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Eugenia uniflora leaves essential oil induces toxicity in *Drosophila melanogaster*: involvement of oxidative stress mechanisms

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Abstract

Eugenia uniflora L. (Myrtaceae family), also known as "pitanga", is a tree species widely used in popular medicine. Despite the well documented beneficial effects of extracts and essential oils from this plant, little is known about its toxicity. We performed a phytochemical fingerprinting and evaluated the toxicity induced by the *Eugenia uniflora* leaves essential oil in a *Drosophila melanogaster* model. In order to understand the biochemical mechanisms involved on *E. uniflora* essential oil toxicity, changes on the Nrf2 signaling as well as hallmarks of oxidative stress were measured. The exposure of adult flies to the essential oil via a fumigant method resulted in increased mortality and locomotor deficits. In parallel, an oxidative stress response signaling, evidenced by changes in ROS production, lipid peroxidation, alterations in the activity of antioxidant enzymes and expression of Nrf2 protein targets occurred. In the light of our findings, it is drawn attention to the indiscriminate use of this plant for medicinal purposes. In addition, a potential bio-insecticidal activity of *Eugenia uniflora* volatile compounds is suggested, a fact that needs to be further explored.

Keywords

Eugenia uniflora, essential oil, toxicity, insect model, natural insecticide, oxidative stress.

1. Introduction

The use of plants in alternative medicine is a common practice by human population since ancient time, and a vast range of new drugs have been developed based on these plants[1,2]. The extensive use of botanical extracts and its derived compounds is favored because they are regarded as safe, easily accessible, affordable and culturally acceptable as a therapeutic alternative by large number of communities[3,4,5,6]. Despite the beneficial effects of plant extracts, there are substantial evidences suggesting their potential toxicity[7,8,9]. Consequently, studies on the toxic effects of plant derivatives are necessary since they are consumed without concerns about their toxic effects[10].

Eugenia uniflora (Myrtacea family), popularly known as "pitanga" in Brazil, is an arboreal tree distributed all over the country[11]. This species have perennial leaves and produce seasonal fruits. In its leaves many secondary metabolites had been characterized as monoterpenes, triterpenes, flavonoids, tannins and leucoanthocyanins[12]. Plants of the genus *Eugenia L.* (Myrtaceae) are used in folk medicine as alternative therapies for diabetes, arthritis, rheumatism and stomach diseases[13,14]. In addition, a variety of biological actions is shown for *Eugenia uniflora* including antioxidant, anti-inflammatory, antimicrobial as well as modulation of symptoms related to depression and mood disorders[15,16,17]. Despite the above mentioned beneficial effects, little is known about the potential toxicity induced by extracts and essential oils from this plant.

The fruit fly *Drosophila melanogaster* is one of modern genetics premier model systems, with an extensive literature ranging from classical and modern genetics, biochemistry, physiology and complex phenotypes. *D. melanogaster* have a rapid reproductive cycle, distinct developmental stage and are easily maintained and handled in the lab with low operational costs. Moreover, *Drosophila,* has no ethical objection as rodents and other vertebrates. Although humans and *D. melanogaster* are only distantly evolutionarily related, almost 75% of disease-related genes in humans have functional orthologs in flies[18], making such organism a valuable model system for the understanding of molecular mechanisms of humans diseases, the mode of action of toxic compounds and prospective studies focusing in new bioactive compounds[19,20].

Our goal with the present study was to evaluate the toxicity of *E. uniflora* leaves essential oil using *D. melanogaster* as model organism. We also searched for potential mechanisms of toxicity by measuring oxidative stress related parameters.

2. Material and Methods

2.1 *Chemical reagents*

All chemicals were of analytical grade or higher and were purchased from Sigma-Aldrich (São Paulo, SP, Brazil). Primary antibodies and ECL chemiluminescent reagent were purchased from Santa Cruz Biotechnology (TX, USA). Secondary antibodies were from Sigma-Aldrich (São Paulo, SP, Brazil). Acrylamide, bis-acrylamide, hybond nitrocellulose were obtained from GE Life Sciences (São Paulo, Brazil).

2.2 *Plant Material*

The botanical material from *Eugenia uniflora L.* was collected in June 2013 at 9:00 ± 00:30 hrs at the Botanical Garden of Crato, CE, Brazil (coordinates: 07 ° 14 ' 28.0 " S and 39 ° 24' 56.7" W). The referred time of collection was chosen because it yielded a higher amount of oil compared to other collection times. After species identification one voucher specimen was deposited in the Herbarium Dárdano de Andrade Lima, Universidade Regional Cariri-URCA under number #3106.

2.3 *Drosophila Stock and Culture*

D. melanogaster (Harwich strain) was obtained from the National Species Stock Center, Bowling Green, OH. The flies were reared in 2.5 × 6.5 cm^2 glass bottles containing 5 mL of standard medium (1% w/v brewer's yeast; 2% w/v sucrose; 1% w/v powdered milk; 1% w/v agar; 0.08% v/w nipagin) at constant temperature and humidity (25 ± 1°C; 60% relative humidity, respectively). All experiments were performed with the same strain.

2.4 *E. uniflora leaves essential oil*

Leaves of *E. uniflora* L. were collected and perforated into pieces of about 1 cm^2. Subsequently, plant material was immersed in distilled water in a 5 liter glass flask, and subjected to extraction with Clevenger apparatus by hydro-distillation, according to the methodology described by Matos[21], obtaining a yield of 0.136%. Such distillations were made in quadruplicates following the addition of Sodium Sulfate anhydrous. Then the essential oil was filtered using a pasteur pipette with cotton and stored in amber glass vials at -20°C.

2.5 *Phytochemical Analysis*

2.5.1 *Gas chromatography (GC-FID)*

The gas chromatography (GC) analyses was performed with Agilent Technologies 6890N GC-FID system, equipped with DB-5 capillary column (30m x 0.32 mm; 0.50 mm) and connected to an FID detector. The thermal programmer was 60°C (1min) to 180°C at 3°C/min; injector temperature 220°C; detector temperature 220°C; split ratio 1:10; carrier gas Helium; flow rate: 1.0 mL/min. The volume injected 1 µL diluted in chloroform (1:10). Two replicates of samples were processed in the same way. Component relative concentrations were calculated based on GC peak areas without using correction factors[22].

2.5.2 *Gas chromatography-mass spectrometry* (GC-MS)

GC-MS analyses were performed on a Agilent Technologies AutoSystem XL GC-MS system operating in the EI mode at 70 eV, equipped with a split/splitless injector (220°C). The transfer line temperature was 220°C. Helium was used as carrier gas (1.0 mL/min) and the capillary columns used were an HP 5MS (30m x 0.35 mm; film thickness 0.50 mm) and an HP Innowax (30m x 0.32mm i.d., film thickness 0.50 mm). The temperature programmer was the same as that used for the GC analyses. The injected volume was 1 µL of the essential oil diluted in chloroform (1:10).

2.5.3 *Identification of the components*

Identification of the constituents was performed on the basis of retention index (RI), determined with reference of the homologous series of *n*-alkanes, C_7-C_{30}, under identical experimental conditions, comparing with the mass spectra library search (NIST and Wiley), and with the mass spectra literature Adams[23]. The relative amounts of individual components were calculated based on the CG peak area (FID response).

2.6 *Essential oil exposure and D. melanogaster assays*

The exposure of flies to the essential oil was performed by a fumigation protocol as described: 20 adult flies (males and females) were placed in 330 cm^3 flasks, containing a filter paper soaked with 1% sucrose in distilled water at the bottom. A counter-lid of polyethylene teraphthalate (PET) was introduced on the screw cap of the flask, to which a filter paper was fixed at the inner side of the cap for application of the different doses of essential oil. By doing this, the flies feed and hydrate on sucrose solution at the bottom of the flasks and the essential oil is allowed to volatilize

from the top in order to reach the fly's respiratory system. The flasks received the following treatments: 1% sucrose (control) and 3, 7.5, 15 23.5 and 30 μg/ml of essential oil. Readings of flies' survivorship were taken at 3, 6, 12, 24 and 48h. Results are presented as percentage (%) of live flies (mean ± SD) obtained from five independent experiments.

2.7 *Locomotor Assay*

The locomotor capacity was evaluated by following the negative geotaxis behavior as described by Coulom and Birman[24] with some modifications. Twenty adult flies (1-4 days old; both genders) were exposed to essential oil exposure as detailed above (2.6 subsection). After treatments were finished, flies were immobilized on ice for 1-2 minutes and placed separately in vertical glass columns (length, 25 cm; diameter, 1.5 cm). After 30 min recovery, flies were gently tapped to 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) were registered. The assays were repeated five times for each fly. Results are presented as number of flies on top (mean ± SD) obtained from three independent experiments.

