OXFORD Toxicology Research

Toxicology Research, 2023, **12**, 913–921
https://doi.org/10.1093/toxres/tfad081
Advance access publication date 21 September 2023
Paper

Effects of *Rosmarinus officinalis* L. (*Laminaceae*) essential oil on adult and larvae of *Drosophila melanogaster*

Aline Lucca Pedroso [1], Miryane Knapp Schonwald [1], Cristiane Lenz Dalla Corte [2], Felix Alexandre Antunes Soares [2], Adriane Sperança [1], Benhur Godoi [3], Nélson Rodrigues de Carvalho [1,*]

[1]Instituto Federal Farroupilha, Campus IFFar, Santo Ângelo, RS 98806-700, Brazil,
[2]Departamento de Bioquímica e Biologia Molecular, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus UFSM, Santa Maria, RS 97105-900, Brazil,
[3]Núcleo de Síntese, Aplicação, e Análise de Compostos Orgânicos e Inorgânicos, Universidade Federal Fronteira Sul, Campus UFFS, Cerro Largo, RS 97900-000, Brazil

*Corresponding author: Instituto Federal Farroupilha, Campus Santo Ângelo, RS 98806-700, Brazil. Email: nelson.carvalho@iffarroupilha.edu.br

Rosmarinus officinalis (_Lamiaceae_ family), also known as "alecrim," is a perennial herb, typical of the Mediterranean region and widely distributed in Brazilian territory. Despite having demonstrated several properties of human interest, insecticide/larvicidal effect of essential oil from _R. officinalis_ on insects remains unclear. In this study, we tested the effects of _R. officinalis_ essential oil on biomarkers of oxidative damage in _Drosophila melanogaster_. Exposure to _R. officinalis_ essential oil increased adult mortality and decreased geotaxis behavior in adult fruit flies. In addition, essential oil increased of larval mortality and impaired the developmental success in _D. melanogaster_. _R. officinalis_ essential oil showed a significant repellent effect, with duration time of about 6 h. To understand the mechanism underlying the toxicity of essential oil both pro-oxidant effects and biomarkers of oxidative damage were evaluated in exposed flies. Exposure to essential oil caused a significant redox imbalance with impairment of both enzymatic and non-enzymatic antioxidant system and increased the lipid peroxidation levels. These results suggest that _R. officinalis essential oil_ can be used as a bioinsecticide and/or larvicide as well as an alternative insect repellent.

Key words: pro-oxidant properties; essential oil; redox imbalance; insecticide; natural product.

Introduction

The use of synthetic insecticides, mainly organophosphate, and pyrethroids, is an important strategy to control the population of insects such as flies and mosquitoes, known as disease vectors.[1,2] Currently, agricultural plantations also suffer from these pests, resulting in economic losses and impairment of product quality.[2,3] The need to use insecticides is often associated with the indiscriminate use of these costly compounds, polluting the environment and promoting pest resistance.[3] Botanical compounds such as essential oils (EOs), which are known as plant secondary metabolites, act in defense against herbivores, microorganisms, and insects, these natural compounds present less toxic effects and the lowest harmful residues in the environment.[4,5] Many studies have been conducted on the use of EOs, and due to their low toxicity to mammals, there is increasing interest in researching these natural compounds as alternative pesticides.[6,7]

Rosmarinus officinalis, popularly known in Europe as Rosmarinus and in Brazil as ¨Alecrim¨, is a perennial herb of the *Lamiaceae* family, typical of the Mediterranean region and widely distributed in the Brazilian territory. This plant is widely used in cooking and has stood out for its biological and pharmacological potential, such as antioxidant, antimicrobial, hepatoprotective, gastroprotective effects, and potential insecticidal activity.[8] However, despite the beneficial effects of plant extracts, there is considerable evidence potential toxicity.[9,10] Previous studies have shown that *R. officinalis* essential oil has insecticidal and larvicidal activity against *Aedes albopctus* and *Drosophila melanogaster.*[2,11] In addition, botanical compounds such as EOs have multiple mechanisms of action that are closely related to both the impairment of the digestive system and neurological enzymes, as well as to the integument of insects causing dehydration and death.[12,13] For this reason, it is important to investigate the mechanisms involved in the toxicity of this essential oil to insects and its value as a bioinsecticide, since the biochemical and behavioral changes of *R. officinalis* essential oil remain unknown.

Drosophila melanogaster, commonly known as the fruit fly, is a valuable experimental model that has contributed to science in many areas, including toxicology.[14] We can highlight the easy maintenance and low cost, as well as, studies on its genome had been shown that approximately 50% of human disease-causing genes have fruit flies orthologues.[15–17] In recent decades, *D. melanogaster* has been used as a convenient experimental model to answer questions about how the organism defends itself against the excess of a toxic agent and for the prospection of new biotechnological compounds that have the objective of combating these pests and represent a danger for public health. Thus, this insect has become a model for testing the toxicity of compounds in vivo and is widely used to assess fumigant activity in "screening."[18]

Considering the above information, the main aim of the present study is to investigate the repellent effects as well as the possible insecticidal and larvicidal effects of *R. officinalis* OE using

Received: May 30, 2023. **Revised:** July 28, 2023. **Accepted:** August 31, 2023

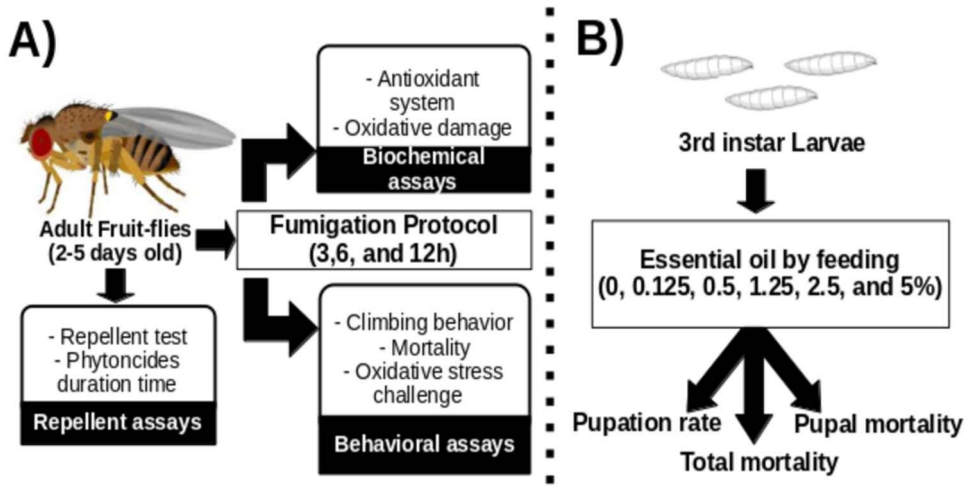

Fig. 1. A) Depicts the fumigation experimental procedure conducted on adult *D. melanogaster*, while B) illustrates the experimental procedure performed on third-instar larvae of the same species.

