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Toxicity and neurophysiological impacts of three plant-derived essential oils against the vineyard mealybug *Planococcus ficus*

R. Brahmi[1)], K. Abdellaoui[1)], A. Harbi[1)], K. Abbes[1)], R. Rahmouni[2)], S. Tounsi[3)], P. Suma[4)] and B. Chermiti[1)]

[1)] University of Sousse, High Agronomic Institute of Chott-Mariem, Department of Biological Sciences and Plant Protection, Sousse, Tunisia
[2)] Technical Centre of Citrus (CTA), Zaouiet Djedidi, Beni Khalled, Nabeul, Tunisia
[3)] Laboratory of Biopesticides, Centre of Biotechnology of Sfax, Sfax University, Sfax, Tunisia
[4)] Department of Agriculture, Food and Environment, University of Catania, Italy

Summary

Many natural products are able to control pests and can be used as alternatives for chemical treatments. Plant essential oils (EOs) have been found to exhibit some biological activity against many insects including mealybugs. This study aimed at studying the insecticidal activity and behavioral and neurophysiological impacts of three plant essential oils against the vine mealybug *Planococcus ficus*. The topical and fumigant toxicity of *Cymbopogon citratus*, *Mentha piperita*, and *Pelargonium graveolens* essential oils was evaluated against *P. ficus* adults. The chemical composition analysis of EOs by gas chromatographic-mass spectrometry (GC-MS) revealed citronellal (31.69 %), menthol (73.78 %), and geraniol (39.6%), as major components, respectively. Bioassays of EOs against vine mealybug adults through fumigation toxicity method revealed lethal concentrations LC_{50} values of 17.01, 26.27 and 24.52 µL·L^{-1} air for *C. citratus*, *M. piperita*, and *P. graveolens*, respectively. In both topical and fumigant bioassays, essential oil from *C. citratus* was the most active altering the behavioral response of treated mealybugs which becomes hyperactive and disoriented. EOs induced general stress in *P. ficus* adults, as evidenced by oxidative stress biomarker analyses. Biochemical analyses showed that the EOs exposure reduced the activity of acetylcholinesterase and significantly induced the glutathione S-transferases and Malondialdehydes accumulation in the vine mealybug tissues. Mortality caused by lemongrass EO positively correlated with the significant decrease in the AChE activity indicating lethal neurological effects. These toxicity bioassays and neurological impact findings provide new informations for formulating effective essential oil based-insecticides to control *P. ficus* in the framework of integrated pest management programs.

K e y w o r d s : botanical insecticide; vine mealybug; toxicity; oxidative stress biomarkers; neurotoxicity.

Introduction

Mealybugs (Hemiptera: Pseudococcidae) constitute a diverse family of insects with nearly 300 genera (Millar 2002). The vine mealybug (VM) *Planococcus ficus* (Signoret) is an economically important pest species of vineyards worldwide including the Mediterranean region, causing extensive damage on leaves and fruits which reduces yield and fruit quality (Mahfoudhi and Dhouibi 2009, Fallahzadeh *et al.* 2011, Beltrà *et al.* 2017, Mansour 2018, Tacoli *et al.* 2018). Damage is due to large amounts of honeydew serving as substrate for sooty mold growth making the fruits unsuitable for marketing (Daane *et al.* 2018). Furthermore, VM is a vector of grapevine leafroll-associated virus 3 (GLRaV-3) causing the grapevine leafroll disease (GLD) (Mahfoudhi *et al.* 2009, Bertin *et al.* 2010, Tsai *et al.* 2010). In some cases, heavy infected vineyards have to be removed (Pietersen *et al.* 2013).

Generally, VM infestations are controlled using repeated synthetic insecticides treatments using organophosphates, neonicotinoids and chitin-biosynthesis inhibitors. Nevertheless, pest management exclusively based on the repetitive applications of insecticide treatments has proven to be not efficient nor sustainable. In case of mealybugs, they are small in size and the colonies are often located beneath the bark of their host plants or underground (Daane *et al.* 2006) so that, together with the waxy body cover, make them difficult to control due to the reduction of the efficacy of any water-based insecticide solutions (Franco *et al.* 2009). Moreover, the use of chemicals has been associated with environmental pollution and adverse effects on non-target organisms and frequent applications of insecticides have led to the occurrence of resistance in many mealybug populations (Franco *et al.* 2009, Mansour *et al.* 2017). This situation has led to an increase of the efforts aiming at finding environmentally-safe pest management techniques to control this pest (Lucchi *et al*. 2019, Franco *et al.* 2021).

The use of natural substances capable to interfere with the physiology of insect pests is a promising alternative to

Correspondence to: Dr. B. Chermiti, University of Sousse, High Agronomic Institute of Chott-Mariem, Department of Biological Sciences and Plant Protection, Sousse, Tunisia. E-mail: chermiti54@yahoo.fr

chemicals in the framework of integrated pest management programmes (Abdellaoui *et al.* 2018a, 2019, La Pergola *et al.* 2017). Plants are a rich source of natural active substances such as nicotine (from *Nicotiana tabacum* L.), pirethrins [from *Tanacetum cinerariifolium* (Trevir.) Sch. Bip.] and rotenone (from *Derris* and *Lonchocarpus* spp.) which were used to control some agricultural pests (Abdellaoui *et al.* 2013). Moreover, plant-based insecticides generally present a relatively low risk to non-target organisms as they are not persistant in the environment and can be easily metabolized by animals receiving sub-lethal doses (Scott *et al.* 2003). Furthermore, botanical insecticides usually comprise a large number of active compounds which minimizes the risks of developing resistance in targeted pests (Hassan Adeyemi 2010); they are abundant in aromatic plants and they can be easily obtained by steam-distillation (Batish *et al.* 2008, Ebadollahi 2011). Therefore, plant essential oils are among the most promising alternatives to pesticides.

The aim of this study is to evaluate, in laboratory experiments, the effects of the EOs extracted from *Mentha piperita* L. (Lamiaceae), *Pelargonium graveolens* L'Herit. (Geraniaceae) and *Cymbopogon citratus* (DC) Stapf. (Poaceae) against the mealybug *P. ficus* through contact and fumigation methods. We also assessed the impacts of these EOs on the behaviour of the pest. The chemical compostion of these EOs was also assessed by gas chromatography-mass spectrometry analysis. Furthermore, we evaluated the oxidative stress biomarkers, the acetylcholinesterase (AChE) and the gluthatione *S*-transferases (GSTs) activities, and the accumulation in the mealybug's tissues of malondialdehydes (MDA). This study aims to implement the knowledge on the toxicity of the selected EOs and to elucidate the mechanisms governing their insecticidal effects.