2.8 Oxidative challenge with Iron (Fe) and Paraquat (PQ)

In order to evaluate the toxicity of the *E. uniflora* essential oil in a comparative way with well known pro-oxidant compounds, twenty flies were exposed to Fe (10mM) and PQ (20mM) alone or in combination with the essential oil (3 μg/ml) and their survivorship and locomotor capacity was evaluated after 12h. Fe and PQ were added to a filter paper with 1% sucrose and *E. uniflora* essential oil was administered to 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 ± SD) and percentage of flies on the top (mean ± SD). The concentrations of Iron and Paraquat were chosen according to previous reports by our group, which were based on literature reports (Cruz et al., 2014; Zemolin et al., 2014).

2.9 *Oxidative stress markers*

From now on, all experiments were performed at 3, 6 and 12h of exposure to 3 μg/ml concentration of the *E. uniflora* essential oil. We have used those time points and concentration based on the results from the mortality and locomotor deficit observed in *D. melanogaster* since we still have live flies after the treatment (Figure 2) allowing us to evaluate oxidative stress related responses.

Oxidative stress was determined by measuring lipid peroxidation, reactive oxygen species formation (ROS), non-protein thiols (NPSH) and protein thiols (PSH). By-products of lipid peroxidation were quantified by the thiobarbituric acid reactive substances method (TBARS) following Ohkawa[25] with few modifications. Briefly, 20 flies from each treatment were homogenized in 0.5 ml of phosphate buffer 0.1 M pH 7.0 with aid of a bead-based homogenizer (Powerlyzer, MO BIO, Carlsbad, CA) at 2,000 rpm, during 20 seconds and centrifuged at 1000g during 10 min at 4ºC. After centrifugation, the supernatant was incubated in acetic acid 0.45 M/HCl buffer pH 3.4, containing tiobarbituric acid 0.28 %, SDS 1.2 %, at 95º C during 60 min for color development and then absorbance was measured at 532nm. Malondialdehide (0-3 nmol) was used as standard. The 2,7-dichlorofluorescein diacetate (DCFDA) oxidation was used as a general index of ROS formation following Perez-Severiano[26]. The fluorescence emission of DCF resulting from DCFDA oxidation was monitored at an excitation wave length of 485 nm and an emission wavelength of 530 nm in a multi mode plate reader (EnsPire®PerkinElmer, USA). Protein and non-protein thiols were determined according to the method described by Ellman[27] and adapted to our lab conditions. In summary, after treatments were finished, flies were homogenized in 0.5M perchloric acid and centrifuged at 5000g for 5 min at 4°C. The NPSH content was determined in the supernatant while the pellet was used for PSH measurement. Total protein was quantified according to Bradford assay[28].

2.10 *Enzymatic assays*

For antioxidant enzymes activity, groups of 20 flies were homogenized in 1 mL 0.1 M phosphate buffer, pH 7.0, and centrifuged at 20,000 g for 30 min. The resulted supernatant was used for determination of glutathione S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD) according to methods described earlier[29]. Glutathione S-transferase (GST; EC 2.5.1.18), activity was assayed following the procedure of Habig and Jakoby[30] using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The assay is based on the formation of the conjugated complex of CDNB and GSH at 340 nm. The reaction was conducted in a mix consisting of 100 mM phosphate buffer pH 7.0, 1 mM EDTA, 1 mM GSH, and 2.5 mM CDNB. Catalase (CAT; EC 1.11.1.6), activity was assayed following the clearance of H_2O_2 at 240 nm in a reaction media containing 50 mM phosphate buffer pH 7.0, 0.5 mM EDTA, 10 mM H_2O_2, 0.012% TRITON X100 according to the procedure of Aebi[31]. Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed following the procedure of Kostyuk and Potapovich[32]. The assay consists in the inhibition of superoxide-driven oxidation of quercetin by SOD at 406 nm. The complete reaction system consisted of 25 mM phosphate buffer, pH 10, 0.25 mM EDTA, 0.8 mM TEMED and 0.05mM

quercetin. All enzyme activities were performed at 25 ± 1ºC using a Cary60 spectrophotometer (Agilent Technologies, New Castle DE) coupled to a peltier-controlled circulating water bath. Total protein was quantified according to Bradford[27].

2.11 *Western Blot analysis of Nrf2/NQO-1/HSP70 signaling pathway*

Protein expression was determined by Western blotting according to Posser[33] with minor modifications. Thirty flies were homogenized at 4°C in 300 μL of buffer (pH 7.0) containing 50 mM Tris, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 20 mM Na_3VO_4, 100 mM sodium fluoride and phosphatase inhibitor cocktail (Sigma, MO). The homogenates were centrifuged at 1000g for 10 min at 4°C and the supernatants (S1) collected. After protein determination (following Bradford[26]) using bovine serum albumin as standard, b-mercaptoethanol and glycerol was added to samples to a final concentration of 8 and 25%, respectively, and the samples frozen until further analysis. Proteins were separated using SDS–PAGE with 10% gels, and then electrotransferred to nitrocellulose membranes as previously described by Posser[32]. Membranes were washed in Tris-buffered saline with Tween (TBST; 100 mM Tris–HCl, 0.9% NaCl and 0.1% Tween-20, pH 7.5) and incubated overnight (4°C) with different primary antibodies (Santa Cruz Biotechnology, Texas, USA), all produced in rabbit (anti-Nrf2, anti-NQO-1, anti-HSP70, anti-β-actin; 1:1000 dilution in TBST). Following incubation, membranes were washed in TBST and incubated for 1 h at 25°C with HRP-linked anti rabbit-IgG secondary specific antibodies (Sigma, MO). Blottings were visualized in the Image Station 4000MM PRO using ECL reagent (Santa Cruz Biotechnology, TX). Immunoreactive bands were quantified using the Scion Image software and expressed as a fold change of the mean relative to control group (treated only with sucrose).

2.12 *Statistical analysis*

Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test when necessary. Differences were considered to be statistically significant when $p < 0.05$.

3 Results

3.1 Chemical compounds in the *E. uniflora* essential oil

The phytochemical fingerprinting of the E. *uniflora* essential oil was performed by GC-MS analysis. Figure 1 shows a chromatogram in which at least 29 compounds could be identified. Regarding the composition and the proportion of the compounds found in the essential oil, the most abundant ones are curzerene (48.06%), γ-elemene (13.49%), atractylone (11.78%) and trans-β-elemenone (8.94%) (Table 1).

3.2 Mortality and locomotor behavior tests

Adult flies were exposed to varying concentrations of *E. uniflora* essential oil (control, 3, 7.5, 15 23.5 and 30 μg/ml) through a fumigation method described earlier and survivorship was evaluated at 3, 6, 12, 24 and 48hs after the initial exposure. We observed a time and concentration dependent increase in mortality (Figure 2). According to results, all oil concentrations tested were able to induce fifty percent or higher mortality rate at 12h time point, except for 3 μg/ml concentration (Figure 2). Besides a milder mortality induction observed at the 3 μg/ml concentration in the first 12h of exposure, it induced around 60% and 75% mortality at 24h and 48h of exposure, respectively ($p<0.05$; $F=2288$). Concentrations higher than 7.5 μg/ml were able to induce 100% mortality between 12 – 24h of exposure.

Similarly, a significant time and concentration dependent locomotor deficit was observed in flies exposed to essential oil (Figure 3). At the first 3h of treatment, significant decreases ($p<0.05$; $F=324.2$) in locomotor ability of flies were evident at higher oil concentrations (23.5 and 30 μg/ml), while at 6 - 12h time points all concentrations tested caused significant decreases in flies' locomotion. Oil concentrations higher than 7.5 μg/ml caused a complete loss of locomotor activity after 24h of exposure (Figure 2). Based on these results, the concentration of 3 μg/ml and time points up to 12h were utilized for the next set of experiments.

3.3 Oxidative stress markers and antioxidant response

Aiming to clarify potential mechanisms by which flies are affected by the *E. uniflora*

essential oil, experimental animals were exposed to 3 μg/ml of oil during 3, 6 and 12h. Then, oxidative stress markers and the activity of antioxidant enzymes were determined. This concentration is below the LC_{50} 12 h (5.56 μg/ml) for *D. melanogaster* as observed in Figure 2. It was possible to observe a significant ($p>0.05$; $F=15.71$) increase in ROS production at 3h exposure to the essential oil, a result that was maintained after 6 and 12h as well (Figure 4 A). Our results also showed an increased ($p<0.05$; $F=17.65$) level of TBARS after 6h and 12h of exposure to essential oil (Figure 4 B). The levels of protein and non-protein thiols (NPSH and PSH) were not changed (Table 2).