D. melanogaster as an experimental platform. In particular, we evaluated the toxic effects of the essential oil on oxidative stress biomarkers such as lipid peroxidation and enzymatic and non-enzymatic antioxidant systems. In addition, behavioral experiments and the developmental changes of fruit fly larvae were evaluated to demonstrate the mechanism of action associated with *R. officinalis* essential oil-induced toxicity in the *Drosophila* model, which may provide information for a more effective insect control approach than agrochemicals.

Materials and methods

Chemical reagents

All chemicals were purchased from Sigma-Aldrich (São Paulo, SP, Brazil). EO was purchased from ViaAroma Indústria de Aromatizadores de Ambientes (Rio Grande do Sul, Brazil) Ltda. Other materials, including fly food ingredients, were purchased from standard commercial suppliers.

Gas chromatography-mass spectrometry

Gas Chromatography-Mass Spectrometry (GC-MS) analyses were performed using Shimadzu GC MS-QP2010 series (GC/MS system): Rtx-5MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness); helium carrier gas at 1.5 mL/min; injector temperature 250 °C; detector temperature 290 °C; column temperature 60–180 °C at 5 °C/min, and then 180–280 °C at 10 °C/min (10 min). Scanning speed was 0.5 scan/s from *m/z* 40 to 350; split ratio (1:200). The injected volume was 1 μL of the essential oil diluted in 1% of ethyl acetate. The mass spectrometer was operated using 70 eV ionization energy. The chromatography assay was tested in triplicate.

Identification of the components

Constituents were identified based on the retention index (RI), determined with reference to the homologous series of n-alkanes, C7–C30, under identical experimental conditions, comparing with the mass spectra library search (NIST and Wiley), and mass spectra previously demonstrated in the literature.[19] The relative amounts of individual components were calculated based on the CG peak area (FID response).

Drosophila melanogaster stock

A study was conducted using Oregon R, a wild-type strain of fruit fly (*D. melanogaster*), which was provided by the Bloomington stock center. Flies were maintained at 25 ± 1 °C, under 12:12 dark-light photoperiod and 60%–70% relative humidity on a basic cornmeal diet composed of cereal flour, corn flour, and water, supplemented with dried yeast and Nipagin added as an antifungal agent, as previously described.[20] All experimental treatments were conducted on two to five-day-old flies. Flies exposed to essential oil from *R. officilanilis* were reared in a separate incubator to prevent the effects of phytoncides on non-exposed flies by diffusion.

Essential oil exposure and *Drosophila melanogaster* assays

As shown in Fig. 1A, the fumigation protocol was performed according to previous work with minor modifications.[18,20] Thirty adult flies (male and female) were placed in 330 cm^3 flasks containing a filter paper soaked with 1% sucrose in distilled water at the bottom. A polyethylene terephthalate (PET) countercap was attached to the screw cap of each flask, with a filter paper attached to the inside of the cap for application of the different doses of essential oil. Each flask received one of the following treatments.

Climbing behavior

Locomotor ability was determined by climbing assay as previously described with some modifications.[21] Briefly, for each assay 10 to 15 flies were placed in vertical glass columns (length, 25 cm; diameter, 1.5 cm). After 30 min of recovery, the flies were gently tapped on the bottom of the column and the number of flies that reached 8 cm of the column (top) and flies that remained below this mark (bottom) was registered. Assays were repeated three times at 1 min intervals with each group of flies and results were expressed as the mean of the triplicate.

Larvae and pupae assay

As shown in Fig. 1B, analyses of larval mortality and pupation rate of flies at different concentration of *R. officinalis* essential oil were performed in banana agar medium (110 g homogenized banana, 6 g agar-agar, 2 g nipagin, 22 g brewer's yeast, 38 g corn glucose and 24 g rye flour). To provide essential oil by feeding *R. officinalis,* the essential oil was added to the medium to produce concentrations of 0%, 0.125%, 0.5%, 1.25%, 2.5%, and 5% of the medium. To test larval mortality and pupation rate in different concentration *R. officinalis* essential oil, approximately 200 adult

flies were transferred to a 300 mL empty bottle that was inverted over a Petri dish containing banana medium (to allow the flies to deposit fresh embryos). After 8 h, the adults were removed and the eggs were left to hatch. The experimental design was entirely randomized with five replicates (10 larvae per replicate) for each concentration ($n = 50$), fruit fly larvae at the L3 stage were placed in transparent polypropylene tubes (29.1 mm diameter × 114.4 mm length) containing 3 mL of banana agar medium with different concentrations of essential oil. Total mortality (TM), pupation rate (PR), and pupal mortality (PM) were assessed daily throughout the developmental stages and the results were expressed as % of control.

Repellent test

To test the repellent activity of *R. officinalis* essential oil, we used a T-maze assay with minor modifications.[11] Briefly, 0.5 μL of essential oil was added to filter strips and placed on one side of the sample chamber, and filter strips of the same size were placed on the other side of the sample chamber as a blank control. Ten fruit flies (male and female) were individually placed in the T-maze and after 2 min the number of flies entering each arm was counted. Three replicates were made for each group and the Preference index was calculated as = (number of flies in test arm − number of flies in control arm)/(number of flies in test arm + number of flies in control arm) × 100.

Measurement of phytoncides duration time

The duration time of repellency was measured by a similar method as the repellent test. T-maze assays were set up with two arms with essential oil (0.5 μL) or distilled water on cotton plugs. Ten fruit flies (male and female), individually, were transferred to T-maze immediately, 3 or 6 h after administration of essential oil and the number of flies moved into each arm was counted for at least 2 min. Three replicates were established for each group.

Oxidative challenge with paraquat

To evaluate the toxicity of the *R. officinalis* essential oil in a comparative manner with known pro-oxidant compounds, thirty flies were exposed to paraquat (PQ; 20 mM) alone or in combination with the essential oil (3 μg/mL) and their survival and locomotor activity were evaluated after 12 h. PQ was added to a filter paper containing 1% sucrose and *R. officinalis* essential oil was administered to the flies by the fumigation method described above. All assays were performed in five independent experiments and the results are presented as percentage of live flies (mean ± SEM) and percentage of flies on the top (mean ± SEM). The concentrations of paraquat were chosen according to previous reports from our group based on literature reports.[21,22]

Ex vivo assays

So, all experiments were performed after 3, 6, and 12 h of exposure to 9 μg/mL concentration of *R. officinalis* essential oil. We used these time points and concentrations based on the results of mortality and locomotor deficit observed in *D. melanogaster,* since we still have live flies after treatment (Fig. 3), allowing us to evaluate oxidative stress-related responses.