Material and Methods

Insect stock: Mealybugs were collected from three vineyards located in the region of Regueb in Central Tunisia. The collected specimens were identified in the Department of Agricultural, food and environment, University of Catania (Italy), using the methodology proposed by Williams and Watson (1988) for the identification of mealybugs. All examined insects were identified as *Planococcus ficus* (Signoret, 1875).

In order to establish a laboratory colony, individuals of the VM were maintained on sprouted potatoes (cultivar Spunta) of 30-40 cm height at the Laboratory of Entomology of the High Agronomic Institute of Chott-Mariem, University of Sousse (Tunisia). Potato tubers were placed in a plastic box measuring 34 x 26 x 12 cm and maintained at 25 ± 2°C, 60-70 % RH, in complete darkness. The introduction of *P. ficus* juvenile stages on sprouted tubers was carried out using leaves of *Schinus molle* L. Lab-reared mealybugs were then used to conduct experiments of the present study.

Plant materials and extraction process: Fresh aerial parts of *M. piperita*, *P. graveolens* and *C. citratus* were collected from the region of Chott-Mariem, costal Tunisia in April 2019. Collected plant parts were washed thorougly with tap water to remove soil and other surface impurities then dried naturally in the shade at room temperature (23-27 °C). The dried materials were ground to a fine powder that was used for the extraction of essential oils. Air-dried aerial parts (100 g) of each plant were hydrodistilled with 1500 mL distilled water for 3 h using a Clevenger apparatus according to the European Pharmacopoeia. The obtained oils were dried over anhydrous sodium sulfate for 24 h and preserved at 4 °C in an amber flask up to use.

Gas chromatography/Mass spectrometry (GC/MS) analyses: The volatile composition of the EOs was assessed using GC/MS (GCMS-QP 2010 Plus Shimadzu, Japan) equipped with RTX-5 ms capillary column (30 m x 0.25 mm x 0.25 µm film thickness). The column temperature was initially programmed at 50 °C for 2 min, then gradually increased by 7 °C/min until reaching the final temperature of 250 °C, where it was held for 5 min. The injector and detector temperatures were respectively 250 and 280 °C, using helium as the carrier gas, at a flow rate of 1.2 $mL \cdot min^{-1}$. The injection volume was 1 µL with a split ratio of 1:50. The identification of the components separated by GC-MS was made by comparing the obtained mass spectra for each component with the values stored in NIST Mass Spectral Library (NIST 08). The percentage composition of the oils was calculated in peak areas using the normalization method.

Efficacy of EOs using contact method: The contact toxicity assay was carried out to understand the mode of action of the studied EOs in liquid phase. The assay was performed in Petri dishes (diameter, 9 cm) that had lids with openings (diameter = 2 cm) covered with fine muslin for ventilation and containing a filter paper disk (Whatman no. 1). Different concentrations of oil (0.02, 0.04, 0.08, 0.15 and 0.3 $\mu L \cdot cm^2$) were prepared by mixing their variable volumes with 0.5 mL of acetone. The obtained solutions were applied and homogeneously distributed on the filter paper using a micropipette. Treated filter papers weres air-dried for 5 min before introduction of the target pest species into the Petri dishes. In the untreated control Petri dishes, the filter paper disks were only impregnated with the same amount of acetone (with the same 5 min-delay before introducing the insects). For each insecticidal bioassay, 30 VM adults (7-10 d old) were placed in the center of each Petri dish. Control and treated groups were kept under the same conditions described above for lab-reared mealybugs. The assay was carried out performing four replicates for each concentration. Mortality of the mealybugs was registered at 24 h post-treatment. Dead insects were identified when no movements were observed after probing with a needle in the cervical region.

Efficacy of EOs using fumigation method: Fumigation assay was performed to assess the activity of EOs in their vapor phase. The assay was performed in a 1-L conical flasks. Cotton swabs impregnated with different concentrations of *M. piperita*, *P. graveolens*, and *C. citratus* oils (12.5, 25, 50, 100 and 200 $\mu L \cdot L^{-1}$ air) were attached to the caps of flasks, while VM adults (n = 30, 7-10 d old) were placed at the bottom surfaces. Four replicates of each oil treatment were performed while control

was treated with acetone. Flasks (control as well as treated one) were kept at the temperature of 25 ± 2 °C and RH of 60-70 %. Mortality was checked up to 24 h.

Behavioral bioassay: In order to assess the impacts of the most toxic EOs on the behaviour of VM, adults of *P. ficus* were released individually in the center of a Petri dish (90 mm diameter) lined with a graph paper to follow behavior and location of the tested mealybug. LC_{50} and LC_{90} concentrations of the essential oil were dispensed on a filter paper disc (2 cm^2) (Whatman No. 1, Sigma-Aldrich) placed on a Petri dish cover at the centre of the arena. One adult of *P. ficus* was introduced in the arena and its movements were continuously monitored for 10 min and the cumulative traveled distance was calculated. The mealybug trajectory was also followed and copied on graph paper before being analyzed on a computer with Adobe Photoshop CS3 software. The results were compared to control experiments (acetone only). For every concentration, 5 replicate observations with different individuals were made.

Oxidative stress biomarkers: The acetylcholinesterase (AChE), glutathione S-transferases (GSTs) and Malondialdehyde (MDA) assays were conducted on 7-day-old exuviated adults sampled from control and EOs-treated groups after 24 h of exposure. The EOs were tested by fumigation at the lethal concentration (LC_{50}) prepared in the acetone. Body-cleaned *P. ficus* adults (n = 10) were homogenized in a glass homogenizer in 100 mM K-phosphate buffer (pH 7.5). The homogenates were centrifuged at 9,000 g for 25 min to generate the S9 fraction. The supernatants were then collected and stored at -80 °C until analysis. All procedures were carried out at 4 °C. Proteins in the S9 fraction were measured according to the Bradford (1976) method using Coomassie Blue reagent. The AChE enzymatic activity was measured according to the method of Ellman *et al*. (1961). Reaction mixture contained 0.1 mol·L^{-1} sodium phosphate buffer (pH 7.5), 8 mmol·L^{-1} 2,4-dinitrothiocyanatebenzene, and the stock cytosolic solution containing acetylcholinesterase fractions. After pre-incubation, the reaction was started by the addition of 8.25 mmol·L^{-1} acetylthiocholine (AtCh) as substrate. AChE activity was determined by kinetic measurement at 412 nm. Results were expressed as nmol AtCh hydrolyzed per minute per milligram proteins.

