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Spilanthol-rich essential oil obtained by microwave-assisted extraction from *Acmella oleracea* (L.) R.K. Jansen and its nanoemulsion: insecticidal, cytotoxic and anti-inflammatory activities

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Abstract

Acmella oleracea (L.) R.K. Jansen, also named jambù, is an edible and medicinal plant native to Brazil but extensively cultivated all over the world due to its diverse utilizations in food, cosmetics, pharmaceuticals, and pest management science. Most of applications are related to the presence of active ingredients, namely alkylamides, among which spilanthol is the most important one. On the other hand, the plant biomass resulting from the large-scale cultivation of *A. oleracea* may be also used to yield an essential oil (EO) which can be used for some industrial purposes, for instance as larvicide against mosquitoes. This EO is mainly characterized by mono- and sesquiterpenes, however also spilanthol can be detected in the mixture. To maximize the content of spilanthol in the *A. oleracea* EO, *A. oleracea* aerial parts obtained from a cultivation in central Italy were subjected to microwave-assisted extraction (MAE). This procedure allowed to obtain a higher yield and spilanthol content when compared with traditional hydrodistillation (0.47 v 0.22%, and 13.31 vs 2.24%, respectively). The EO terpene fraction was mainly represented by β -pinene (10.8%), myrcene (12.3%), (*E*)-caryophyllene (19.4%) and α -humulene (1.1%). The *A. oleracea* EO was encapsulated into a nanoemulsion (NE) containing 6% EO using a high-energy method. The formulation physical stability was assessed by optical microscope and DLS analyses at different timepoints showing stability up to one year of storage. The *A. oleracea* EO and its NE, along with the isolated spilanthol, were evaluated for acute toxicity against the 3rd instar larvae of *Culex quinquefasciatus*, a filariasis vector of public health importance, achieving LC₅₀ values of 16.1, 407.5, and 3.1 μ L/L, respectively. The larvicidal activity seemed to be not dependent on the interaction with the cholinergic system, as shown by the high IC₅₀ values of *A. oleracea* EO and spilanthol exhibited in the anti-acetylcholinesterase (AChE) assay. Testing LC₃₀ values, the *A. oleracea*-borne products showed a significant impact on larval development, fecundity, fertility and potential natality of *C. quinquefasciatus*. Testing LC₃₀ values, the *A. oleracea* EO-NE provided a higher efficacy than spilanthol,

reducing the adult fertility, in terms of egg hatchability (%) and the overall abundance of F₁ larvae. These results can be considered as promising for the development of new mosquito larvicides. The safety use of *A. oleracea* EO, NE and spilanthol was demonstrated in assays on mammalian fibroblasts and microglia cells, showing low level of cytotoxicity coupled with protective effects against inflammation.

Keywords: essential oil nanoemulsion; jambù; Culicidae; *Culex quinquefasciatus*; larvicide; mosquito control

1. Introduction

Acmella oleracea (L.) R.K. Jansen (syn. *Spilanthes acmella* (L.) L.), encompassed in the Compositae (Asteraceae) family, is an aromatic and medicinal plant typical of Brazil and Perù. Nowadays, it is also spread worldwide, as in Asia (China, India and Taiwan), Central America (Mexico), and Africa (Dubey et al., 2013). Common names of this plant are jambù, ‘paracress’ or ‘toothache plant’, referring to its well-recognised uses in the treatment of tooth pain (Jayaweera, 1981). Being a perennial herb in the mediterranean and tropical areas, if the winter temperatures don't go below the 0°C, 30-60 cm in height, *A. oleracea* shows decumbent or erect stems, which are tomentose only at the top. The leaves are simple, opposite, oval to deltate and with dentate borders and glabrous petiole. The yellow inflorescences are discoid with pedunculate flower heads which bloom from July to October (Benelli et al., 2019a; Matyushin et al., 2017).