Homogenate preparation

At the end of the treatment period, flies that were still alive were anesthetized in ice. Of particular importance, we took care to maintain a constant ratio of males to females (i.e. 50% males and 50% females) for homogenate preparation for all experiments. Briefly, 50 flies from each treatment were homogenized in 500 μL of Tris-HCl buffer (50 mM, pH 7.4). The homogenates were centrifuged at 3,000 rpm for 10 min, and the supernatant was used for biochemical assays. All biochemical assays were performed in duplicate independent experiments.

Measurement of lipid peroxidation

Lipid peroxidation (LPO) was measured by the thiobarbituric acid reactive substances (TBARS) assay, which was performed according to the method described previously.[23] An aliquot of homogenate (100 μL) was incubated in water bath at 37 °C for 1 h. The samples were incubated in acetic acid 0.45 M/HCl buffer pH 3.4, containing thiobarbituric acid 0.8%, SDS 8.1% at 100 °C for 120 min for color development, and then the absorbance was measured at 532 nm, and the results were expressed as % of control after normalization by the protein content.

Determination of non-protein thiol and total thiol levels

The Non-protein Thiol (NPSH) level was determined in the control and treated flies according to the method previously described.[24] For the NPSH assay, 200 μL homogenates were precipitated with 200 μL TCA 10% (1:1, v:v) followed by centrifugation at 3,000 rpm for 10 min. The reaction system was made up of 350 μL of 0,5 M phosphate buffer, pH 7.4, 100 μL of the sample, and 50 μL of 5 mM DTNB. For total thiol, the reaction system was 0.5 M phosphate buffer, pH 7.4, 50 μL of the sample, and 50 μL of 5 mM DTNB. For both assays, 10 min incubation at room temperature (25 °C), the absorbance was measured at 412 nm. The results were expressed as % of control after correction by the protein content.

Glutathione-S-transferase activity

The activity of glutathione-S-transferase (GST) was assayed according to a previously published procedure,[25] using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The assay reaction mixture was 30 μL of the sample with 100 mM phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM GSH and 2.5 mM CDNB. The reaction was monitored for 2 min (15 s intervals) at 340 nm at room temperature (25 °C). GST activity was standardized per total protein level and expressed as % of control after correction by the total protein content.

Catalase activity

Catalase (CAT) activity was measured spectrophotometrically as previously described.[26] Briefly, 30 μL of each tissue homogenate samples (1:5) was reacted with 30 μL of 2 M H_2O_2 in the presence of 200 μL phosphate buffer (10 mM, pH 7.0). The reaction was stopped by the addition of 400 μL dichromate acetic acid. The absorbance of the reaction mixture was read at 620 nm in a spectrophotometer after 5 min at room temperature (25 °C). Results were expressed as a percentage of control after correction by protein content.

Superoxide dismutase activity

Superoxide dismutase (SOD) activity was measured by evaluating the inhibition of quercetin auto-oxidation, according to the previously described procedure,[27] with some modifications.[28] Briefly, the reaction medium contained 40 μL of the sample with 25 mM phosphate buffer (pH 10), 0.1 mM EDTA, 1 μM TEMED, and 2 nM quercetin. The reaction was analyzed at time 0 and after 2 min at 406 nm at room temperature (25 °C). The results were expressed as a percentage of control after correction by the protein content.

Table 1. Chemical composition of *Rosmarinus officinalis* essential oil. Relative proportions of the essential oil constituents are expressed as percentages. Experimental retention indices (based on the homologous series of n-alkane C7–C30).

Nº	RI	Compounds	%
1	905	tricyclene	0.9
2	909	α-tujene	0.1
3	915	α-pinene	14.9
4	927	camphene	7.0
5	952	β-pinene	6.2
6	965	β-myrcene	1.9
7	978	α-phellandrene	0.3
8	990	δ-3-carene	0.2
9	998	*p*-Cymene	2.5
10	1,003	limonene	4.8
11	1,006	1,8-cineole	31.6
12	1,034	Υ-terpinene	0.7
13	1,066	terpinolene	0.2
14	1,078	linalool	1.2
15	1,083	fenchol	0.2
16	1,126	camphor	13.9
17	1,140	citronellal	0.4
18	1,144	Isopulegol	0.1
19	1,150	*trans*-Verbenol	2.2
20	1,159	borneol	0.2
21	1,163	terpinen-4-ol	0.4
22	1,178	α-terpineol	1.9
23	1,186	myrtenal	0.3
24	1,199	*cis*-dihydrocarveol	0.1
25	1,285	bornyl acetate	1.1
26	1,426	β-caryophyllene	2.9
27	1,460	α-humulene	0.2
28	1,500	α-muurolene	0.1
29	1,502	β-bisabolol	0.2
30	1,531	*trans*-cardina-1,4-diene	0.1
31	1,588	caryophyllene oxide	0.1
32	1,601	humulene epoxide II	0.7
33	1,695	α-bisabolol	0.6
34	1,784	benzyl benzoate	0.6
		other	1.0

Protein determination

Protein contents were determined as described previously,[29] using bovineserumalbumin as standard.

Statistics

Statistical analysis was performed using GraphPad (version 5.0 for Macintosh OS X, GraphPad Software, San Diego, CA). Significance was assessed by one-way analysis of variance (ANOVA), followed by Bonferroni's test for post hoc comparison. Values of $p \leq 0.05$ were considered statistically significant.

Results

Chemical compounds in the *Rosmarinus officinalis* essential oil

The phytochemical fingerprinting of the *R. officinalis* essential oil was performed by GC-MS analysis and presented in Table 1. Thirty-four compounds were identified in the essential oil. Results demonstrated that the oil contained mainly monoterpenes, oxygenated monoterpenoids, and sesquiterpenoid hydrocarbons, and the major constituents (> 4%) in the essential oil were found to be 1,8-cineole (31.6%), α-pinene (14.9%), camphor (13.9%), camphene (7%), β-pinene (6.2%) and limonene (4.8%).

Effects of *Rosmarinus officinalis* essential oil on survival of *Drosophila melanogaster*

Adult flies were exposed to varying concentrations and were monitored for 12 h to determine the effects of treatment with essential oils. After this period, treatment with 9, 12, and 24 μg/mL of *R. officinalis* essential oil presented a significant mortality which was 54, 85 e 100%, respectively (Fig. 2A). According to the results, after 12 h the cumulative mortality was significant with 6, 9, 12, and 24 μg/mL of *R. officinalis* essential oil (Fig. 2B).