We also evaluated the activity of three enzymes involved in the antioxidant cell defense: GST, SOD and CAT as well as the expression of protein targets involved in stress response and antioxidant signaling (Nrf2, NQO-1 and HSP70). When flies were exposed to essential oil, a significant increase ($p<0.05$; $F=42.18$) in the activity of GST was evident when compared to non-exposed controls at 6 and 12h (Figure 5A), whilst, the activity of SOD was increased ($p<0.05$; $F=14.51$) at 12h time point (Figure 5B). Catalase activity was unchanged at all time points evaluated (Figure 5C). As demonstrated in Figure 6, flies exposed to the essential oil presented a significant increase ($p<0.05$; $F=189.8$) in the expression of NQO-1 at 3h of exposure (Figure 6 A and C). The protein levels of HSP70, a stress responsive chaperone, did not changed after the first 3 and 6h of treatment but had a significant increase ($p<0.05$; $F=16.4$) in flies exposed to essential oil at 12h time point (Figure 6 A, B and D). The basal levels of Nrf2 transcription factor remained unaltered in all time points tested (Figure 6 A, B).

3.4 Susceptibility to oxidative challenge with iron and paraquat

Since markers of oxidative stress were apparent in flies exposed to *E. uniflora* essential oil, then we asked whether it would increase flies susceptibility to oxidative stress inducers: iron (Fe) and Paraquat (PQ). These oxidative stressors are widely used to induce a pro-oxidative condition in a range of animal models including *Drosophila*[34,35]. The exposure of flies to 3 μg/ml of oil during 12h did not cause significant changes in mortality, while PQ (20 mM) and Fe (10 mM) induced an increase in mortality of approximately 30% (Figure 7A and Figure 8A respectively). The combination of essential oil with PQ ($p< 0.05$; $F=104.4$) or Fe ($p< 0.05$; $F=98.82$) substantially potentiated the mortality effect (Figure 7A and Figure 8A). A similar result was observed in the locomotor behavior test in which both PQ or Fe alone had a mild potency in impairing flies locomotor ability, but when in combination with essential oil, a potentiated effect was observed

(PQ: $p<0.05$; $F=35.94$; Fe: $p<0.05$; $F=103.1$) (Figure 7B and Figure 8B). In general, the administration of essential oil concomitantly to PQ or Fe caused a higher effect in flies mortality and locomotor performance when compared with PQ and Fe alone (Figure 7B and Figure 8B).

4. Discussion

Even though the use of plant extracts and essential oils have been reported to exert a variety of pharmacological actions, there is evidence that some botanical derivatives can cause toxicity[30,32]. Therefore, it is imperative to explore the toxicity potential of plant extracts and essential oils popularly used in folk medicine. Considering the crescent interest in the pharmacological properties of *E. uniflora* derived compounds[15,16,17], the understanding of it potential toxicity is needed. In the present study, we investigated the potential toxicity of the essential oil extracted from the leaves of *E. uniflora* in a *D. melanogaster* model, and identified some phytochemicals present in the oil.

The beneficial effects of *E. uniflora* extracts and essential oils are well documented in literature[13,14,15,16,17]. However, little is known about its potential toxicity. Here, we demonstrated that in a short period of exposure, low concentrations of *E. uniflora* leaves essential oil are able to induce mortality and locomotor deficits in *Drosophila*. As a mechanism for the observed toxicity, it is suggested the establishment of a pro-oxidant condition after flies were in contact with oil derived volatile compounds. Such an effect is confirmed by increased production of reactive species and accumulation of lipid peroxidation end products. Additionally, a clear adaptive response to oxidative stress was apparent in the oil exposed flies, since it was possible to observe an activation of antioxidant signaling pathways and increased activity of key cellular antioxidant enzymes. Considering the rapid induction of mortality and locomotor deficit phenotypes in flies exposed to low concentrations of *E. uniflora* essential oil, we draw attention for the important toxicity imposed by the oil, which in turn, may be of interest in terms of a natural insecticide.

Plant derived compounds are reported to induce toxicity to a wide range of insects and may interfere directly with all developmental stages of the fruit flies and cockroaches[36,37]. Compounds such as terpenes, flavonoids, alkaloids, steroids and saponins are important phytochemicals when considering the insecticide activity of plant derivatives[38]. In addition to acute toxicity and mortality, terpenoids and flavonoids have been also studied for their insect repellent activity[38,39]. In this study, the major compounds found in the *E. uniflora* essential oil were mono and sesquiterpenoids (Table 1), an observation that is partially in agreement with previous reports[15,40,41,42,43]. The most abundant compound was curzerene, making approximately 48% of oil's chemical constitution. Although we

did not performed assays to evaluate the toxicity of each compound in our model, the presence and high abundance of curzerene suggests that it may present major importance in the toxicity of the essential oil. We also observed a significant impairment in the negative geotaxis behavior of flies treated with low concentrations of *E. uniflora* oil, which reflects in a locomotor deficit. The locomotor effect of the oil occurred in a very short period of time, causing flies to present loss of locomotor performance at time points as earlier as 3h of exposure. Compounds from essential oils as terpenoids and phenylpropanoids can alter the insect neurotransmitters system, including the dopaminergic and cholinergic apparatus[44,45,46]. It has been shown that many terpenes are inhibitors of the acetylcholinesterase (AchE)[46]. In general, the terpenoid compounds found in *E. uniflora* are suggested be involved in the oil's observed biocide effect. Studies are ongoing in order to clarify the role of different compounds found in the essential oil tested here.

In parallel to the induced mortality and locomotor deficits, flies exposed to *E. uniflora* leaves essential oil also showed signs of oxidative stress, including ROS and TBARS formation as well as changes in important antioxidant response systems. The cellular response to oxidative stress is mostly regulated by the Nrf2 nuclear transcription factor[47]. ROS/xenobiotics-induced alterations in the cellular redox state constitute an important signal to promote adaptive responses mediated by Nrf2[48,49]. The up-regulation of detoxifying enzymes by natural compounds appears to be related to activation of Nrf2-ARE pathway[48,49]. The Nrf2 nuclear translocation and subsequent binding to DNA sequence known as the "antioxidant response element; ARE" may be triggered by dissociation from the inhibitory protein Keap1 as well as by phosphorylation of serine residues at the Nrf2 protein by upstream kinases such as PKC and MAPK[49]. Among proteins that are usually involved in response to oxidative stress-driven Nrf2 activation, the NAD(P)H dehydrogenase, quinone 1 oxidoreductase (NQO-1), glutamate cysteine ligase (GCL), GST and CAT plays central role[50]. Our results showed a time dependent activation of key factors on the regulation of an antioxidant response. Since a high mortality rate was evident at all doses of essential oil at the first 24h of exposure, we measured oxidative stress markers up to 12h, in order to have a profile of the antioxidant response in animals under *E. uniflora* oil treatments. Apparently, in response to the toxicity induced by oil compounds, flies presented increased ROS levels and a peak of NQO-1 expression at the first 3h of treatment, a phenomenon that is consistent with an early activation of the Nrf2-ARE pathway[51]. While ROS continued to increase from 3h up to 12h, lipid peroxidation took place at 6 and 12h time point (Figure 4). The antioxidant enzyme GST was activated after 6h of oil exposure and continued to increase up to 12h of treatment. In addition, a later antioxidant response also can be observed by the increased activity of SOD at 12h after essential oil treatment, a fact that occurred in parallel to an increase in the expression of HSP70. These results clearly

suggest a two-phased adaptive response to oxidative stress induced by *E. uniflora* leaves essential oil. An early phase triggered by ROS induction, resulting in activation of the master regulator of cellular antioxidant response, the Nrf2 transcription factor, and a late phase, characterized by oxidative damage and increased ROS/xenobiotic detoxifying enzymes (CAT and GST) in parallel to increased HSP70 expression, a stress responsive chaperone. Later on, the toxicity induced by the essential oil was accomplished by the expression of mortality and locomotor deficits phenotypes.