Acmella oleracea finds several applications in horticulture, cuisine, arthropod pest management, beside also in cosmetic and pharmaceutical field. It is classified as safe by the European legislation (EFSA Panel on Food Contact Materials, Enzymes Flavourings and Processing Aids, 2015). It is a botanical that can be also used in the preparation of food supplements from the Italian Ministry of Health (<http://www.gazzettaufficiale.it/eli/gu/2018/09/26/224/sg/pdf>), being also included in the BELFRIT list

(<https://www.trovanorme.salute.gov.it/norme/renderNormsanPdf?anno=0&codLeg=48636&parte=3&serie>). The traditional uses of jambú go from local anesthetic, diuretic, analgesic, aphrodisiac, fungicidal to insecticidal agent (Paulraj et al., 2013; Rondanelli et al. 2020; Uthpala and Navaratne, 2020). The main biological activities of this plant are linked to its major secondary metabolite, which is (2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrienamide, named also spilanthol, which belongs to the chemical class of alkylamides. Spilanthol displayed several biological properties such as antioxidant, antimicrobial, antinociceptive and insecticidal activities (Dias et al., 2012; Matyushin et al., 2017; Molina-Torres et al., 2004; Paulraj et al., 2013; Ratnasooriya et al., 2004; Silveira et al., 2018). For the above-mentioned reasons, several uses of this molecule can be found at the industrial level. The major increase of its applications rose in the years 2005 and 2007; then 2014 was characterized by the highest number of patents related to spilanthol, demonstrating its large employment in cosmetic, food and pharmaceutical industries (Silveira et al., 2018). Moreover, the intake of spilanthol as flavoring agent in the European industry is of 24 mg/capita/day (EFSA, 2015). Spilanthol is usually obtained from plants, but is also commercially available, e.g. for US\$ 125.00/5 mg (ChromaDex[®], USA, 2017).

Besides the promising alkylamide found in *A. oleracea*, an essential oil (EO) can also be obtained from this plant especially from inflorescences, leaves and stems. One of the main extraction technique used to obtain EOs is hydrodistillation (HD), but, despite its large use, displays some disadvantages. Firstly, it is a time-consuming process, needing energy and water consumption, and often causing the loss of thermosensitive compounds (Filly et al., 2014). Recently, microwave-assisted extraction (MAE) resulted as a novel, solvent-free and green extraction procedure able to enhance the yield of volatile organic compounds (VOCs) from matrices of different nature (Lucchesi et al., 2004). This extraction process relies on microwave radiation, which causes the vibration of polar molecules and of water within the cells of the matrix. This vibration leads the achievement of higher temperatures and to the vaporization of water that disrupt the cell membranes, with the consequent release of VOCs. This process usually leads to higher yields and

also lower costs (Filly et al., 2014). In addition, the selection of the optimal operative conditions can usually modulate the chemical profile of the obtained EO (Fiorini et al., 2020). The EO of *A. oleracea* is gaining more and more attention for its biological properties, especially for its insecticidal activity. Even if it has been poorly investigated before, its chemical composition and biological activity have been recently studied (Benelli et al., 2019a), showing the effectiveness of the EO as insecticide against the southern house mosquito, *Culex quinquefasciatus* Say (Diptera: Culicidae), the African cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), and the common housefly, *Musca domestica* L. (Diptera: Muscidae).

Currently, mosquito control is facing many key challenges, including the fast spread of highly invasive species (Wilke et al., 2020), the onset of insecticide resistance in several vectors (Hemingway et al., 2016; Benelli et al., 2021), anthropogenic habitat changes (Wilke et al., 2019; Orta-Pineda et al., 2021), and serious non-target effects of broad spectrum pesticides (Desneux et al., 2007; Bolzonella et al., 2019; Taning et al., 2019; Varikou et al., 2019, 2020). In this alarming scenario, the number of botanical-based insecticides, with special reference to EO-based ones, available for mosquito control programs remains low (Petrovic et al., 2019; Benelli, 2020a; Isman, 2020a). This is partly linked to regulatory barriers at national and international level (Isman, 2006; Pavela and Benelli, 2016), as well as to the limited stability of EO active ingredients in real-world conditions, thus needing the development of proper micro- and nanoformulations to magnify the EO effectiveness and stability (Campolo et al., 2017; Pavoni et al., 2019).

On the above, in the present study we evaluated different products extractable from the *A. oleracea* plant biomass obtained from a central-Italy cultivation. The EO and its encapsulated form (nanoemulsion, NE), as well as the hexane extract and its main alkylamide spilanthol, were tested for their efficacy as larvicides against *C. quinquefasciatus* Say (Diptera: Culicidae), an important vector of lymphatic filariasis and of several arboviruses, for which Integrated Vector Management (IVM) tools with proven epidemiological impact need to be urgently implemented (Wilke et al., 2020). Furthermore, we evaluated the sublethal effects of the above-mentioned *A. oleracea*-borne

botanicals on *Cx. quinquefasciatus* larval mortality, as well as on the emergence rates, fecundity, fertility and natality of the new generation adults. Lastly, to support safety of these products for possible use in real world applications related to IVM the cytotoxicity on a panel of mammal cells, the anti-inflammatory effects in microglial cells and the anti-acetylcholinesterase activity were determined as well.