Effect of *Rosmarinus officinalis* essential oil on locomotive activity

We next examined the climbing of flies in response to *R. officinalis* essential oil exposure. Climbing is a natural behavior of flies, which is known as negative geotaxis. After *R. officinalis* essential oil treatment, it can be noticed that all oil concentrations tested were able to reduce the performance index of negative geotaxis behavior of flies at the 3, 6, and 12 h (Fig. 3).

Effects of *Rosmarinus officinalis* essential oil on avoidance behavior

Following the survival assay, we evaluated the repellent activity of the essential oil in the fruit fly. Based on the T-maze assay, a choice

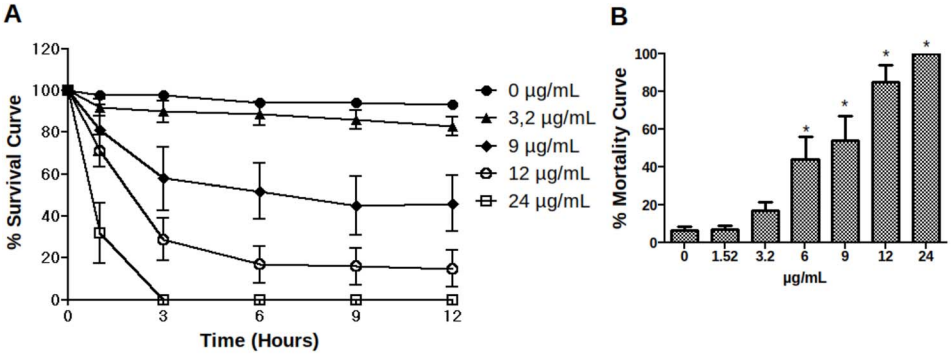

Fig. 2. Effects of treatment with *R. officinalis* essential oil on the mortality of *D. melanogaster*. A) Shows the survival curve and B) cumulative mortality in 12 h. Fruit flies were exposed through the fumigation describe in the Material and methods. Mortality was analyzed at the indicated time points. Results are expressed as mean ± S.E.M. from 5 independents experiments. *p ≤ 0.05 indicates statistical difference from control group by one-way ANOVA, followed by compared to control.

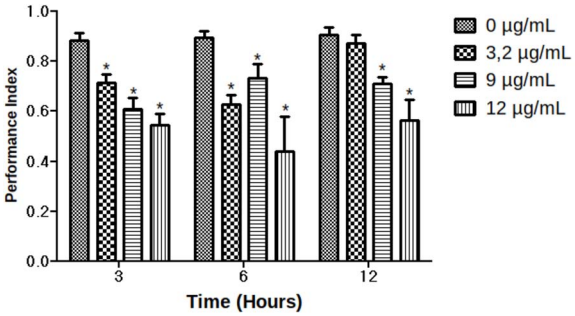

Fig. 3. Determination of locomotor activity in *D. melanogaster* exposed to *R. officinalis* essential oil through the fumigation protocol at 3 h, 6h, and 12 h. Results are presented as means ± S.E.M, from 5 different preparations. *p ≤ 0.05 indicates statistical difference from control group by one-way ANOVA, followed by Bonferroni's post hoc test.

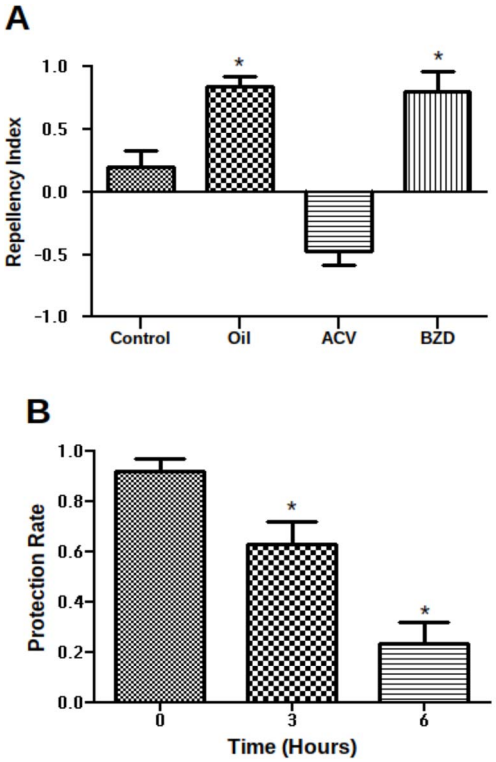

Fig. 4. Effects of Repellency activity in *D. melanogaster* exposed to *R. officinalis* essential oil. A) Represents the avoidance behavior and B) duration time of essential oil. Results are presented as means ± S.E.M, from five different preparations. *p ≤ 0.05 indicates statistical difference from control group by one-way ANOVA, followed by Bonferroni's post hoc test.

chamber was designed to give flies two irreversible choices, one with essential oil and one without. The number of flies choosing each arm was recorded (Fig. 4A). First, the positive and negative controls were evaluated to demonstrate the positive and negative quimiotaxis in fruit flies of apple cider vinegar (ACV, 5×10^{-2} dilution) and benzaldehyde (5×10^{-2} dilution), respectively. As expected, ACV attracted the fruit flies, while benzaldehyde had a significant repellent effect. When the flies were exposed to essential oil, the essential oil was able to promote an avoidance effect similar to the classic benzaldehyde chemorepellent.

Duration time of essential oil fumigants

Since *R. officinalis* essential oil presented repellent effects in repellency assay, we examined the duration of repellency of essential oil using a T-maze assay (Fig. 4B). Fruit flies were transferred to the T-maze and given the choice immediately, 3 or 6 h after 0.1 μL *R. officinalis* essential oil administration. The protection rate was approximately 92% after the immediate essential oil administration. After 3 and 6 h the protection rate was 63% and 23% of protection rate, respectively.

Oxidative stress markers and antioxidant response

To elucidate the mechanism of essential oil toxicity, we examined biomarkers of oxidative stress. Since 9 μg/mL essential oil reduced fly survival by 64% at 12 h and impaired locomotor activity (i.e. climbing ability) by 31, 17, and 21% at 3, 6, and 12 h, this concentration was chosen for further experiments. Fruit flies were treated with 9 μg/mL essential oil and the lipid peroxidation (LPO), reactive oxygen spices (ROS), and enzymatic antioxidant and non-enzymatic antioxidants were evaluated at 3, 6, and 12 h. It is possible to notice a significant increase of LPO at 6 and 12 h exposure to essential oil (Fig. 5A). Our results showed a significant reduction of protein (PSH) and non-protein thiols (NPSH) at 3 h exposure to essential oil, a result that was also maintained at 6 and 12 h (Fig. 5B and C).