Glutathione S-transferase is an important antioxidant enzyme involved in phase II detoxification systems[52]. GSTs belong to a family of multifunctional enzymes that catalyze the conjugation of GSH to various other molecules and play a role in mechanisms of intracellular detoxification of endo and xenobiotic compounds[53,54]. The observed increase of GST activity in *Drosophila melanogaster* exposed to *E. uniflora* oil may be related to an adaptive response related to elimination of toxic plant derivatives[55,56]. Singh and co-workers demonstrated that natural compounds are able to increase the expression of GST that together with endogenous GSH favors the elimination of plant metabolites from organisms[57]. SOD plays a crucial role in the clearance of superoxide radical from cells as well as for oxidative stress defense[31]. Our results demonstrated a significant increase in SOD activity in flies treated with the essential oil (Figure 5). This was concomitant to a rise in ROS production. The observed rise in GST and SOD activity by *E. uniflora* oil exposure in fruit flies may be explained by a potential activation of the Nrf2 signaling pathway. In fact, an early activation of this signaling pathway was noted in flies exposed to the essential oil, by means of increased NQO-1 expression, which is well documented as a main target of Nrf2 transcription factor[48,51]. Corroborating with results discussed above, we found *E. uniflora* leaves essential oil to increase the susceptibility of flies to oxidative challenge with the pro-oxidants Fe and PQ. Paraquat is a widely used nonselective broad spectrum herbicide with toxicological importance to animal and human health[58]. It has been also employed as a Parkinsonism inducer in animal models due to its structural similarities to 1-methyl-4-phenylpyridinium ion (MPP+), which is frequently used to induce Parkinson's disease-like[58] phenotypes in several models including *Drosophila*[59]. Oxidative stress induction is reported as the main mechanism of toxicity induced by PQ in *Drosophila*[60]. Exposure to high levels of Fe is also reported to increase oxidative stress in flies[20]. In light of our results and literature evidences, we can hypothesize that the increased susceptibility of *E. uniflora* leaves essential oil exposed flies to Fe and PQ is related to the presence of pro-oxidant compounds in the essential oil constitution.

The molecular mechanisms by which exposure to *E. uniflora* leaves essential oil induces oxidative stress in our model still needs further clarification. Although we have not addressed this issue in the present study, literature reports have been published supporting our findings. Usually,

natural compounds are studied for their antioxidant ability. However, depending on concentration, hormetic-like effects may arise, mainly due to metabolizing by-products[61]. For instance, Martins and collaborators[62] observed an increased lipid peroxidation induced by high doses of quercetin, indicating a pro-oxidant effect of this natural compound. It was also showed that natural compounds can either induce or inhibit hydroxyl radical formation via Fenton's reaction[63]. In addition, interferences with mitochondrial function have also been shown as a mechanism involved in natural compounds-induced toxicity[9,64]. Apparently, the anti or pro-oxidative fate of natural compounds depends on concentration and model of study[62,64,65].

5. Conclusion

In summary, our results showed that the *E. uniflora* leaves essential oil induced a highly toxic effect in *D. melanogaster*, causing substantial mortality and locomotor deficits. As a mechanism associated to the essential oil induced-toxicity, oxidative stress appears to play major role. The deleterious effects caused by the essential oil in flies were prominent; therefore, a potential application of the *E. uniflora* leaves essential oil as an alternative to synthetic insecticides is suggested. Furthermore, our findings point to the urgent need for additional toxicological evaluations of *E. uniflora* derived natural compounds previous to its pharmacological use.

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7. Conflict of interest

Authors declare no conflict of interest

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Figure Legends:

Figure 1 - Representative GC-MS chromatogram profile of *E.uniflora* leaves essential oil. The most abundant compounds are curzerene (peak 19), γ-elemene (peak 12), atractylone (peak 28) and trans-β-elemenone (peak 27).

Figure 2 - Effect of the *E.uniflora* leaves essential oil on the survivorship of *D. melanogaster*. Flies were exposed through the fumigation method according to described in Material and Methods section. The survivorship was analyzed at the indicated time points. Results are expressed as Mean±SD of the percentage (%) of live flies after each exposure times. * $p < 0.05$ compared to control.

Figure 3 - Effect of the *E.uniflora* leaves essential oil on the locomotor ability of *D. melanogaster*. Flies were exposed through the fumigation method according to described in Material and Methods section. The locomotor activity was determined as negative geotaxis behavior. Results are expressed as Mean±SD of the number of flies able to climb a marked glass column as described earlier at each exposure times. * $p < 0.05$ compared to control.

Figure 4 - Measurement of ROS (A) and TBARS (B) production in D. melanogaster exposed to *E.uniflora* leaves essential oil. Flies were exposed to essential oil at 3 µg/ml concentration during 3, 6 and12h, through the fumigation method to according to described in Material and Methods section. After treatments were finished, ROS and TBARS were determined in while flies homogenates.ROS production is expressed as percentage of DCFDA oxidation (Mean±SD) in which control was considered 100%. TBARS levels are expressed as nmol TBARS/mg total protein (Mean±SD). * p
<0.05 compared to control.

Figure 5 - Activity of antioxidant enzymes in D. melanogaster exposed to *E.uniflora* leaves essential oil. GST (A), SOD (B) and CAT (C) were determined in flies homogenates after exposure to essential oil at 3 µg/ml concentration during 3, 6 and 12h, through the fumigation method to according to described in Material and Methods section. Enzyme activity is expressed as mU/mg of total protein (Mean±SD). * $p < 0.05$ compared to control.

Figure 6 - Analysis of the Nrf2/NQO-1/HSP70 signaling pathway in *D. melanogaster* exposed to a

3 μg/ml *E.uniflora* leaves essential oil concentration. After treatments samples were collected at each time point indicated and processed for western blot evaluation of each protein target. (A) Representative immunoblots for protein targets at 3 (A), 6 and 12h (B) of exposure to the essential oil. (B) Optical densitometry of immunoreactive bands of NQO-1 (C) and HSP70 (D). Results are expressed as arbitrary units (mean±SD). *p<0.05 compared to control.

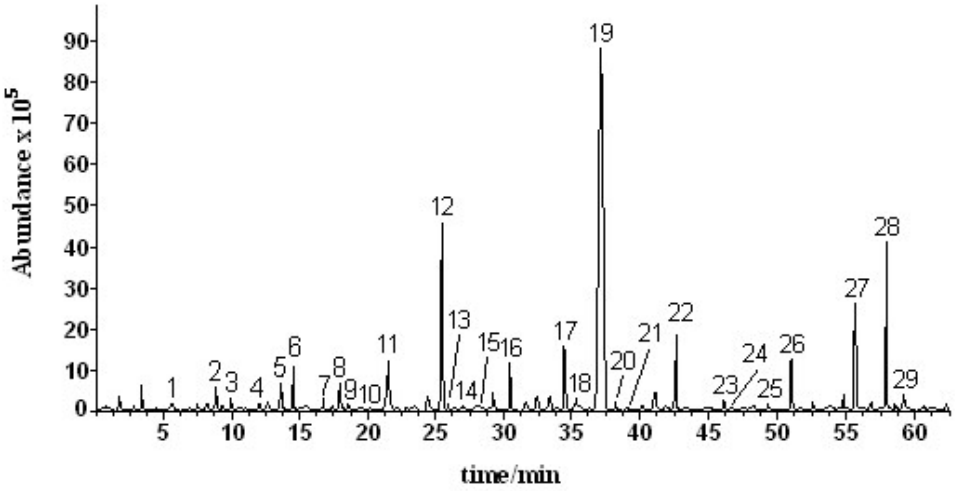
Figure 7 – Susceptibility of *D. melanogaster* exposed to *E.uniflora* leaves essential oil to Paraquat (PQ), an oxidative stress inducer. Flies were co-exposed to essential oil (3μg/ml) and PQ 20 mM during 12h. After treatments were finished, survivorship and locomotor activity were determined. (A) Number of dead flies exposed to essential oil and PQ. (B) Negative Geotaxy behavior of flies exposed to essential oil and PQ 20 mM. Essential oil was administered by fumigation, as described earlier. PQ was given to flies as a solution prepared in sucrose 1%. Controls received only sucrose1%. Results are expressed as Mean±SD. *p<0.05 compared to control. # p<0.05 comparing PQ and PQ + oil group.