2. Materials and Methods

2.1. Chemicals and reagents

Analytical standards used for the characterization of *A. oleracea* EO and extract were acquired from Merck (Milan, Italy). Alkanes standard mixture (C₇-C₃₀) and solvents were all obtained from Merck. High glucose Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin, glutamine, LPS from *Escherichia coli* serotype O127:B8, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), L-glutamine, H₂O₂, dimethyl sulfoxide (DMSO), were purchased from Merck. Low-Endotoxin FBS was purchased from Euroclone (Milan, Italy).

2.2. Plant material

A. oleracea var. *oleracea* plants in nursery plant tray were planted in the 2nd decade of May 2019 in the flat ground of the Middle-end section of the riverbed of the Musone river, in the Municipality of Castelfidardo (AN) at 38 m a.s.l. The plantation was exposed to the South, in a sandy-silty ground. The station was characterized by a sub-Mediterranean-low mesotemperate climate. The fertilization was moderate, using organic, enzymatic and microbiological fertilizers, but also NPK water-soluble organic fertilizer. The cultivation required medium-high irrigation, with a periodic cadence every 1-2 days in presence of water stress. The plantation was protected by floral biomass removal. The harvest was manual and was conducted at 70% of the flowering phase, as the colour of the flowers turned red. It was performed cutting the flower stems and harvesting a limited leaf biomass, from September to October 2019, with a harvesting time every 10-14 days. The plant material was then

dried in open environment (hangar) with protection from solar irradiation and in open environment with no solar protection (greenhouse with plastic coating). The drying duration was approximately of 5-6 days.

2.3. Microwave-assisted extraction (MAE)

The MAE was conducted with a Milestone ETHOS X (Milestone, Italy) advanced microwave extraction system. Frozen plant material (1.750 kg) was left thawing in the 12 L glass reactor (Pyrex) for 30 min before the extraction process, which was then conducted at 1800 W for 160 min using the 'Fragrances set-up'. These conditions were previously set up according to the manufacturer's instructions (e.g., 1 W/g fresh matter) and time until no condensation and internal combustion occurred. After the extraction, the EO was separated from water, collected in a PTFE-silicon septum vial, and kept at 4°C. The EO was obtained in 0.47% yield (w/w dw). The moisture percentage in the biomass was calculated putting the plant in oven at 110°C for 24 h.

2.4. Hydrodistillation (HD)

For the isolation of the EO through HD, 2 kg of fresh plant material were distilled with 12 L of distilled water and subjected to hydrodistillation with a Clevenger-type apparatus for 4 h until no more EO was obtained. The distillation system was heated in a mantle system Falc MA (Falc Instruments, Treviglio, Italy). After the extraction time, the EO was dehydrated with Na₂SO₄ and stored at 4°C in PTFE/silicon cap vial. The EO was isolated in 0.22% yield (w/w dw). The moisture content was calculated as previously reported at Sect. 2.3.

2.5. Preparation of *A. oleracea* extract

Dried and crushed flowers, stems and leaves of *A. oleracea* were extracted with *n*-hexane (1:10 w/v), using an ultrasound bath (Analogic ultrasonic bath Mod. AU-220, ARGOLAB, Carpi, Italy) at 40°C for 3 h. Afterwards, the extract was filtered on cotton and then under vacuum using a Buchner funnel equipped with filter paper. The solution was evaporated to dryness using a rotary evaporator (Buchi Rotavapor R-200, Büchi Labortechnik AG, Flawil, Switzerland), keeping the bath temperature under 40°C; then it was weighted and treated with 50% (w/w) of activated

charcoal. The residue (12.9 g) was dissolved into *n*-hexane (50 mL), added with activated charcoal (6.45 g) and then placed into a warm bath at 30°C with constant stirring for one hour. After that time, the mixture was filtered on Celite[®] and then evaporated to dryness. The extract was stored at 4°C until use.