In relation to the antioxidant enzymatic system, catalase activity was increased at a 12 h time point (Fig. 6A). When flies were treated with essential oil, a significant reduction in the SOD activity was evident when the control group at 6 and 12 h (Fig. 6B).

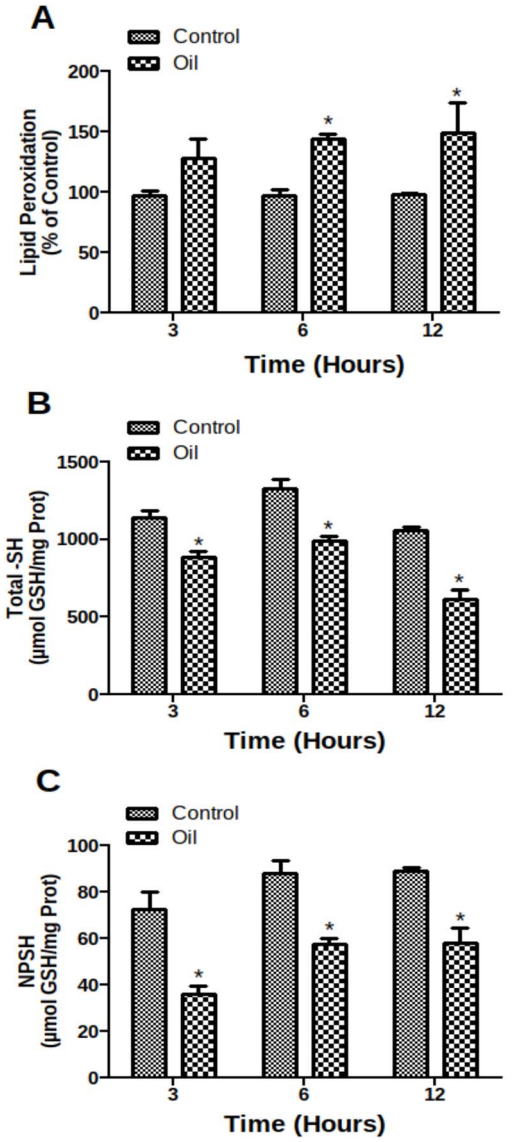

Fig. 5. Levels of oxidative stress biomarkers in *D. melanogaster* exposed to *R. officinalis* essential oil. A) Shows the lipid peroxidation, B) Shows the total SH levels and C) Shows the non-protein SH levels. Results are presented as means ± S.E.M, from 5 different preparations. *$p \leq 0.05$ indicates statistical difference from control group by one-way ANOVA, followed by Bonferroni's post hoc test.

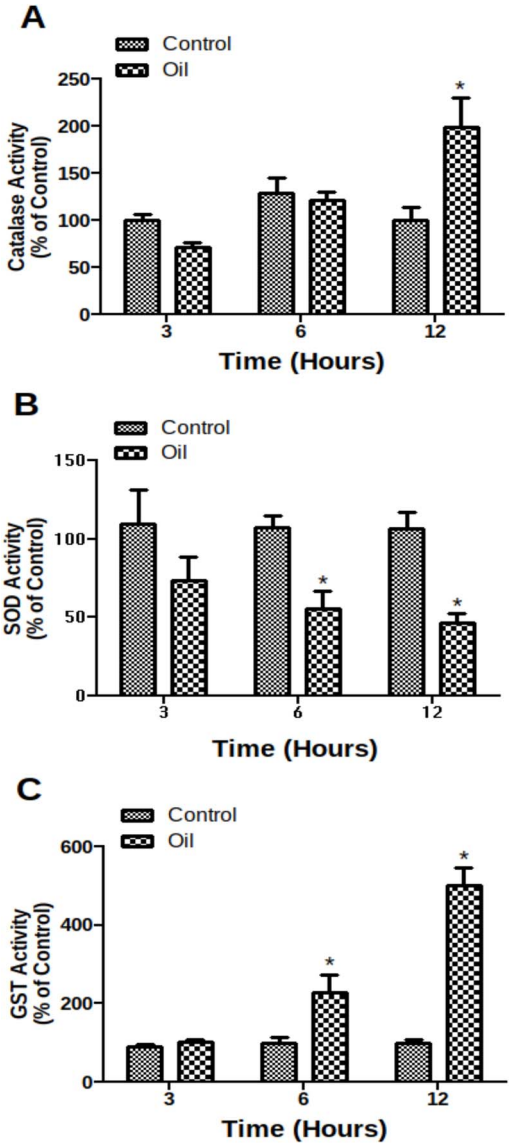

Fig. 6. Activity of enzyme antioxidant in *D. melanogaster* exposed to *R. officinalis* essential oil. A) Shows the catalase (CAT) activity, B) Shows the supreoxide dismutase (SOD) activity and C) Glutathione-S-transferase (GST) activity. Results are presented as means ± S.E.M, from 5 different preparations. *$p \leq 0.05$ indicates statistical difference from control group by one-way ANOVA, followed by Bonferroni's post hoc test.

Our results also showed an increased level of GST activity after 6 and 12 h (Fig. 6C).

Susceptibility to oxidative challenge with paraquat

Because of increase in oxidative damage, we examined the susceptibility to the pro-oxidant paraquat. PQ promoted significant mortality and locomotor impairment (Fig. 7A and B). Exposure of fruit flies to the 3.2 μg/mL oil and PQ for 12 h promoted higher mortality compared to the control and PQ group (Fig. 6A). On the other hand, when the climbing activity was evaluated PQ and PQ + Oil showed a similar reduction compared to the control group (Fig. 7B).

Effects of *Rosmarinus officinalis* essential oil on development

The effect of *R. officinalis* essential oil on fruit fly development was assessed by measuring larval mortality and pupation rate. Figure 8A illustrates the larvicidal effect of the essential oil, showing a progressive increase in mortality with increasing concentration against third instar larvae of *D. melanogaster*. In addition, the pupation rate, which represents the successful transformation from larvae to pupae, showed a significant decrease after treatment with essential oil at concentrations of 1.25%, 2.5%, and 5% (Fig. 7B) compared to control. In addition, all concentrations except 0.125% resulted in an increase in pupal mortality, (Fig. 8C). These results suggest that *R. officinalis* essential oil has a significant effect on mortality and developmental progression of fruit flies.