The GSTs activity was measured in DG cytosol as described by Habig *et al*. (1974) using 10 mg of cytosolic protein, 1 mM 1-chloro- 2,4-dinitrobenzene (CDNB) (Sigma-Aldrich, Saint Louis, MO, USA) as a substrate, and 4 mM glutathione (reduced form; GSH) in 100 mM sodium phosphate buffer, pH 7.5. GST activity was determined by kinetic measurement at 20 °C using a Jenway 6105 spectrophotometer (λ = 340 nm). The results were expressed as nomol GSH-CDNB produced per minute per milligram proteins. MDA accumulation was evaluated according to Livingstone *et al*. (1990) using 0.67 % thiobarbituric acid (TBA) and 20 % trichloroacetic acid (TCA) with 200 µL of the S9 fraction. The product of cell membrane's degradation reacts with the mixture TCA/TBA to generate a pink product read on (λ = 532 nm). Results are expressed as µmol of produced MDA per mg of proteins.

Statistics: All mortality data were corrected using Abbott's formula (Abbott 1925). The lethal concentrations LC_{50} and LC_{90}, chi-square, and 95 % confidence intervals for each regression coefficient were calculated using probit analysis (Finney 1971). The results of enzymatic assays are presented asmeans ± standard deviations (mean ± SD). GraphPad Prism 6 (San Diego, California, USA) was used to test the statistical significance of the observed differences. The normality was tested using the Shapiro-Wilk test. For multiple comparisons, a parametric one-way analysis of variance (anova) was performed on data followed by Tukey's as a *post-hoc* test. Principal component analysis (PCA) was performed using R software (R Core Team 2015) and the package ADE4TkGUI (Thioulouse and Dray 2007). Moreover, a correlation matrix was generated using the R software and the package CORRPLOT.

Results and Discussion

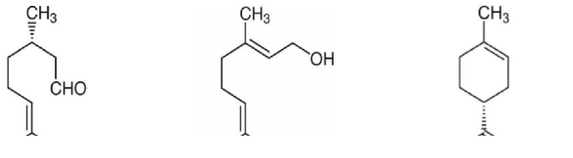
Chemical constituents of EOs: Chemical composition of EOs obtained from *C. citratus*, *M. piperita*, and *P. graveolens* leaves were identified and quantified by GC/MS analysis. The identified compounds, their percentages as well as the retention time are listed in Tabs 1, 2 and 3, respectively. Characterization of *C. citratus* essential oil showed the presence of 40 compounds representing 98.9 % of the total oil composition. The three major compounds of *C. citratus* essential oil were Citronellal (31.69 %), Geraniol

Table 1

Chemical constituents of the essential oils from *Cymbopogon citratus* aerial parts collected from Chott-Mariem, Tunisia

Peak No.	Compound	RT	%
1	Phenylmethanal	7.36	1.21
2	DL-Limonene	8.16	3.2
3	L-Limonene	9.16	9.18
4	Citronellal	12.45	31.69
5	β-Citronellol	14.83	7.76
6	Geraniol	15.67	18.63
7	Citronellyl acetate	18.19	2.62
8	Geranyl acetate	19.02	4.33
9	2-Methyl-6-methylene-1,7-octadiene	19.17	1.01
10	β-cubebene	21.41	1.1
11	Delta-cadinene	22.41	1.05
12	10-epi-Elemol	23.21	3.04
13	Palmitic acid	32.31	1.09
14	Oleic acid	35.65	2.84
Other compounds (26)			10.17
Total (40 compounds)			98.97

Major components structure



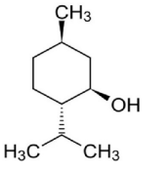
Citronellal Geraniol L-Limonene

Table 2

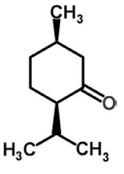
Chemical constituents of the essential oils from *Mentha piperita* aerial parts collected from Chott-Mariem, Tunisia

Peak No.	Compound	RT	%
1	α-Pinene	5.89	1.68
2	β-Pinene	7.1	2.17
3	L-Limonene	8.75	4.69
4	Isomenthone	12.06	22.45
5	(+)-Neomenthol	13.25	12.12
6	Menthol	14.08	43.78
7	α-Terpineol	14.3	1.73
8	Cis-isopulegone	15.33	1.46
9	L-Menthyl acetate	16.75	6.05
Other compounds (9)			3.82
Total (18 compounds)			99.95

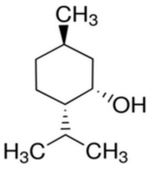
Major components structure



Menthol



Isomenthone



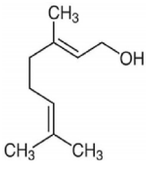
(+)-Neomenthol

Table 3

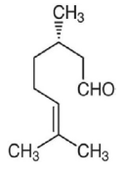
Chemical constituents of the essential oils from *Pelargonium graveolens* aerial parts collected from-Chott Mariem, Tunisia

Peak No.	Compound	RT	%
1	L-Limonene	9.09	9.36
2	L-Linalool	11.26	1.25
3	α-Terpineol	13.93	1.25
4	β-Citronellol	15.09	17.49
5	Geraniol	15.99	39.6
6	(-)-Nopol	16.44	1.16
7	Cis-2,6-dimethyl-2,6-octadiene	18.22	11.71
8	Geranyl acetate	19.04	14.66
9	Oleic acid	35.55	1.36
Other compounds (3)			2.1
Total (12 compounds)			99.94

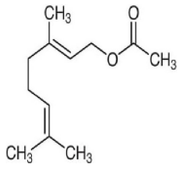
Major components structure



Geraniol



β-Citronellol



Geranyl acetate

(18.63 %), and L-Limonene (9.18 %) (Tab. 1). EOs profile of *M. piperita* and *P. graveolens* through GC/MS showed the presence of 18 and 12 compounds forming respectively, 99.95 and 99.94 % of the total composition of oils (Tabs 2 and 3). Essential oils of *M. piperita* were dominated by Menthol (73.78 %), Isomenthone (22.45 %), and (+)-Neomenthol (12.12%) (Tab. 2). The main components of *P. graveolens* oils were Geraniol (39.6 %), β-Citronellol (17.49 %), and Geranyl acetate (14.66 %) (Tab. 3).