2.6. Isolation and characterization of spilanthol

The purification of spilanthol from *A. oleracea* extract was performed through a flash silica gel column chromatography (230-400 mesh, Merck), using a gradient solvent system of hexane/ethyl acetate 90:10 to 80:20. In particular, 1 g of the extract obtained from dry leaves of *A. oleracea* was purified, yielding 158 mg of pure spilanthol, whose chemical structure was confirmed by NMR and MS analyses, which were in line with the data of Alonso et al. (2018). For the assessment of the purity of spilanthol obtained from the flash column chromatography, HPLC-DAD analysis was performed. In particular, 3.6 mg of spilanthol were dissolved in 2 mL of methanol. The analysis was performed using a Phenomenex Luna C₁₈ column (4.6 x 150 mm), the injection volume was 0.5 µL and the flow rate was of 1 mL/min; while the mobile phase used was CH₃CN:H₂O = 70:30. The purity was assessed using different wavelengths (210, 220, 230, 240, 250, 270 nm), obtaining a purity of 100% at 270 nm and a purity of 96.60% at 210 nm.

2.7. Gas chromatography–mass spectrometry (GC–MS) analysis of *A. oleracea* essential oil

The chemical analysis of the two EOs obtained from *A. oleracea* by MAE and HD was performed with a gas chromatograph coupled to a mass spectrometer detector (GC-MS, Agilent 6890N equipped with a 5973N single quadrupole detector). The analysis conditions used and the identification of the EO chemical constituents were linear with the work of Benelli et al. (2019a).

2.8. Quantification of the *A. oleracea* essential oil marker compounds by GC-FID analysis

Eos obtained from HD and MAE together with the extract were analysed through GC coupled to flame ionization detector (FID) to quantify α -pinene, β -pinene, myrcene, 1,8-cineole, (*E*)- β -ocimene, terpinolene, (*E*)-caryophyllene, humulene, germacrene D, caryophyllene oxide and spilanthol. The analysis was conducted using an Agilent 6850 GC series GC-FID. EO (50 µL) was

dissolved in 5 mL of analytical grade *n*-hexane and the solution (0.5 μ L) injected into the GC. Instead, for the analysis of the extract a preliminary transmethylation was performed. The extract (15 mg) was dissolved in 1 mL of analytical grade *n*-hexane. Then, 2 N KOH solution in methanol (0.1 mL) was added and the solution mixed vigorously with the help a vortex device for 2 min. The reaction was then quenched by the addition of 1.5 mL of a saturated brine, and the mixture shaken for 2 min and then centrifuged for 5 min (5000 rpm). The organic phase was finally recovered analysing each sample in duplicate, injecting 0.5 μ L. For all the analysed samples the GC method was the same. A generator PGH2-250 (DBS Analytical Instruments, Vigonza, Italy) was used for the production of the carrier gas (hydrogen) which flowed at 3.7 mL/min. The injector was set at 300°C using the split mode (1:30). The analytical conditions used for separation and quantification of *A. oleracea* EO main constituents were the same of those previously reported (Fiorini et al., 2020), with the additional analysis of spilanthol. Whenever analytical standards were not available, calibration curves of representative compounds for each chemical classes have been used for quantitative purposes.

2.9. Chiral chromatography

The enantiomeric distribution of α -pinene, β -pinene, limonene, β -phellandrene, (*E*)-caryophyllene, and caryophyllene oxide in the two EOs was determined by GC-MS using a chiral column (HP 20B, 30 m l. x 0.25 mm i.d., 0.25 μ m f.t.) from Agilent. The GC-MS analytical conditions were the same of those reported in section 2.7. The identification of the enantiomers of the above compounds was performed comparing RT and RI (calculated respect to a mixture of C₇-C₃₀ *n*-alkanes, Merck) with those of analytical standards (Merck) and with literature data.