Discussion

Currently, the use of agrochemicals to control pests in agriculture has reached alarming numbers and consequently caused damage to the environment, non-target organisms, and human

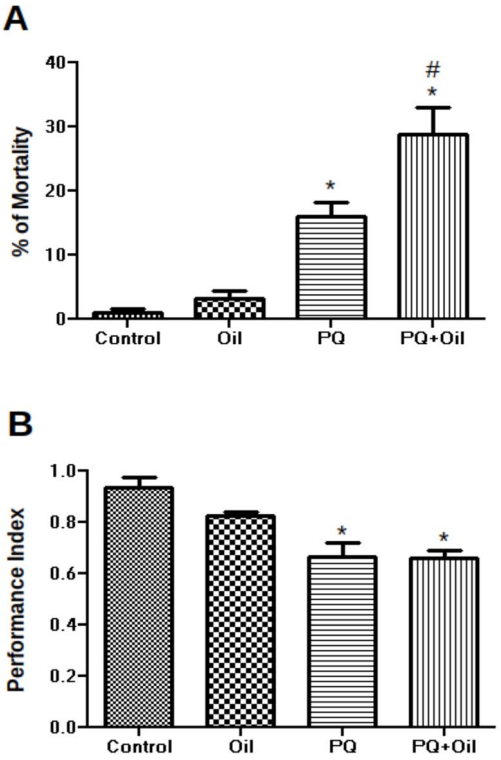

Fig. 7. Susceptibility of *D. melanogaster* exposed to *R. officinalis* essential oil to paraquat (PQ) and oxidative stress inducer. Flies were coexposed to essential oil (3 μg/mL) and PQ for 12 h. After completion of treatment, mortality and locomotor activity were evaluated. A) Shows the mortality and B) Shows the geotaxis behavior. Results are presented as means ± S.E.M, from 5 different preparations. *$p \leq 0.05$ indicates statistical difference from control group by one-way ANOVA, followed by Bonferroni's post hoc test.

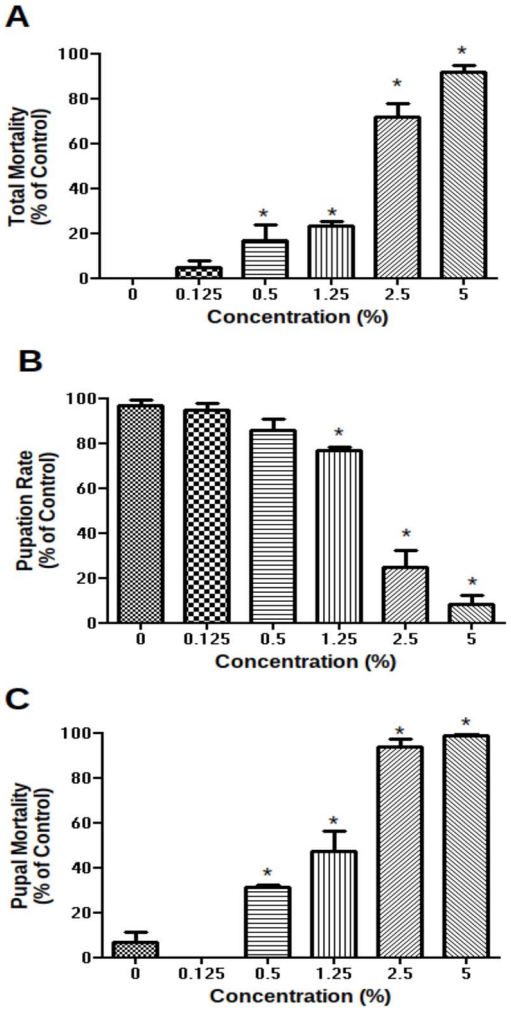

Fig. 8. Larvicidal effects in *D. melanogaster* exposed to *R. officinalis* essential oil. A) Shows the total mortality of third instar larvae of fruit-flies, B) Shows the transformation from larvae to pupae and C) Shows the pupal mortality. Results are presented as means ± S.E.M, from 5 different preparations. *$p \leq 0.05$ indicates statistical difference from control group by one-way ANOVA, followed by Bonferroni's post hoc test.

health. The exacerbated agrochemical usage population of organisms with resistance has been demonstrated in the literature.[3,30] So, due to the adverse effects of agrochemicals, investigating the botanical compounds with bioinsecticide potentials, such as essential oils (EOs), is important to highlight. Therefore, this study has been achieved to valorize plant EOs, such as *R. officinalis* EO, by evaluating their insecticidal and larvicidal effect against *D. melanogaster*. In that investigation, both adults and larvae of flies were exposed to the essential oil via fumigation and food, respectively. *R. officinalis* EO causes mortality and impairs geotaxis (i.e. climbing capability), and most importantly, shows a repellent effect similar to classical repellent in the *D. melanogaster* model. We also showed for the first time that essential oil can promote oxidative damage and imbalance in the antioxidant defenses of adult fruit flies, and a significant larvicidal effect causing mortality and impairment of larval development. In addition, the essential oil (3.2 μg/mL) and Paraquat synergistically compromised both survival and geotaxis. So, our finding indicated a pro-oxidant mechanism of *R. officinalis* EO, which would be associated with oxidative damage and impairment of enzymatic and non-enzymatic systems.

R. officinalis EO exhibits both bioinsecticidal and larvicidal potential against adult and third instar larvae of *D. melanogaster*, with LC_{50} values for bioinsecticide of approximately 6.9 μg/mL and larvicidal of 1.81%, respectively. These results corroborated the previous study and demonstrated significant larvicidal properties against third-instar larvae of *Drosophila suzukii* and *Aedes aegypti* larvae.[11,13] Our results show that Rosmarinus EO contains many organic compounds such as monoterpenes, oxygenated monoterpenoids, and sesquiterpenoid hydrocarbons. It is essential to highlight the 1,8-cineole (31.6%), α-pinene (14.9%), camphor (13.9%), camphene (7%), β-pinene (6.2%) and limonene (4.8%). The increase in mortality of third-instar larvae may be closely related to the higher concentration of 1,8-cineole and camphor, as these compounds may promote damage to the digestive tract and impair larval development by damaging and destroying the cuticular layer. Our results showed that EO reduced the pupation rate and increased pupal mortality; these results suggest that EO affects the successful transition from larva to adult. Morphogenesis in holometabolous insects is metabolically costly with high bioenergetics demands. *R. officinalis* EO could promote metabolic impairment by reduction of caloric intake, thereby limiting metabolite pools in the organism and reducing driving processes ranging from cell proliferation and differentiation to tissue remodeling and even cell death.