Contact toxicity at 24 h: Acetone-diluted essential oils were tested on adults of VM to determine their contact toxicity. Results reported in Tab. 4 showed that *C*. citratus*, M. piperita* and *P. graveolens* EOs exhibited insecticidal activity against *P. ficus*. *Cymbopogon citratus* EO was relatively more toxic with LC_{90} values of 0.01 µL·cm^2 (Tab. 4) while the lowest effect was noted with the *P. graveolens* EO. The corresponding LC_{50} and LC_{90} values were respectively 0.23 and 0.34 µL·cm^2. Probit analysis also showed that the EOs toxicity followed this order: *C. citratus* > *P. graveolens* > *M. piperita* (Tab. 4).

Fumigant toxicity at 24 h: Essential oils obtained from the three plant species were tested in fumigation bioassays against *P. ficus* adults. Data on toxicity after 24 h exposure are presented in Tab. 5. Probit analysis showed that *P. ficus* adults were more susceptible to *C. citratus* EO. The corresponding LC_{50} and LC_{90} values were respectively 17.01 µL·L^{-1} air (95 % confidence limits = 5.45-26.11 µL·L^{-1} air) and 106.59 µL·L^{-1} air (95 % confidence limits = 94.4-

Table 4

Contact toxicity of *Mentha pipertia*, *Pelargonium graveolens* and *Cymbopogon citratus* EOs against *Planococcus ficus* adults

Essential oils	LC_{50} (µL·cm$^{2\text{-}1}$)	LC_{90} (µL·cm$^{2\text{-}1}$)	χ^2	df	P*	95 % CI*
M. piperita	0.23	0.34	93.86	22	0.000	10.25-11.90
P. graveolens	---	0.14	366.58	22	0.000	6.83-9.43
C. citratus	---	0.01	95.89	22	0.000	4.13-8.56

Table 5

Fumigant toxicity of *Mentha pipertia*, *Pelargonium graveolens* and *Cymbopogon citratus* EOs against *Planococcus ficus* adults

Essential oils	LC_{50} (µL·L^{-1} air)	LC_{90} (µL·L^{-1} air)	χ^2	df	P*	95% CI**
M. piperita	26.27	114.66	107.06	18	0.000	0.013 - 0.016
P. graveolens	24.52	115.4	100.16	18	0.000	0.012 - 0.016
C. citratus	17.01	106.59	43.06	18	0.001	0.011 - 0.015

* Significance level based on Chi-square test (Pearson goodness-of-fit).
** 95 % confidence intervals. Each treatment contained four replicates with 30 adults each and mortality was recorded after 24 h.

123.42 µL·L^{-1} air) (Tab. 5). Insects exposed to vapors of *P. graveolens* and *M. piperita* EOs showed LC_{50} and LC_{90} values of 24.52 (95 % confidence limits = 8.52 - 36.32) and 115.4 (95 % confidence limits = 96.2 - 148.76) and 26.27 (95 % confidence limits = 10.51 - 38.03) and 114.66 µL·L^{-1} air (95 % confidence limits = 95.3 - 148.7), respectively (Tab. 5).

Intoxication symptoms (non-quantitative) in vine mealybug treated with plant EOs: Observation of EOs-treated-insects showed typical neurotoxic symptoms such as hyperactivity, uncoordinated movement and wandering behavior. Some VM adults were also paralyzed, *i.e.*, they were unable to walk. Leg tremors were also observed in *C. citratus* Eo-treated insects. Results of this bioassay also showed other intoxication symptoms such as malformations that can affect the whole body of the insect (Fig. 1D) and change in color that becomes darker with necrotic areas (Fig. 1B, C). The most marked effect was noted with *C. citratus* EO. We also noted that the exposure to tested EOs seems to affect wax production as evidenced by the small amount of the waxy secretions on the body surface of treated insects compared to the control group (Fig. 1B, C). Some females were unable to form their ovisacs and the eggs were deposited without a waxy protection (Fig. 2B). This is a very interesting result and requires further investigations and more detailed studies.

Behavioral bioassay: Representative walking tracks of *P. ficus* adults released in the center of the arena are shown in Fig. 3. They show that *C. citratus* EO affected the behavioural response of treated mealybugs which becomes hyperactive and disoriented as evidenced by the cumulative distance traveled and the trajectory of walking activity. The most marked result was noted with the LC_{50} concentration where treated insects moved rapidly in arena and toward the end of 10 min, the mean distances travelled were 19.83 ± 1.6 cm (Fig. 3). Mealybugs exposed to the highest concentration (LC_{90}) were less active than those treated with LC_{50} and accumulate a distance of 15.16 ± 1.04 cm during 10 mn. In the control group, the calculated distance for the same period was 13.5 ± 0.86 cm and untreated adults exhibited normal behavior and regular movements compared to untreated ones. ANOVA showed a significant difference among treatments (F = 21.96, d.f. = 2, P < .00017) (Fig. 3). Monitoring of mealybugs showed also that *P. ficus* adults exposed to lemongrass EO take resting time, preferably against the walls of the arena which can be reached in 5 min (Fig. 3).