Figure 8 – Susceptibility of D. melanogaster exposed to *E.uniflora* leaves essential oil to Iron (Fe), an oxidative stress inducer. Flies were co-exposed to essential oil (3μg/ml) and Fe 10 mM during 12h. After treatments were finished, survivorship and locomotor activity were determined. (A) Number of dead flies exposed to essential oil and PQ. (B) Negative Geotaxy behavior of flies exposed to essential oil and Fe 10 mM. Essential oil was administered by fumigation, as described earlier. Fe was given to flies as a solution prepared in sucrose 1%. Controls received only sucrose 1%. Results are expressed as Mean±SD. *p<0.05 compared to control. # p<0.05 comparing PQ and PQ + oil group

Supplementary Figure 1. Treatment scheme. (A) Adult Drosophila (male and females) were exposed to the essential oil by a (B) fumigation protocol as described: 20 adult flies (males and females) were placed in 330 cm^3 flasks, containing a filter paper soaked with 1% sucrose in distilled water at the bottom (blue bar). A counter-lid of polyethylene teraphthalate (PET) was introduced on the screw cap of the flask, to which a filter paper was fixed at the inner side of the cap for application of the different doses of essential oil (red bar). By doing this, the flies feed and hydrate on sucrose solution at the bottom of the flasks and the essential oil is allowed to volatilize from the top in order to reach the flies' respiratory system. The flasks received the following treatments: 1% sucrose (control) and 3, 7.5, 15 23.5 and 30 μg/ml of essential oil. When Iron or Paraquat were

present, solutions of each oxidative stressors were applied a filter paper at the bottom of the flask (blue bar). (C) Readings of flies' survivorship and locomotor activity were taken at 3, 6, 12, 24 and 48h. After treatments were finished, flies were used for sample preparation for further biochemical analysis.

Figure 1.



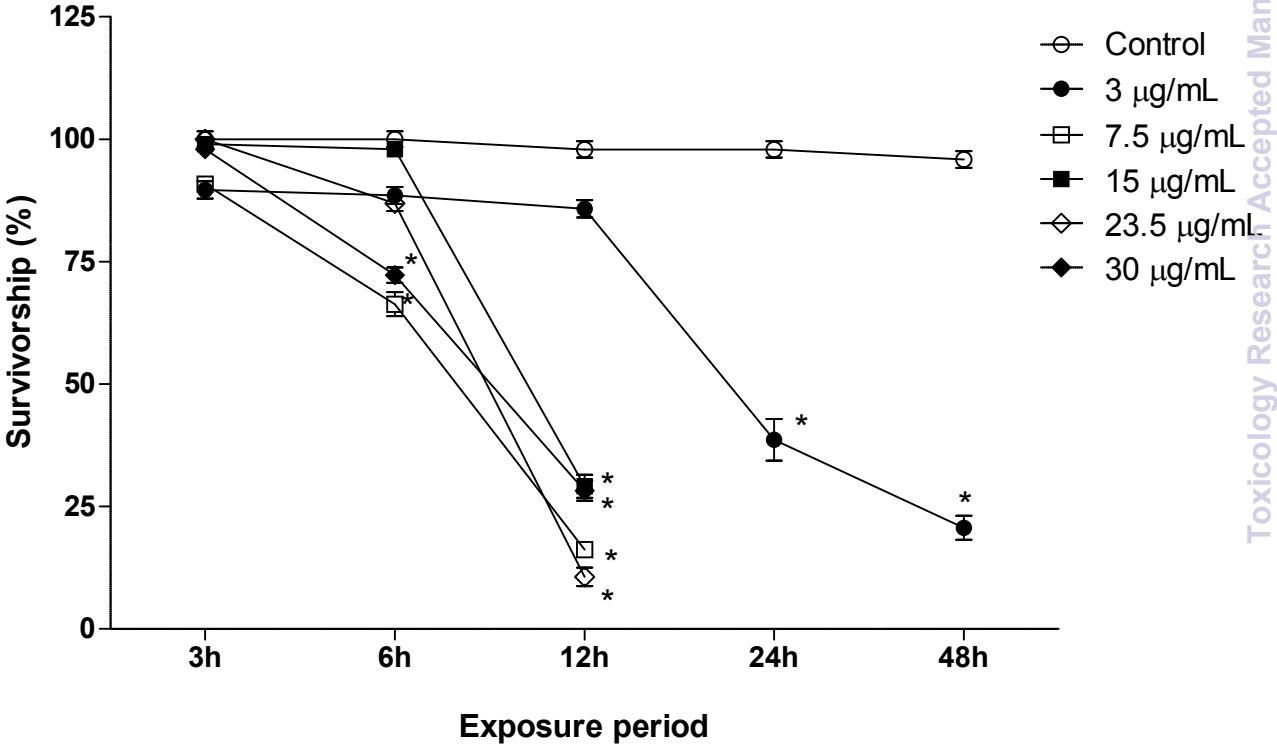
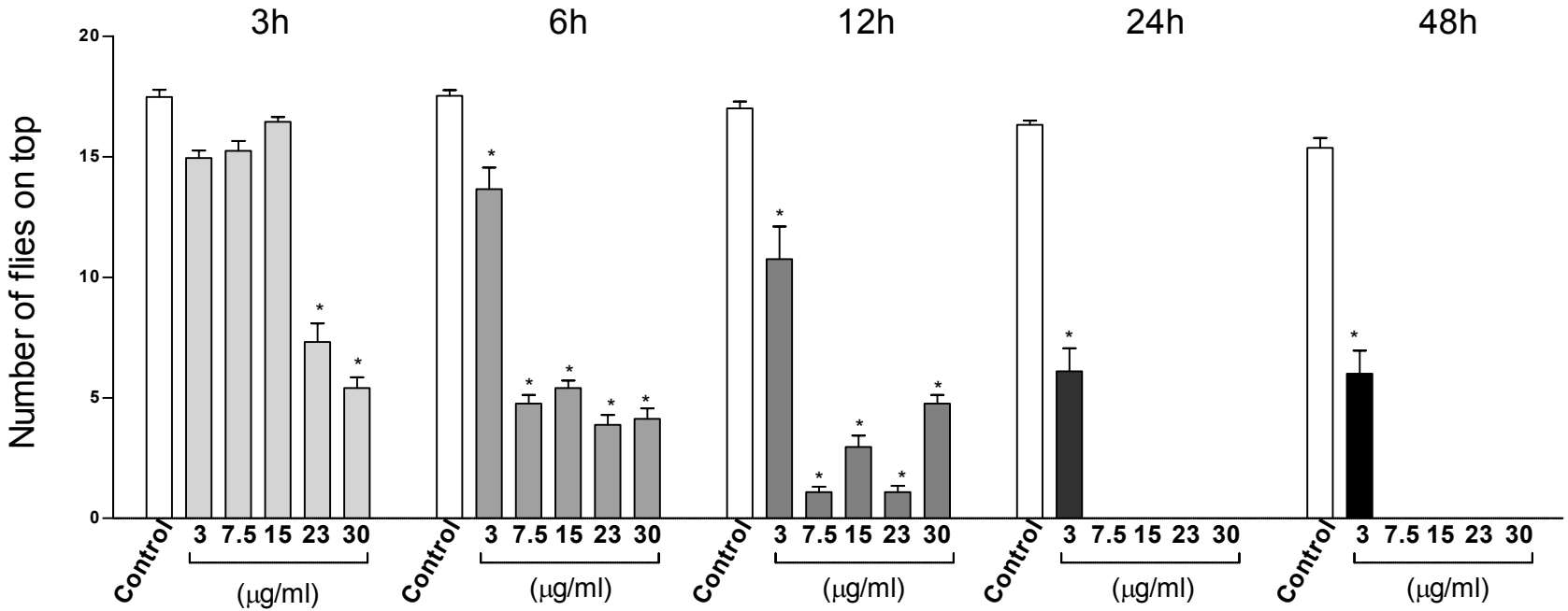


Figure 3.