D. melanogaster is a fundamental experimental model for screening and bioprospecting new compounds, being useful for elucidating mechanisms involved in the toxicological genesis

of numerous xenobiotics and pathologies. Due to the genetic similarity of its genome with humans, it has been widely used in the understanding of human diseases.[31–33] There are limited reports of studies in the literature evaluating the mechanism of the toxicity of the *R. officinalis* EO on the *D. melanogaster* arthropod model, this study being the first to report this effect. Similar to the *R. officinalis* EO, we could highlight the *Eugenia uniflora* and *Psidium guajava* essential oils, which also have bioinsecticide potential,[18,20,22] however, no larvicidal potential was demonstrated with both studies of essential oils.

Essential oils are products of plant metabolism used as a defense mechanism against predators and parasites. As such, essential oils contain a myriad of bioactive compounds, some of which have pro-oxidant potential. Because of the pro-oxidant potential previously noted in the literature, we have evaluated a non-toxic concentration of essential oil in combination with paraquat, so, the non-toxic concentration of *R. officinalis* (3.2 μg/mL) and paraquat presented a synergetic effect of toxicity on *D. melanogaster*, confirming a pro-oxidant potential. Our results show that *R. officinalis* essential oil promoted an increase in lipid peroxidation, which was accompanied by an increase in catalase (CAT) activity and a reduction in superoxide dismutase (SOD) activity, resulting in an imbalance in the SOD/CAT activity ratio and, consequently, an inability of the enzymatic antioxidant system to contain the exacerbated production of reactive oxygen species (ROS). In addition, the impairment of thiol group-dependent antioxidant defenses was also evidenced as a reduction in total and non-protein thiols; in this regard, our work also demonstrated an increase in glutathione-s-transferase (GST) enzyme activity, a strong indication of the accumulation of toxic metabolites in the model organism and possibly resulting in reduced levels of thiol groups.

Fumigation using *R. officinalis* essential oil exhibited strong repellent effects against fruit flies, making it a promising natural fly repellent comparable to classical repellents like benzaldehyde. However, our results findings also revealed that the protective effects diminished significantly after 6 h, consistent with existing literature. The insecticidal efficacy of essential oils can be attributed to their diverse chemical constituents, but their volatile nature results in a short half-life. Considering the evolutionary similarities between arthropods and other medically important organisms, *R. officinalis* essential oil could serve as an effective prophylactic measure without causing substantial harm to non-target organisms and the environment. It has the potential to function as a bioinsecticide and larvicide against fruit flies. From our results, we can draw two important observations: 1) the essential oil acts as a repellent, but, its effect are short-lived, and 2) the essential oil can influence behavioral and toxicological changes, likely related to its pro-oxidant properties that lead to an increase in oxidative biomarkers. However, further research is necessary to gain a better understanding of how *R. officinalis* essential oil induces biochemical injury. In this context, *R. officinalis* essential oil shows promise as a natural and eco-friendly approach for fruit fly control. It effectively repels fruit flies but may require frequent application due to its volatile nature. Additionally, its impact on oxidative biomarkers warrants further investigation to fully grasp its potential as a bioinsecticide and larvicide without causing harm to non-target organisms and the environment.

Conclusion

Our study shows that exposure of flies to *R. officinalis* essential oil results in significant mortality at both adult and larval stages. Essential oil exposure induces pro-oxidant effects, inducing an increase in oxidative damage while impairing the antioxidant defense system. These effects are likely due to a combination of compounds present in the oil, such as monoterpenes, sesquiterpenes, and phenylpropanoids. The disruption of cellular redox homeostasis leading to lipid peroxidation appears to involve multifactorial mechanisms, including pro-oxidant properties, ROS generation, and toxic metabolites. These findings underscore the potent insecticidal and larvicidal activities of *R. officinalis* essential oil in Drosophila, highlighting its potential as an effective natural insecticide.

Acknowledgments

We would like to thank to Dr. Luis Loose for his dedication and commitment to promoting research on our campus, and to our laboratory technician, Julierme Lopes, we express our gratitude for his assistance in laboratory maintenance.

Author contributions

I would like to highlight that whole author presented a meaningful contribution for performation of this work. Aline Lucca Pedroso was responsible for treatment, mortality assay, climbing assay and biochemical assays; Miryane Knapp Schonwald was responsible for larvae and pupae assay and biochemical assays; Adriane Sperança and Benhur de Godoi were responsible for the phytochemical analyses; Nélson R. de Carvalho, Cristiane Lenz Dalla Corte and Felix Alexandre Antunes Soares were responsible for results interpretation, elaboration of experimental protocols and the preparation of the paper.

Funding

This work was supported by a grant from the Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (011/2022). Pró-Reitoria de Pesquisa, Pós-Graduação e Inovação do Instituto Federal Farroupilha - PRPPGI IFFAR for the financial support (346/2022).

Conflict of interest statement: None declared.

References

1. van den Berg H, Velayudhan R, Yadav RS. Management of insecticides for use in disease vector control: lessons from six countries in Asia and the Middle East. *PLoS Negl Trop Dis*. 2021:**15**(4):e0009358.
2. Dusfour I, Vontas J, David JP, Weetman D, Fonseca DM, Corbel V, Raghavendra K, Coulibaly MB, Martins AJ, Kasai S, et al. Management of insecticide resistance in the major Aedes vectors of arboviruses: advances and challenges. *PLoS Negl Trop Dis*. 2019:**13**(10):e0007615.
3. Belz RG, Carbonari CA, Duke SO. The potential influence of hormesis on evolution of resistance to herbicides. *Curr Opin Environ Sci Health*. 2022:**27**:100360.
4. War AR, Buhroo AA, Hussain B, Ahmad T, Nair RM, Sharma HC. Plant defense and insect adaptation with reference to secondary metabolites. *Ref Ser Phytochem*. 2020, pp.795–822.
5. Elshafie HS, Camele I, Mohamed AA. A comprehensive review on the biological, agricultural and pharmaceutical properties of secondary metabolites based-plant origin. *Int J Mol Sci*. 2023:**24**(4):3266.