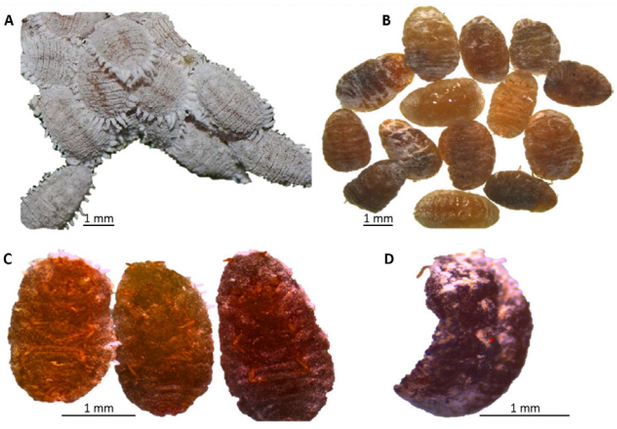


Fig. 1: Toxicity effects of *C. citratus* essential oils on *P. ficus* mealybug. (**A**) control insects with normal morphology and a body surface completely covered by the waxy layer which play an important role in protecting the pest especially from desiccation and parasites contamination. (**B** and **C**) Treated adult females showing a change in body color that becomes darker with small amount of the waxy secretions on the body surface. (**C**) malformations and severe desiccation of adult female body after exposure to the EO.

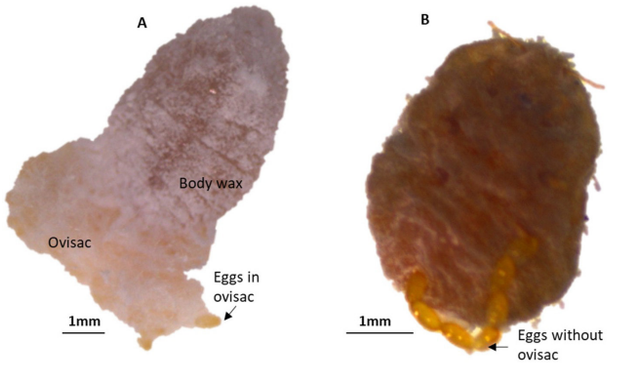


Fig. 2: Control (**A**) and *C. citratus* essential oils-treated (**B**) adult females after 24 h of exposure. Note the presence of eggs in a normal formed ovisac in the control female. Eggs of treated female are without a waxy protection and the insects are unable to form their ovisacs.

Oxidative stress biomarkers

Neurological impacts by acetylcholinesterase activity measurements: AChE measurement after 24 h exposure to the LC_{50} concentration of EOs are illustrated in Fig. 4. Results revealed significant inhibition of the enzymatic activity compared to control. The analysis of variance considering the EO used as classification criteria showed a significant difference among treatments ($F_{(3, 8)}$ = 86.24, ddl = 3, P < 0.0001) as indicated by the heterogeneous groups generated using the Tukey's test (Fig. 4). The most neuroinhibitory effects was noted with the *C. citratus* essential oils where AChE activity reached 1.77 ± 0.22 nmol·min^{-1}·mg of protein^{-1} *versus* 7.94 ± 0.84 nmol·min^{-1}·mg of protein^{-1} in control group after the same exposure period. The AChE activity recorded in the *M. piperita* and *P. graveolens* EOs-treated groups are 5.99 ± 0.31 and 5.52 ± 0.25 nmol·min^{-1}·mg of protein^{-1}, respectively (Fig. 4).

Effect on gluthatione S-transferases activities: A significant increase in the GSTs activity was observed in the *P. ficus* adults after 24 h of exposure period compared to the control insects ($F_{(3, 8)}$ = 6.17, ddl = 3, P = 0.017 (Fig. 5). No significant changes in GSTs activities were detected (Fig. 5). GSTs activities recorded in the treated groups at the LC_{50} concentration were 17.91 ± 1.2, 18.27 ± 1.81, and 18.02 ± 2.1 nmol·min^{-1}·mg of protein^{-1} after 24 h of exposure to *C. citratus*, *M. pipertia*, and *P. graveolens* essential oils, respectively. GSTs value of the control group was 13.67 ± 0.48 nmol·min^{-1}·mg of protein^{-1} in the same experimental conditions (Fig. 5).

Effect on lipid oxidative alteration (MDA accumulation): Lipid oxidative alteration was also studied by measuring the MDA accumulation