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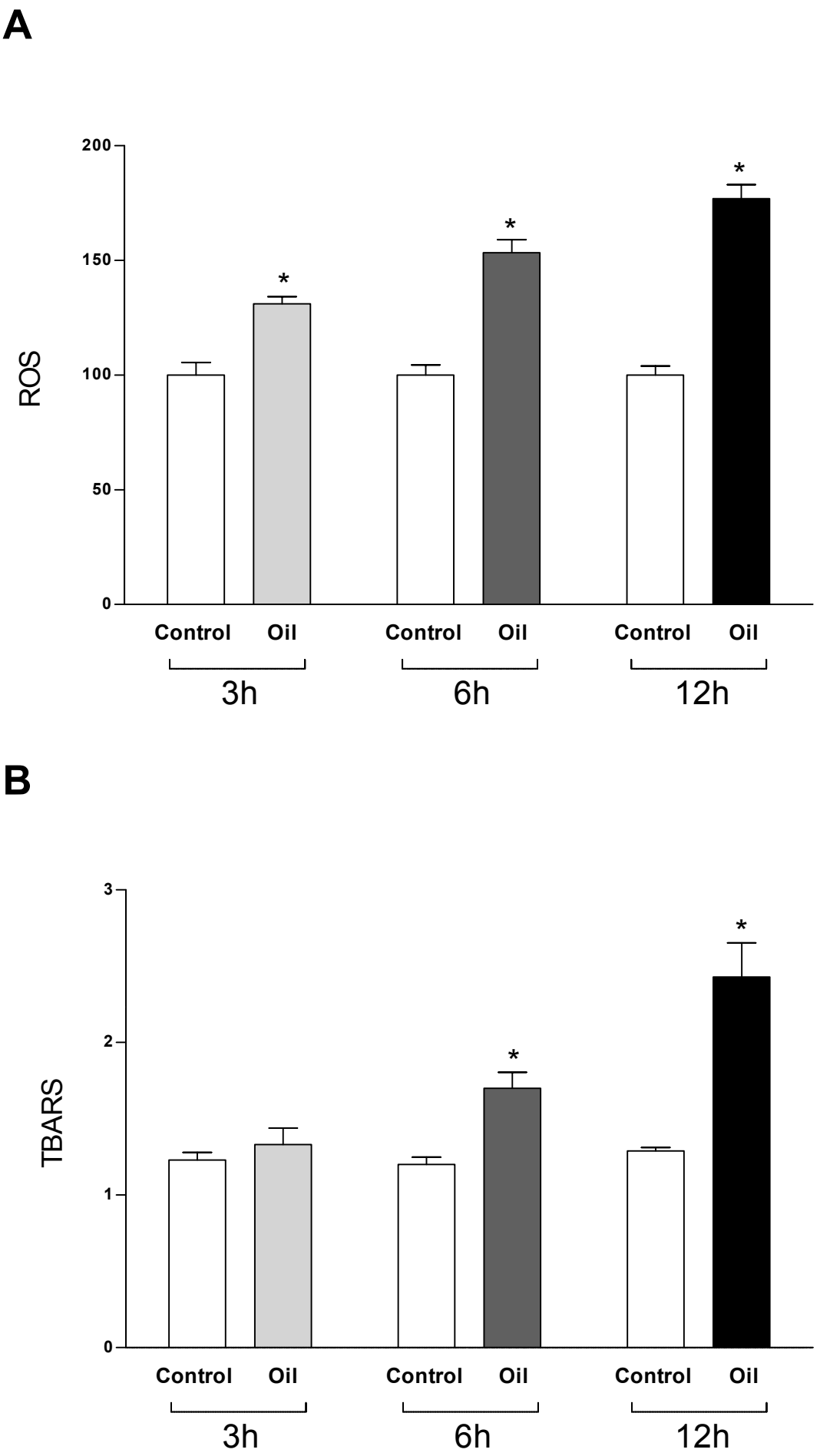
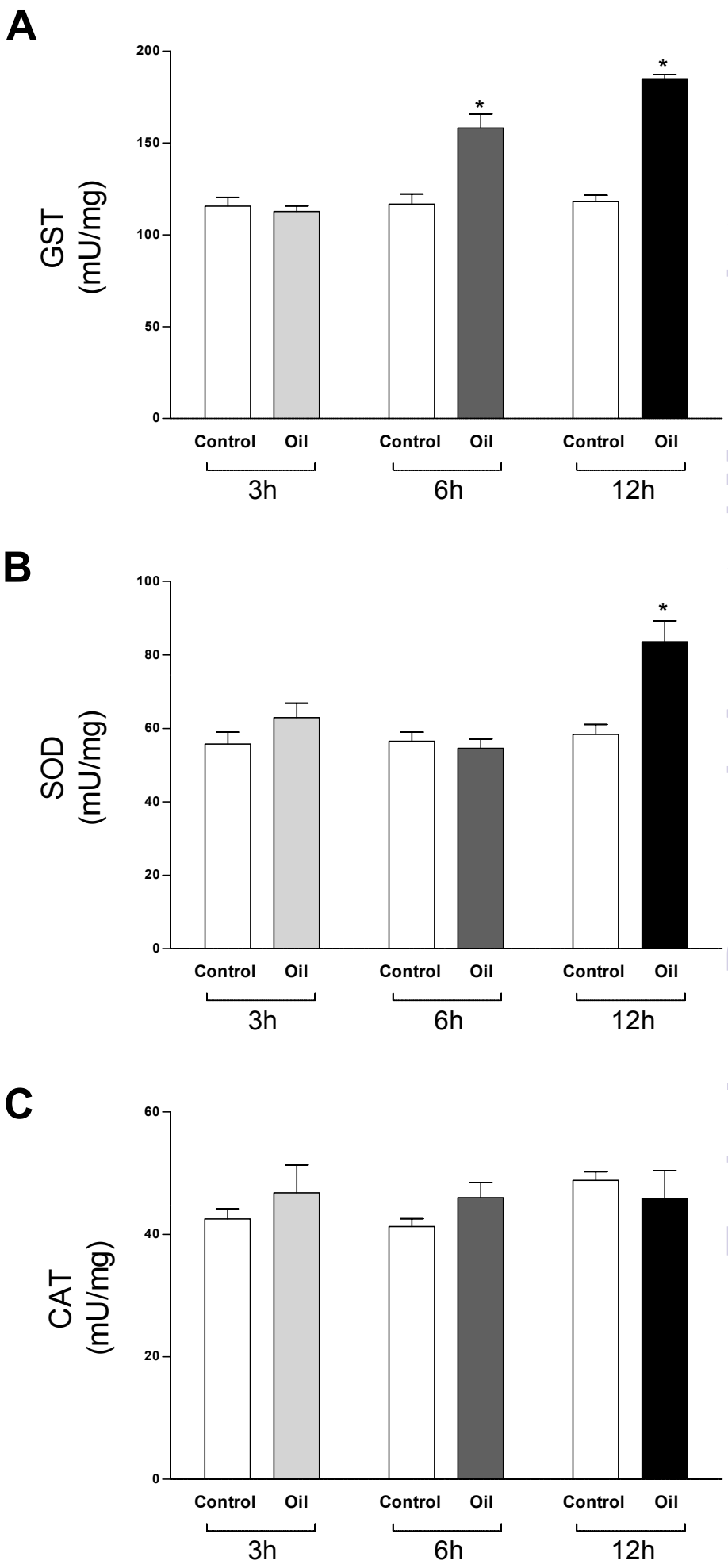
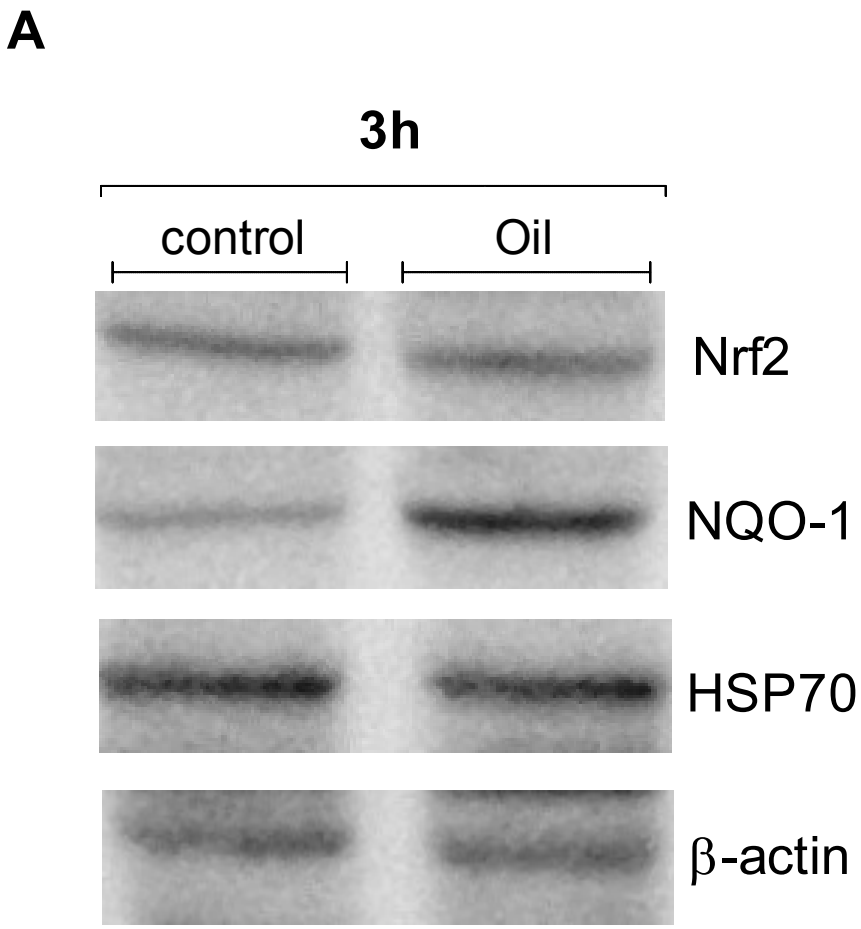
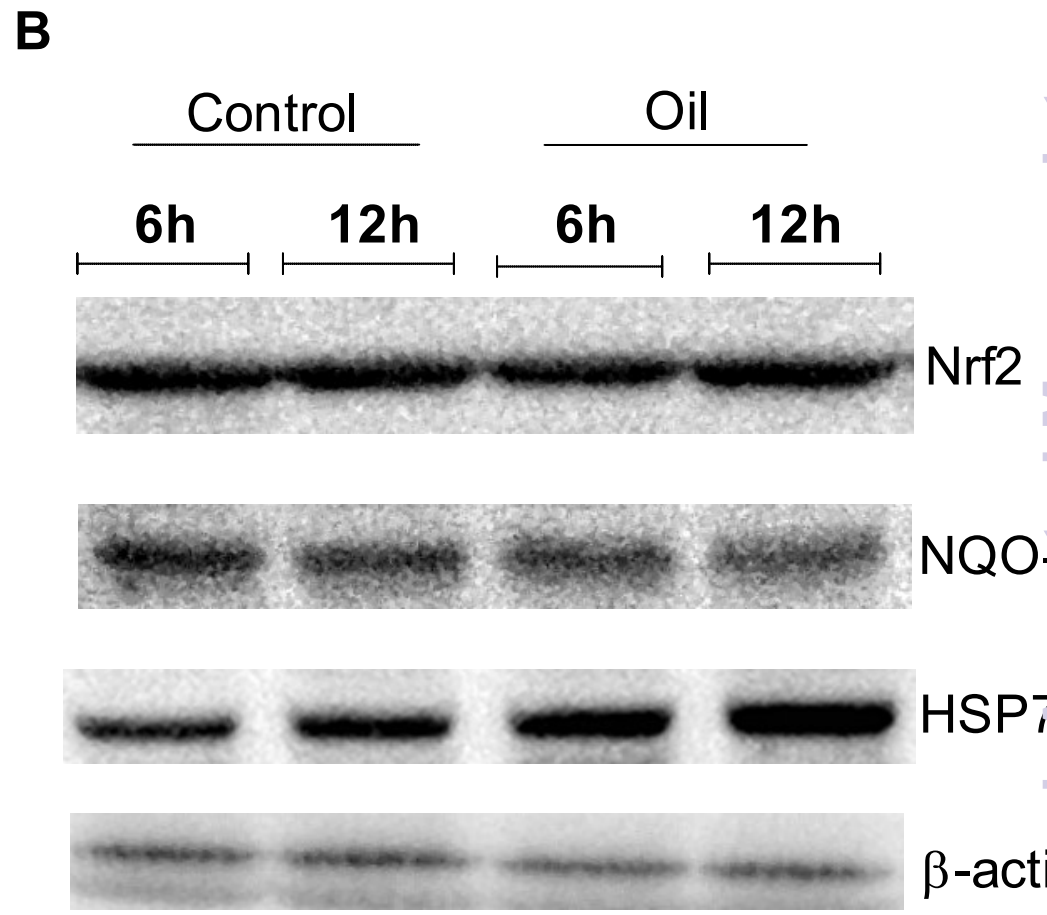
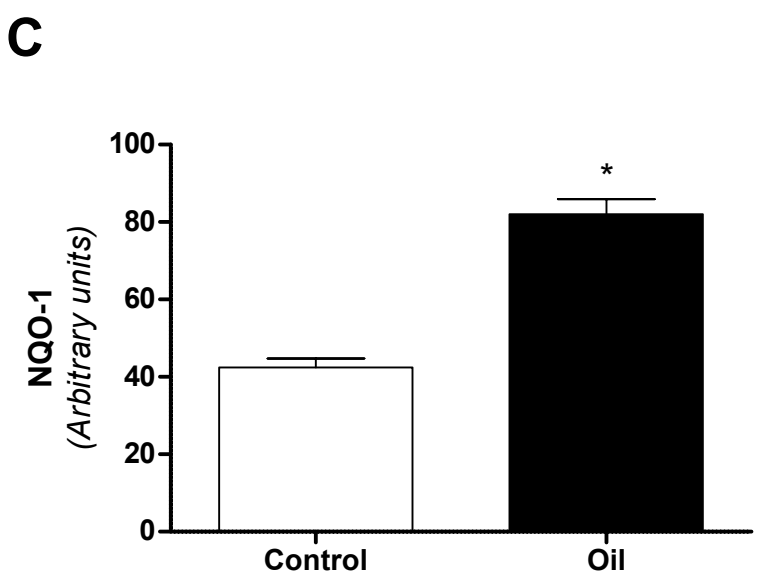