6. Baptista-Silva S, Borges S, Ramos OL, Pintado M, Sarmento B. The progress of essential oils as potential therapeutic agents: a review. *J Essent Oil Res*. 2020:**32**(4):279–295.
7. Benelli G, Pavela R, Maggi F, Nkuimi Wandjou JG, Yvette Fofie NGB, Koné-Bamba D, Sagratini G, Vittori S, Caprioli G. Insecticidal activity of the essential oil and polar extracts from Ocimum gratissimum grown in Ivory Coast: efficacy on insect pests and vectors and impact on non-target species. *Ind Crop Prod*. 2019:**132**:377–385.
8. Amaral GP, de Carvalho NR, Barcelos RP, Dobrachinski F, Portella RDL, da Silva MH, Lugokenski TH, Dias GRM, da Luz SCA, Boligon AA, et al. Protective action of ethanolic extract of *Rosmarinus officinalis* L. in gastric ulcer prevention induced by ethanol in rats. *Food Chem Toxicol*. 2013:**55**:48–55.
9. Penha-Silva N, Firmino CB, de Freitas Reis FG, Huss JC D C, de Souza TMT, de Freitas MV, Netto R D CM. Influence of age on the stability of human erythrocyte membranes. *Mech Ageing Dev*. 2007:**128**(7–8):444–449.
10. Saleh AM, Aljada A, Rizvi SAA, Nasr A, Alaskar AS, Williams JD. In vitro cytotoxicity of *Artemisia vulgaris* L. essential oil is mediated by a mitochondria-dependent apoptosis in HL-60 leukemic cell line. *BMC Complement Altern Med*. 2014:**14**(1):226.
11. Lee SH, Do HS, Min KJ. Effects of essential oil from *Hinoki cypress*, *Chamaecyparis obtusa*, on physiology and behavior of flies. *PLoS One*. 2015:**10**(12):e0143450.
12. Jankowska M, Rogalska J, Wyszkowska J, Stankiewicz M. Molecular targets for components of essential oils in the insect nervous system—a review. *Molecules*. 2018:**23**(1):34.
13. Trombin de Souza M, Trombin de Souza M, Bernardi D, Oliveira D D C, Morais MC, de Melo DJ, Richardi VS, Zarbin PHG, Zawadneak MAC. Essential oil of *Rosmarinus officinalis* ecotypes and their major compounds: insecticidal and histological assessment against *Drosophila suzukii* and their impact on a nontarget parasitoid. *J Econ Entomol*. 2022:**115**(4):955–966.
14. Leão MB, Gonçalves DF, Miranda GM, da Paixão GMX, Dalla Corte CL. Toxicological evaluation of the herbicide Palace® in *Drosophila melanogaster*. *J Toxicol Environ Health A*. 2019:**82**(22):1172–1185.
15. MacPherson RA, Shankar V, Anholt RRH, Mackay TFC. Genetic and genomic analyses of Drosophila melanogaster models of chromatin modification disorders. *Genetics*. 2023:**224**(4):iyad061.
16. Nitta Y, Sugie A. Studies of neurodegenerative diseases using Drosophilaand the development of novel approaches for their analysis. *Fly (Austin)*. 2022:**16**(1):275–298.
17. Lathen DR, Merrill CB, Rothenfluh A. Flying together: Drosophila as a tool to understand the genetics of human alcoholism. *Int J Mol Sci*. 2020:**21**(18):1–29.
18. Da Cunha FAB, Wallau GL, Pinho AI, Nunes MEM, Leite NF, Tintino SR, Da Costa GM, Athayde ML, Boligon AA, Coutinho HDM, et al. Eugenia uniflora leaves essential oil induces toxicity in *Drosophila melanogaster*: involvement of oxidative stress mechanisms. *Toxicol Res (Camb)*. 2015:**4**(3):634–644.
19. SparkmanOD. *Identification of essential oil components by gas chromatography/mass spectroscopy Robert P. Adams. J. Am. Soc. Mass Spectrom*. 1997:**8**(6):671–672.
20. de Carvalho NR, Rodrigues NR, Macedo GE, Bristot IJ, Boligon AA, de Campos MM, Cunha FAB, Coutinho HD, Klamt F, Merritt TJS, et al. Eugenia uniflora leaf essential oil promotes mitochondrial dysfunction in Drosophila melanogaster through the inhibition of oxidative phosphorylation. *Toxicol Res*. 2017:**10**:e0120600.
21. Zemolin APP, Meinerz DF, de Paula MT, Mariano DOC, Rocha JBT, Pereira AB, Posser T, Franco JL. Evidences for a role of glutathione peroxidase 4 (GPx4) in methylmercury induced neurotoxicity in vivo. *Toxicology*. 2012:**302**(1):60–67.
22. Pinho AI, Wallau GL, Nunes MEM, Leite NF, Tintino SR, da Cruz LC, da Cunha FAB, da Costa JGM, Douglas Melo Coutinho H, Posser T, et al. Fumigant activity of the Psidium guajava Var. Pomifera (Myrtaceae) essential oil in *Drosophila melanogaster* by means of oxidative stress. *Oxidative Med Cell Longev*. 2014:**2014**:696785.
23. Bianchini MC, Gularte COA, Nogara PA, Krum BN, Gayer MC, Bridi JC, Roos DH, Roehrs R, Fachinetto R, Pinton S, et al. Thimerosal inhibits *Drosophila melanogaster* tyrosine hydroxylase (DmTyrH) leading to changes in dopamine levels and impaired motor behavior: implications for neurotoxicity. *Metallomics*. 2019:**11**(2):362–374.
24. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys*. 1959:**82**(1):70–77.
25. Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. *J Biol Chem*. 1974:**249**(22):7130–7139.
26. Sinha AK. Colorimetric assay of catalase. *Anal Biochem*. 1972:**47**(2):389–394.
27. Kostyuk VA, Potapovich AI. Superoxide-driven oxidation of quercetin and a simple sensitive assay for determination of superoxide dismutase. *Biochem Int*. 1989:**19**(5):1117–1124.
28. Portela JL, Bianchini MC, Boligon AA, Carriço MRS, Roehrs R, Soares FAA, de Gomes MG, Hassan W, Puntel RL. Ilex paraguariensis attenuates changes in mortality, behavioral and biochemical parameters associated to methyl malonate or malonate exposure in *Drosophila melanogaster*. *Neurochem Res*. 2019:**44**(9):2202–2214.
29. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976:**72**(1–2):248–254.
30. Comont D, Lowe C, Hull R, Crook L, Hicks HL, Onkokesung N, Beffa R, Childs DZ, Edwards R, Freckleton RP, et al. Evolution of generalist resistance to herbicide mixtures reveals a trade-off in resistance management. *Nat Commun*. 2020:**11**(1):1–9.
31. Aryal B, Lee Y. Disease model organism for Parkinson disease: *Drosophila melanogaster*. *BMB Rep*. 2019:**52**(4):250–258.
32. Mirzoyan Z, Sollazzo M, Allocca M, Valenza AM, Grifoni D, Bellosta P. *Drosophila melanogaster*: a model organism to study cancer. *Front Genet*. 2019:**10**:51.
33. Ng CT, Yu LE, Ong CN, Bay BH, Baeg GH. The use of *Drosophila melanogaster* as a model organism to study immune-nanotoxicity. *Nanotoxicology*. 2019:**13**(4):429–446.