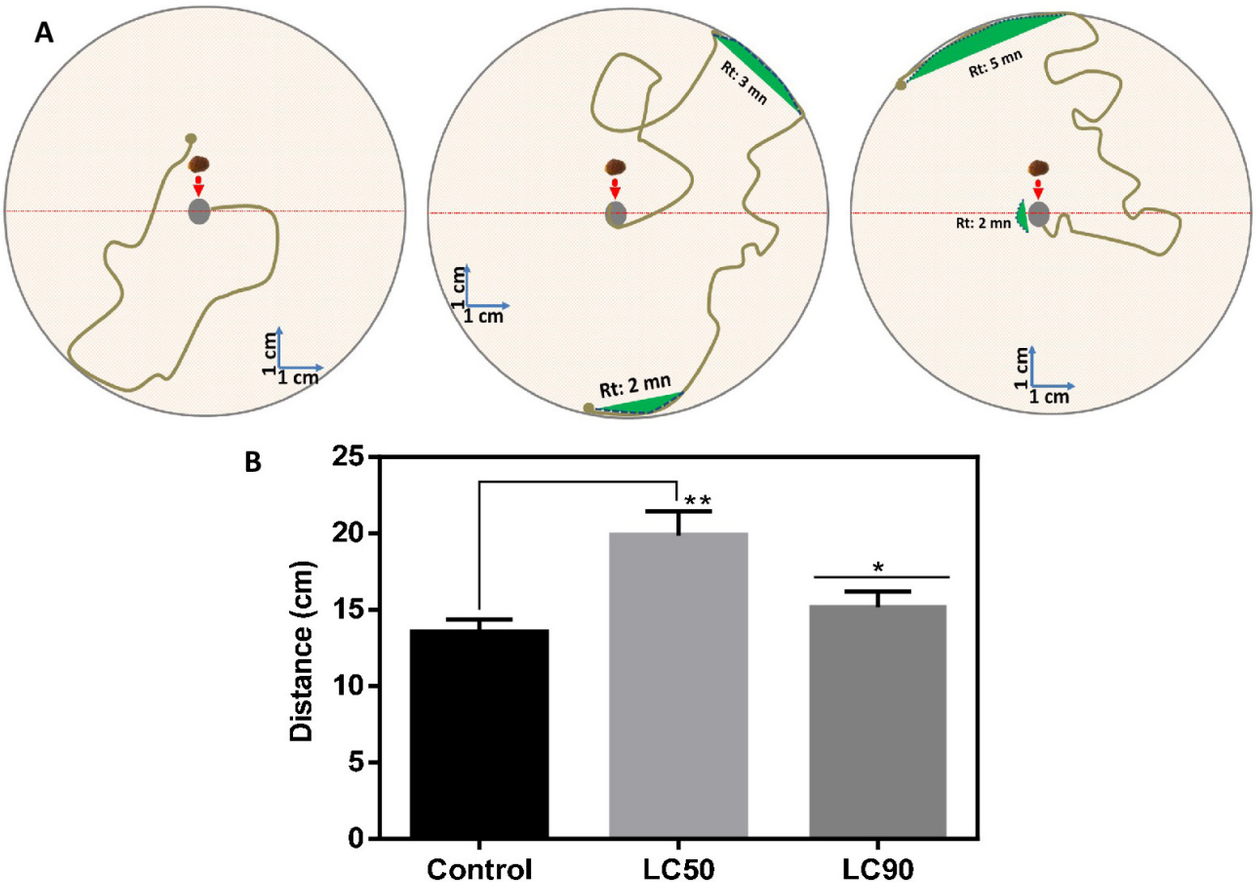


Fig. 3: (**A**) Representative tracks showing the walking activity of *P. ficus* over a 10 min period on graph-paper filter arena with lemongrass (*C. citratus*) essential oil at the concentrations of LC_{50} and LC_{90}. Brown tracks indicate the trajectory of walking activity, and green tracks indicate the resting time. The point with the red arrow indicates the initial position of the mealybug. (**B**) The cumulative travelled distance calculated for 10 mn. Data represent mean ± SD of 5 replicates per treatment. Statistical analysis consisted of one-way ANOVA. Significant differences ($P < 0.01$ and $P < 0.001$) are flagged with asterisks (* and **, respectively) (Tukey's test). Rt: resting time.

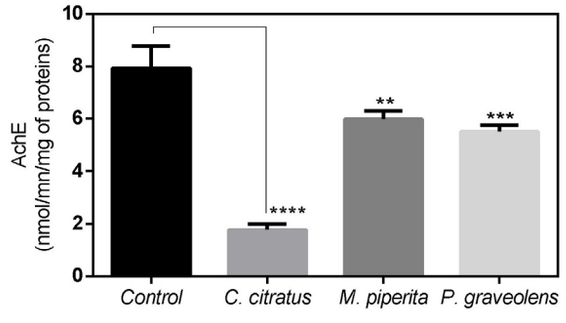


Fig. 4: Specific activities of acetylcholinesterase in *P. ficus* adults after 24 h of exposure to the *lethal* concentration LC_{50} of *C. citratus*, *M. pipertia*, and *P. graveolens* essential oils. Insects were treated using fumigation method. The data represent mean ± SD of 10 insects per treatment. Statistical analysis consisted of one-way ANOVA. Significant differences ($P < 0.01$, $P < 0.001$ and $P < 0.0001$) are indicated flagged with asterisks (**, *** and ****, respectively) (Tukey's test). Asterisks represent comparison between control and different tested plants for the same concentration.

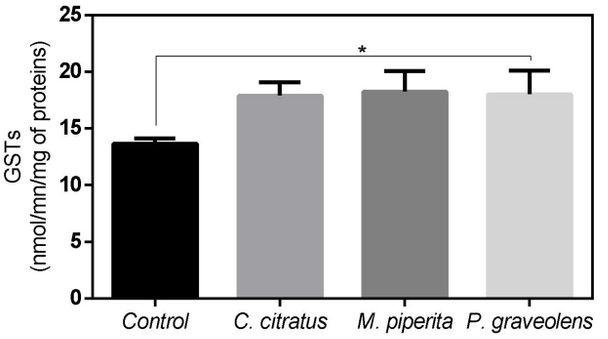


Fig. 5: Specific activities of gluthatione *S*-transferases in *P. ficus* adults after 24 h of exposure to the *lethal* concentration LC_{50} of *C. citratus*, *M. pipertia*, and *P. graveolens* essential oils. Insects were treated using fumigation method. Data represent mean ± SD of 10 insects per treatment. Statistical analysis consisted of one-way ANOVA. Asterisk (*) indicate significant difference ($P < 0.01$) (Tukey's test). Asterisks represent comparison between control and different tested plants for the same concentration.

(Fig. 6). Results showed significant difference after exposure of the adult-stadium *P. ficus* to the EOs lethal concentration compared to the control group. MDA increased significantly after 24 h of exposure especially with *C. citratus* EOs reaching 3.77 ± 0.23 µmol·mg of protein^{-1}·g of tissue^{-1} *versus* 2.69 ± 0.037 µmol·mg of protein^{-1}·g of tissue^{-1} in control insects at the same exposure time (Fig. 6) with significant statistical differences ($F_{(3, 8)} = 16.26$, ddl = 3, P = 0.0009) (Fig. 6). Data analysis also revealed that MDA accumulation did not differ significantly between the insects exposed to *M. piperita*, and *P. graveolens* essential oils and those of the control group (Fig. 6).

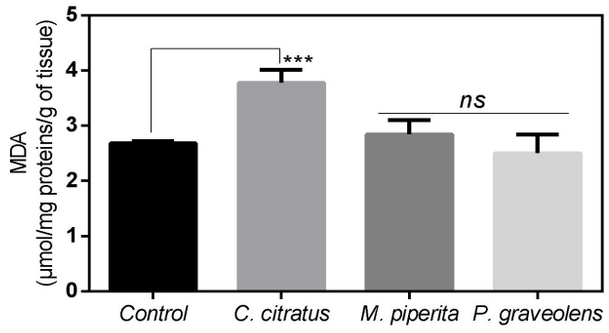


Fig. 6: Malondialdehydes accumulation in *P. ficus* adults after 24 h of exposure to the *lethal* concentration LC_{50} of *C. citratus*, *M. pipertia*, and *P. graveolens* essential oils. Insects were treated using fumigation method. Data represent mean ± SD of 10 insects per treatment. Statistical analysis consisted of one-way ANOVA. Asterisks (***) indicate significant difference ($P < 0.001$) (Tukey's test). Asterisks represent comparison between control and different tested plants for the same concentration.

