diameter smaller than d (0.1 µm), 50% of the particles have a diameter smaller/larger than *d* (0.5 µm), 90% of the particles have a diameter smaller than the *d* (0.9 µm), and *d* (4.3 µm) represents the mean diameter of all the diameters in the sample (Figure 1).

The formulations based on rapeseed oil + emulsifier (RE) and sunflower oil + emulsifier (SE) showed no effect on the oviposition over the four days (Table 3) since the average number of larvae on berries treated with RE formulation and on untreated berries were very similar during all four days. Similar results were obtained with SE for-

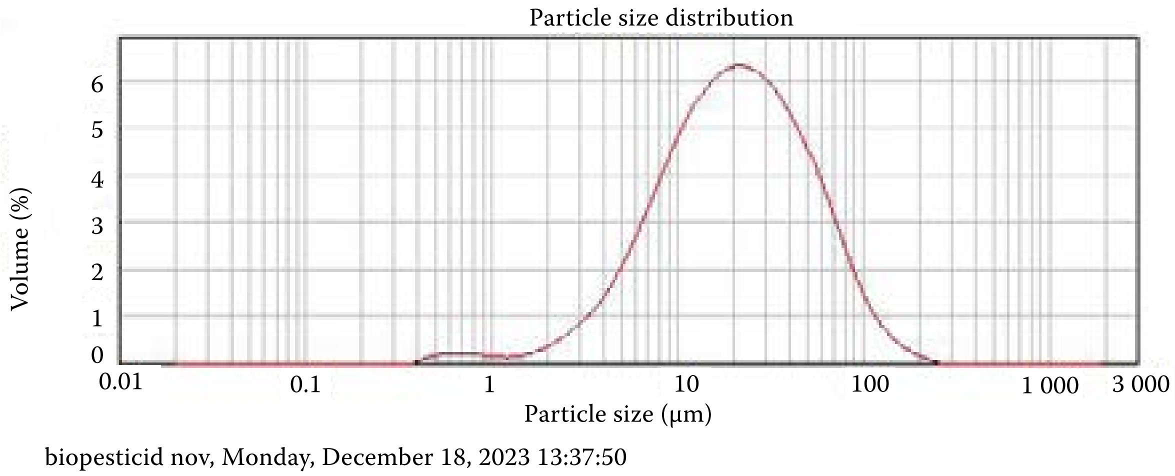

Figure 1. The particle size distribution of formulated bioinsecticide

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Table 3. Mean number of hatched larvae in two-choice bioassay

MIX1	SUM C	Mean C	SD	SUM T	Mean T	SD	*t*-test	df	*P*-value
1st day	33	8.25	2.63	8	2.00	0.82	15.1168	6	0.0000
2nd day	35	8.75	1.71	11	2.75	0.96			
3rd day	29	7.25	1.71	7	1.75	0.96			
4th day	31	7.75	1.71	8	2.00	0.00			
RE	SUM C	Mean C	SD	SUM T	Mean T	SD	*t*-test	df	*p*-value
1st day	15	3.75	0.96	17	4.25	2.75	0.1076	6	0.9178
2nd day	19	4.75	0.50	23	5.75	1.71			
3rd day	22	5.50	1.29	18	4.50	0.58			
4th day	16	4.00	0.82	15	3.75	0.96			
SE	SUM C	Mean C	SD	SUM T	Mean T	SD	*t*-test	df	*p*-value
1st day	15	3.75	1.26	16	4.00	0.82	0.1295	6	0.9012
2nd day	21	5.25	1.71	18	4.50	1.73			
3rd day	22	5.50	1.29	20	5.00	0.82			
4th day	18	4.50	2.38	21	5.25	1.71			
MCTE	SUM C	Mean C	SD	SUM T	Mean T	SD	*t*-test	df	*p*-value
1st day	27	6.75	2.75	19	4.75	0.96	2.0494	6	0.0865
2nd day	19	4.75	1.71	12	3.00	1.41			
3rd day	24	6.00	2.45	20	5.00	0.82			
4th day	22	5.50	1.29	20	5.00	0.82			