Figure 5.









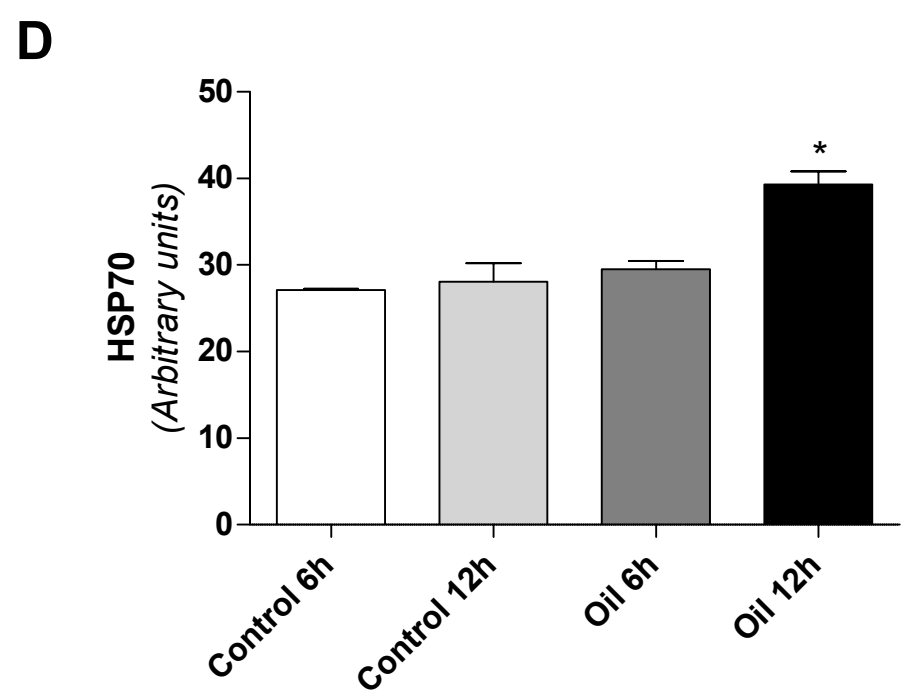
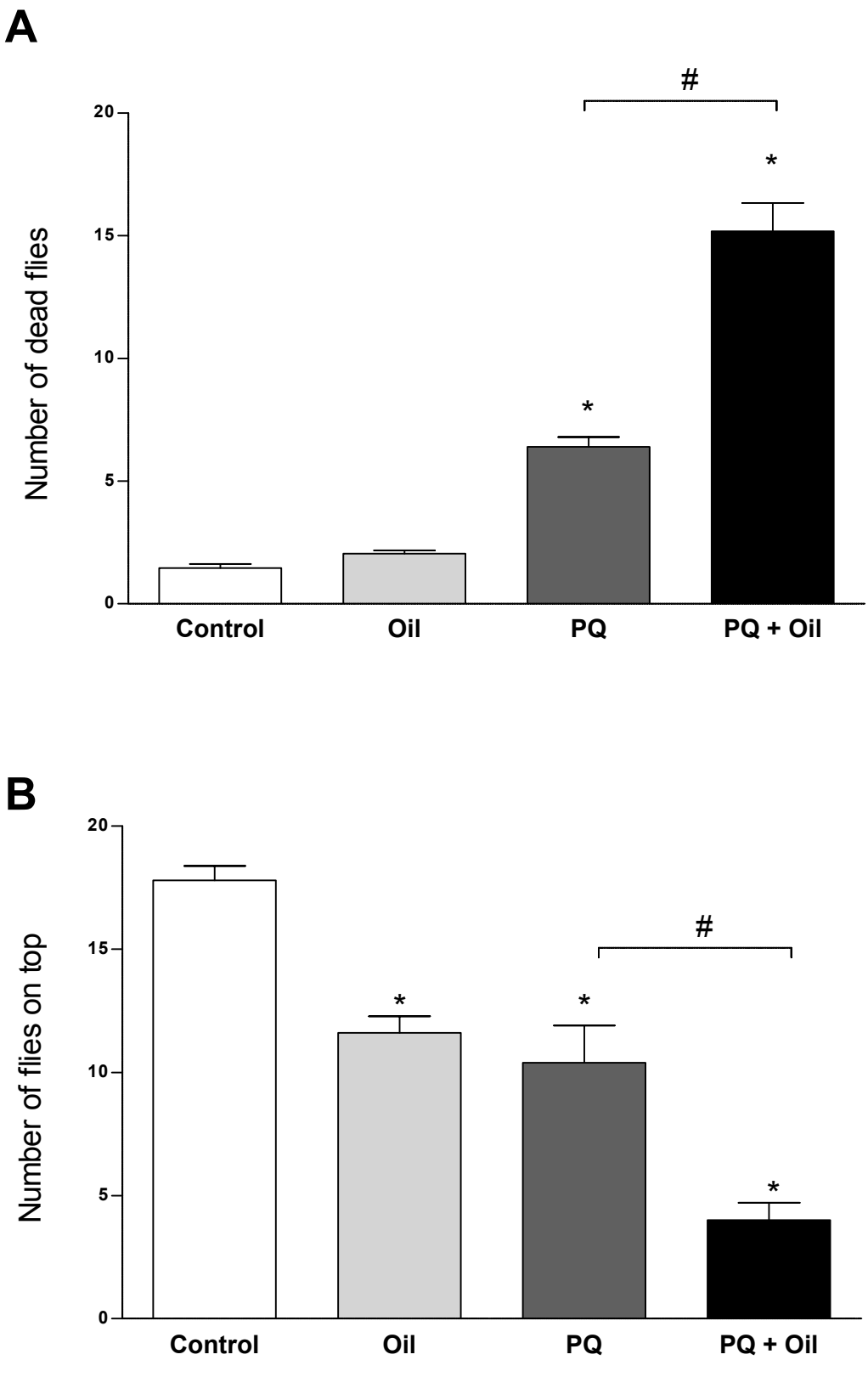