PCA using biochemical biomarkers: Results of PCA using biochemical biomarkers data of *P. ficus* adults exposed to the lethal concentration (LC_{50}) of EOs revealed that the first axis (69.16 %) was mainly influenced by AChE and MDA, while GSTs mainly composed the second axis (24.3 %). Insects treated with *C. citratus*, *M. pipertia*, and *P. graveolens* were clearly separated from control one. Moreover, insects treated with *C. citratus* EO also constitute a separate group. Adults exposed to the EOs were characterized by a significant activation of GSTs activity, a high MDA accumulation, and an inhibition of AChE. It can be concluded that the adulticidal activity of *C. citratus* is due essentially to neurotoxicity following inhibition of AChE, and hyperaccumulation of MDA induced by oxidative stress following treatment (Fig. 7).

Correlation between biomarkers and mortality: Tested EOs seems to induce general stress in *P. ficus* adults, as evidenced by oxidative stress biomarker assays. The most significant result was the neurotoxic action exerted by EOs especially those extracted from *C. citratus*. To further identify the mechanisms of action of these substances, a correlation matrix showing the relationship between insect mortality upon treatment with *C. citratus* EO and oxidative stress biomarkers, was performed using the corrplot function in R software. The correlation matrix showed that the adulticidal property of *C. citratus* EO was negatively correlated with AChE activity. Indeed, as illustrated in the correlogram plot, the mortality rate correlates well with the significant decrease in the AChE activity of EO-treated adults. It appears that exposure to *C. citratus* EO affect the nervous system of treated VM resulting in a lethal neurotoxic action (Fig. 8).

This study investigated the chemical composition of three plant essential oils and assessed their insecticidal and physiological impacts on adults of the vine mealybug *P. ficus* under laboratory conditions. Our findings revealed that in both contact and fumigant bioassays, *C. citratus*, *M. piperita*, and *P. graveolens* EOs exhibited a toxic effect against *P. ficus* adults. Probit analysis showed that the highest insecticidal effects were recorded using the lemongrass *C. citratus* EO where LC_{50} values reached 17.01 $\mu L \cdot L^{-1}$ air against 24.52 and 26.27 $\mu L \cdot L^{-1}$ air for *P. graveolens* and *M. piperita*, respectively. *Cymbopogon citratus*-derived EO was previously reported as being effective, with contact and fumigant toxicity against several insect pests including mealybugs (Pumnuan and Insung 2016). Furthermore, chemical composition analysis of *C. citratus* oil by GC-MS revealed citronellal, geraniol, and limonene as principal components in agreement with previous studies on terpenoids from lemongrass essential oil (Kumar *et al.* 2013).

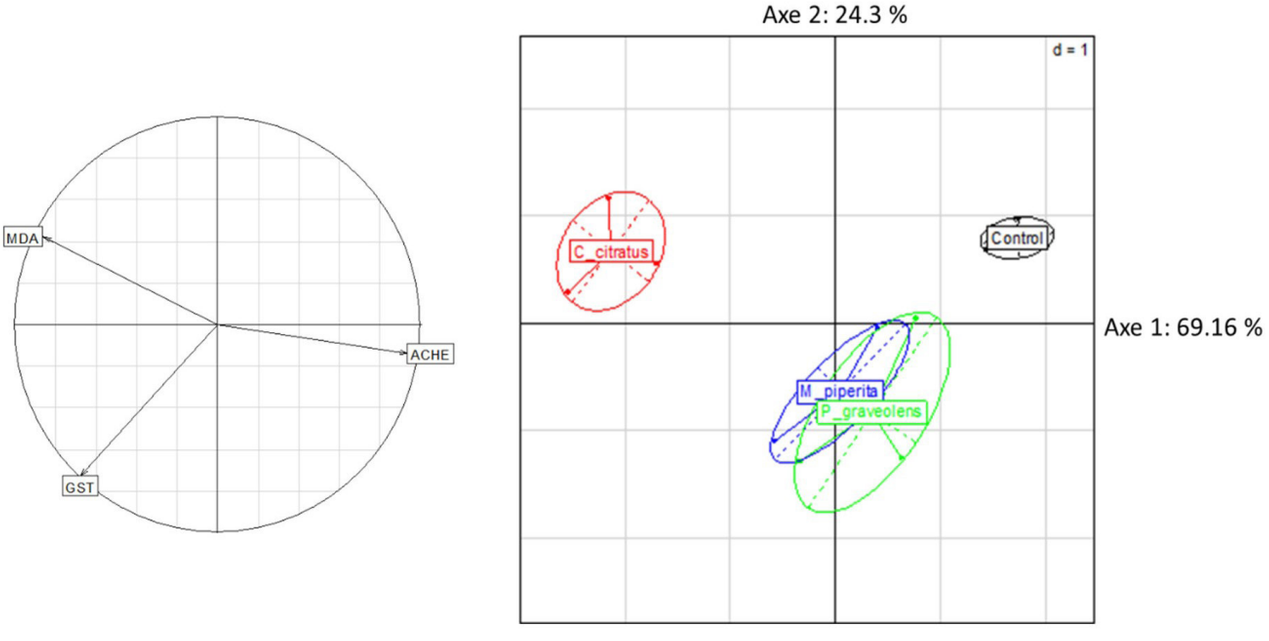


Fig. 7: Principal component analysis of oxidative stress biomarkers evolution in the vine mealybug *P. ficus* adult tissues after 24 h of exposure to the *lethal* concentration LC_{50} of *C. citratus*, *M. pipertia*, and *P. graveolens* essential oils. Insects were treated using fumigation method. The plot of the oxidative biomarkers (left) and the plot of the different treatments (right) are represented.