SD – standard deviation; df – degrees of freedom; C – control; T – treatment

mulation, with a similar number of hatched larvae between treated berries with SE and control. However, the formulation with MCT oil + emulsifier (MCTE) resulted in fewer larvae than control. After the first day, the number of larvae on treated berries was significantly lower than the control. The same effect was observed after the second day. With time, the difference between the number of larvae in treatment and control decreased. Considering all the above, the formulation based on rapeseed as a solvent (MIX 1) was chosen to determine the effect of EOs in the two-choice bioassay. Also, a formulation based on rapeseed showed slightly better physicochemical properties regarding foam and emulsion stability. The berries treated with bioinsecticide (MIX 1) enabled fewer larvae to develop compared to the untreated berries after all four days. Using the t-test, it was demonstrated that statistically significant differences were observed between control and treatment in MIX 1 ($P < 0.05$). No significant differences were observed between control and treatment in RE ($P = 0.92$) and control and SE ($P = 0.90$). The difference between MCTE and control treatment is also considered not significant ($P = 0.09$).

DISCUSSION

Geranium, dill, and scots pine EOs have been documented to have insecticidal effects on oviposition deterrence, mortality and repellency Bošković et al. (2023). Comparing their chemical composition, which is different in the percentage of dominant components, and the mode of action that typically results from the synergistic effects, a decision was made to formulate a blend of these compounds and evaluate their collective impact on *D. suzukii*. Using a mixture of different EOs can often be more effective than using EOs individually. When EOs are combined, they can have a synergistic effect, enhancing their overall efficacy because different EOs may target different aspects of an insect's biology or behaviour. Combining EOs can help broaden the spectrum of pests that can be targeted. Insects may have varying sensitivities or resistance to specific EOs, so using a mixture can increase the likelihood of affecting them.

Based on the results obtained, MCT oil had an effect on oviposition in berries compared to the control. This effect could be potentially considered as an additional repellent effect in the formula-

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tion. However, it is important to note that the focus of the study was specifically on the deterrence effect related to the combination of EOs.

A wealth of information is available on various types of pesticide formulations that utilise EOs, designed to enhance the effectiveness of pesticides. An emulsion is a mixture of two immiscible liquids, typically oil and water, stabilised with an emulsifying agent. Emulsions tend to have larger droplet sizes Sneha and Kumar (2022), which diminishes as the oil-to-surfactant ratio decreases Campolo et al. (2020). In the available literature, TWEEN 80 or 20, Span 20 or 80 are commonly used as emulsifiers for bioinsecticide formulations. These emulsifiers are often used alone or combined with various glycols and alcohols (Lucia & Guzmán 2021). In this study, an emulsifier was used alone. Emulsions can be developed through a self-emulsifying process or a combination with sonication, employing different surfactants (Tween 20, Tween 80, Span 20, Span 80). The sonicated nano-formulations exhibited reduced droplet size and greater homogeneity than their non-sonicated formulation. Emulsions formulated with Tween 80 generally demonstrated superior outcomes Campolo et al. (2020). Milićević et al. (2022) formulated EC based on Clove bud EOs, three types of carriers, rapeseed oil and Tween 20. The ratio of these compounds showed good physicochemical properties. The emulsion based on EOs can be formed using water, Tween 80 and ethanol. Shi et al. (2021) reported that a cinnamon EO microemulsion can be prepared by adding 4 mL of EO to 6 mL of distilled water, followed by the addition of a mixture of surfactant and anhydrous ethanol. Continuous stirring at 1 500 rpm resulted in a clarified emulsion. The repellent effect was determined using a two-choice bioassay to assess the effect of lavender and catnip oil in a laboratory. Still, the lower levels of SWD infestation in treatments with these oils were not determined in field conditions Gullickson et al. (2020). It becomes imperative to leverage the tools afforded by emerging biotechnological methods to address the challenge associated with their direct application.

CONCLUSION

In conclusion, this research has shown that a formulated bioinsecticide based on a combination of emulsifier, solvent and a mixture of EOs has the potential to act as a repellent for *D. suzukii*, since there was a noteworthy decrease in the number of larvae on the berries when compared to the control and it exhibited favourable physicochemical properties. The field of pest control utilising botanical insecticides containing EOs is rapidly developing. However, further studies must be conducted under field conditions to better understand their performance when applied in outdoor settings. Therefore, conducting future studies in this field is of utmost importance to delve deeper into their effectiveness in practical pest control scenarios.

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