Figure 7.



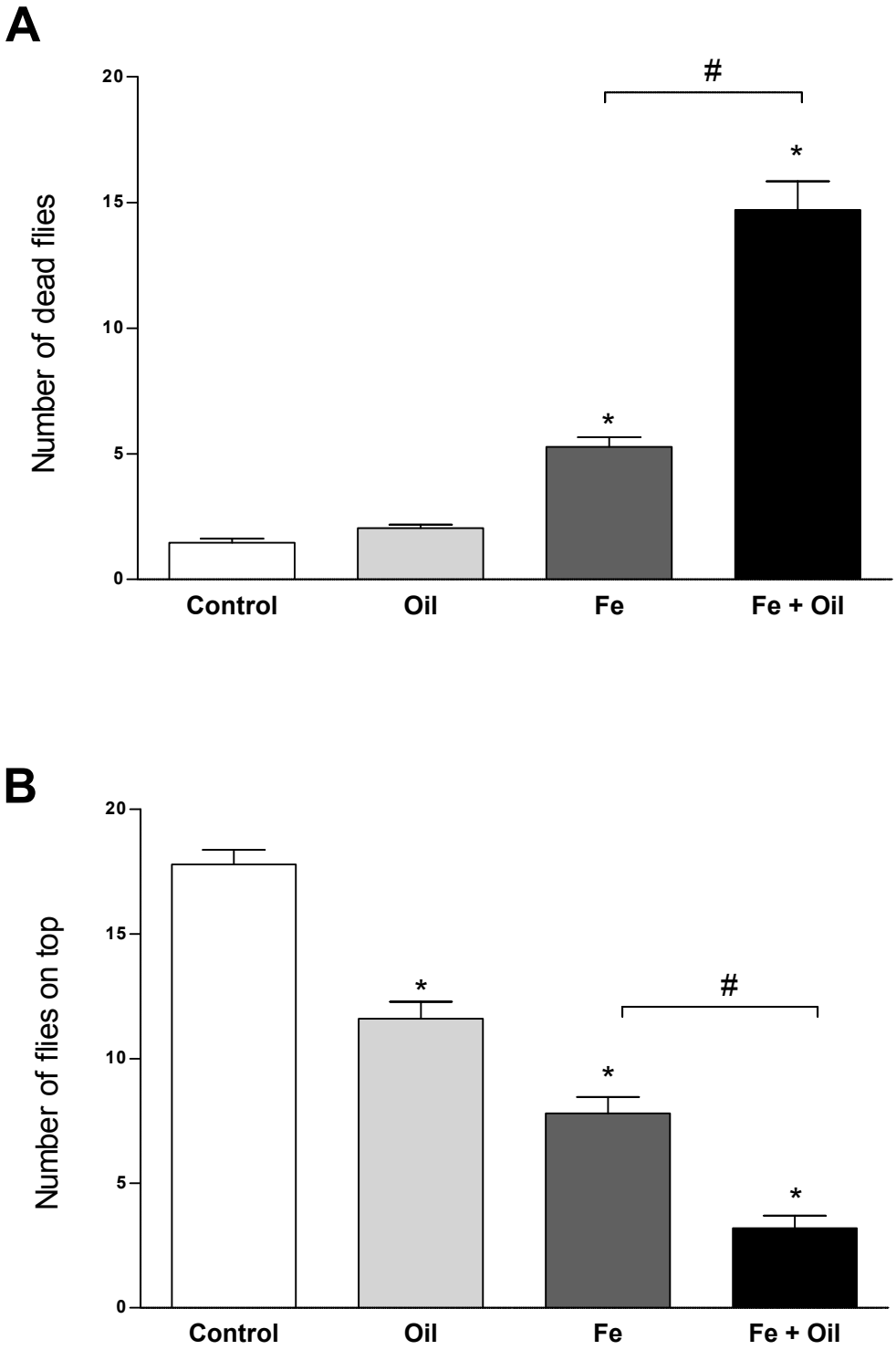


Table 1: Composition of *Eugenia uniflora* essential oil.

No	Compounds	RI[a]	RI[b]	oil
				%
1	α-pinene	937	939	0.05
2	β-pinene	980	980	1.27
3	β-myrcene	994	991	0.36
4	p-cymene	1025	1029	0.18
5	limonene	1033	1031	1.56
6	γ-terpinene	1062	1061	2.09
7	linalool	1098	1098	0.49
8	δ-Elemene	1335	1338	1.03
9	α-Cubebene	1349	1345	0.16
10	α-copaene	1376	1376	0.08
11	β-caryophyllene	1417	1418	2.57
12	γ-elemene	1435	1433	13.49
13	aromadendrene	1439	1439	0.51
14	α-humelene	1451	1454	0.35
15	alloaromadendrene	1465	1462	0.16
16	γ-muurolene	1476	1477	2.85
17	germacreno D	1483	1480	3.21
18	β-selinene	1484	1485	0.78
19	curzerene	1498	1496	48.06
20	γ-cadinene	1509	1513	0.32
21	α-cadidene	1540	1538	0.17
22	germacrene B	1556	1556	4.93
23	spathulenol	1577	1576	1.09
24	caryophyllene oxide	1580	1581	0.08
25	globulol	1585	1583	0.25
26	viridiflorol	1590	1590	3.16
27	trans-β-elemenone	1599	1601	8.94
28	atractylone	1654	1653	11.78
29	germacrone	1691	1693	1.24
	Total identified (%)			**97.43**

Relative proportions of the essential oil constituents were expressed as percentages. [a]Retention indices experimental (based on homologous series of *n*-alkane C_7-C_{30}). [b]Retention indices from literature (Adams, 1995[21]).

Table 2 – Thiol status of *D. melanogaster* exposed to the *E. uniflora* leaves essential oil.

	PSH	NPSH
Control 3h	6.0±0.1	1.9±0.3
Oil 3h	5.8±0.3	2.2±0.3
Control 6h	6.2±0.9	1.9±0.1
Oil 6h	5.8±0.4	2.0±0.2
Control 12h	5.6±0.3	2.1±0.1
Oil 12h	5.5±0.2	2.1±0.1

PSH and NPSH: $\mu mol\ mg^{-1}\ protein^{-1}$
Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test when necessary. Differences were considered to be statistically significant when $p< 0.05$.