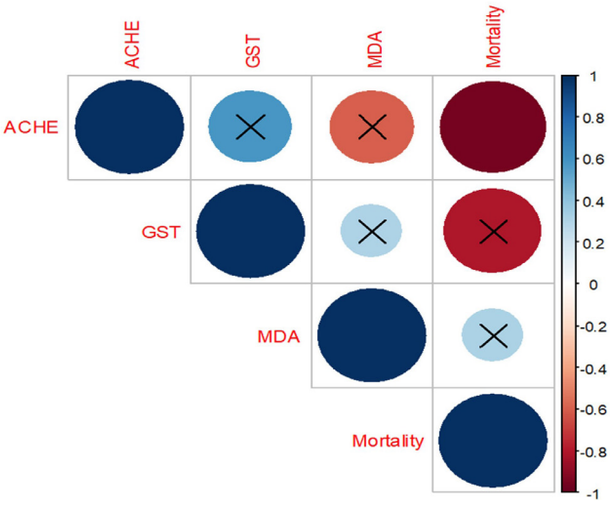


Fig. 8: Correlation matrix highlighting the relationship between the vine mealybug *P. ficus* adult mortality and the different oxidative stress biomarkers analysed. The visualization of the correlation matrix was made by corrplot method in R software. Positive correlations are displayed in blue and negative correlations in red color. Color intensity and the size of the circle are proportional to the correlation coefficients.

Concerning insecticidal activity, Plata-Rueda *et al*. (2020) showed that lemongrass essential oil was toxic to the adults of the tenebrionid beetle *Ulomoides dermestoides* (Chevrolat) and exerted a strong effect by topical application (LD_{50} = 5.17 µg·insect^{-1} and LD_{90} = 19.1 µg·insect^{-1}). *C. citratus*-derived EO was also evaluated against *Musca domestica* L. (Diptera: Muscidae) through contact and fumigant toxicity assays. Results showed LC_{50} value of 0.41 µL·cm^2 and of percentage inhibition rate (PIR) of 77.3 %. Fumigation assay was comparatively more effective with LC_{50} of 48.6 µL·L^{-1} air against housefly larvae, and a PIR value of 100 % against pupae. Similarly, *C. citratus* EO was reported to be effective against the mealybug *Pseudococcus jackbeardsleyi* Gimpel and Miller (Hemiptera: Pseudococcidae) with an LC_{50} of 1.58 µL·L^{-1} air (Pumnuan and Insung 2016).

The mode of action of lemongrass essential oil have not been fully elucidated, but it is probable that its neurotoxic effect on *P. ficus* is due to the presence of terpenoids, resulting in rapid insect mortality, as reported for other insects treated with plant essential oils (Tak *et al.* 2017, Brügger *et al.* 2019, De Souza Alves *et al.* 2019).

Results obtained in this study also demonstrate the adulticidal activity of *M. piperita* and *P. graveolens* EOs as potential VM control agents. The compositional analysis of oils showed that menthol and geraniol are the major bioactive components, respectively. Jeon *et al*. (2009) studied the acaricidal activities of geraniol from the oil of *P. graveolens* against the storage food mite, *Tyrophagus putrescentiae* (Schrank) (Acari: Acaridae) compared to the commercial acaricide, benzyl benzoate. Results revealed that geraniol was more effective than benzyl benzoate with the 50 % lethal dose value being 1.95 µg·cm^3 and 1.27 µg·cm^3, respectively. The mechanism of the insecticidal effects of geraniol was investigated by testing its neurophysiological effect on the American cockroach *Periplaneta americana* (L.) and on the discoid cockroach *Blaberus discoidalis* (Serville). Similarly, Samarasekera *et al*. (2008) showed the insecticidal activity of essential oil of *M. piperita* against local mosquitoes [*Culex quinquefasciatus* (Say), *Aedes aegypti* L. and *Anopheles tessellatus* (Theobald)] and was linked to the presence of menthol as major compound. Menthol insecticidal activity was also demonstrated against stored-products pests by inducing central nervous system excitation (Lin *et al*. 2002). Several other plant-derived EOs were also reported to have high toxicity against the VM. For instance, Karamaouna *et al*. (2014) found that essential oils from citrus, peppermint and thyme-leaved savory were more or equally toxic compared to the paraffin oil considered as reference product. Furthermore, Peschiutta *et al*. (2017) assessed the toxicity of Eos extracted from *Minthostachys verticillata* (Griseb) Epling (Lamiaceae) and *Eucalyptus globulus* Labillardiere (Myrtaceae) on females of *P. ficus* and concluded that the former was more toxic than *E. globulus* EO, while pulegone was more toxic than the other constituents of the EO studied. In field assays, Tacoli *et al*. (2018) also observed a reduction in VM population density on grape leaves sprayed with a citrus EO-based insecticide. Neem-derived oil-based insecticides are also used in several countries against this pest to control young instars, which are less protected by waxy covering (Flint 2016). Essays with other EOs extracted from eucalyptus, lavender and basil were not effective against the VM (Karamaouna *et al*. 2013, Peschiutta *et al*. 2017).

Results of the current study showed also that the EOs particularly from *C. citratus* affect the nervous system of VM adults as evidenced by the significant inhibition of AChE the activity. In similar studies, EOs treated insects had muscle contractions and altered locomotion ability, followed by irrecoverable paralysis indicating neurotoxic effects (Wang *et al.* 2004, Rattan 2010). The neurotoxic modes of action on insects are mainly related to AChE levels and several studies demonstrated the interference of essential oils and monoterpenes with AChE enzyme activity in insects (Yeom *et al.* 2013).

We found that EOs significantly increased the Glutathione *S*-transferases activity in treated VM. GSTs are involved in detoxification of xenobiotics and physiological processes such as intracellular transport, biosynthesis of hormones and protection against oxidative stress (Enayati *et al.* 2005). Numerous studies reported that GST is activated in many insects exposed to chemical insecticides, plant allelochemicals and pathogens (Qin *et al.* 2011, Acheuk *et al.* 2018). This is consistent with our results showing high GST activities in *P. ficus* adults exposed to plant EOs (Qin *et al.* 2013). Moreover, our findings showed a significant alteration in the cell membrane of treated *P. ficus* adults as recorded by MDA contents. Indeed, MDA is the major aldehyde metabolite of lipid peroxidation rate that reflects the degree of membrane alterations from free radicals (Wang *et al.* 2016). Overall, the bioassays established insecticidal efficacy of plant-derived essential oils for vineyard mealybug *P. ficus* control through contact toxicity as well as fumigation assay. The study demonstrates potentiality of EOs for the development of alternative plant protection strategies against mealybugs. They can be used alongside chemical

insecticides to reduce impacts on non target organisms and to reduce the emergence of resistant populations while reducing residues in fruits.

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Conflict of interest

The authors declare that they have no competing interests.

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