2.10. Nuclear Magnetic Resonance (NMR) and High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) analysis of the *A. oleracea* hexane extract

The main alkylamides occurring in *A. oleracea* hexane extract were determined by NMR using a protocol recently published (Sut et al., 2020). Briefly, exactly weighted amount of 10-15 mg of *A. oleracea* extract were dissolved in 1 ml of deuterated chloroform, adding exact amount (1.0-1.5 mg)

of caffeine as internal standard. ^1H NMR was acquired at room temperature using calibrated P1 and optimized spectral width, using 6 s of D1 to allow complete relaxation of the different proton signals. Data were used to calculate the amount of total alkylamides (mostly represented by spilanthol) using the integral of H-2 of alkylamides (δ 5.81, CH, d) and one of the signal of methyl group of caffeine (δ 3.61, CH₃, s). Integrals were normalised on the base of signal multiplicity (3H for methyl group, ^1H for the alkylamide CH) and molecular weight of the compounds. For the quantification of alkylamides, spilanthol (MW 221) was considered as the most abundant compound and used as reference to calculate the total amount. On the other hand, for the analysis of alkylamides by LC-MS, the extract was dissolved in methanol (10 mg/10 mL) by 10 min sonication, then centrifuged at 13000 g for 10 min; supernatant was used for analysis. For the analysis, a Phenomenex RP-MAX 150 x 3.0 mm (4 μm particle size) column was used as stationary phase. As mobile phases acetonitrile (A) and water 0.1% formic acid (B) were used, with the following gradient: min 0 (15% A, 85% B); min 33 (85% A, 15% B); min 33.5 (15% A, 85% B) with 4 min of re-equilibration time. Flow and injection volume were 0.4 mL/min and 10 μL , respectively. The MS 500 ion Trap was used in positive ion mode for the identification of alkylamides in a range of 100-700 Da in TDDS mode (Turbo Detection Data Scanning) that allows the acquisition of fragmentation pathway of each ion. Fragments of ions were compared with that reported by Boonen et al. (2010) and each peak was assigned. Amount of each compound was calculated on the base of the relative peak area and the total alkylamide derivatives were calculated by NMR.

2.11. *Acmella oleracea* essential oil formulation

2.11.1. Encapsulation of the *A. oleracea* essential oil in nanoemulsions (NEs)

Acmella oleracea EO NEs were prepared using a high-energy method. EO and ethyl oleate at a ratio of 0.5 were added drop by drop to a polysorbate 80 (P80, Sigma-Aldrich) water solution under stirring at high-velocity (Ultraturrax T25 basic, IKAfi Werke GmbH & Co.KG, Staufen, Germany) for 5 min at 9500 rpm. A French Pressure Cell Press (American Instrument Company, AMINCO, MY, USA) was used to homogenize the obtained emulsion using a pressure of 130 MPa for four

times. NEs were prepared by changing the percentage of the oil phase from 6 to 9% (w/w) and the percentage of P80 from 0.2 to 0.5% (w/w) (Table 1).

The visual inspection of formulation and the assessment of the formation of NEs were performed by a MT9000 polarizing optical microscope (Meiji Techno Co Ltd., Japan) endowed with a CMOS camera (Invenio 3S, DeltaPix, Denmark) at 10X magnification. The internal phase droplet size was evaluated by a Zetasizer nanoS (Malvern Instruments, Worcestershire, UK) coupled with a backscattered light detector working at 173°. One mL of formulation was analyzed thrice at 25°C using an equilibration time of 180 s (Benelli et al., 2020b; Kavallieratos et al., 2021). Observation by optical microscope and DLS analyses were performed at different intervals (i.e. 0, 15, 30, 90 and 360 days, T0, T15, T30, T90, T360, respectively) to evaluate the physical stability at room temperature of the formulations.

2.11.2. Head Space-Gas-Chromatography/Mass spectrometry (HS-GC/MS) analysis

To study the stability of the *A. oleracea* EO NEs over time, we analysed the vapor phase of pure and encapsulated *A. oleracea* EO, using an autosampler (Perkin-Elmer Headspace Turbomatrix 40) coupled to a GC-MS Clarus 500 (Waltham, MA, USA) (Garzoli et al., 2020). For the determination of VOCs, 2 mL of the sample were placed into 20 mL vials equipped with headspace PTFE-coated silicone septa. The headspace parameters were applied as follows: 80°C thermostating temperature for 20 min; 3.0 min pressurization time and 0.3 min of injection time. A Varian Factor Four VF-1 fused-silica capillary column (length 60 m x 0.32 mm ID x 1.0 µm film thickness) was used for separation using helium (1.0 mL/min) as carrier gas. The column temperature was programmed at 60°C and increased to 220°C at a rate of 5°C/min, and held 30 min and the injector temperature was 280°C; the ionization energy was 70 eV, ion source temperature was 200°C and with mass range of 40.0–500.0 AMU. The components were identified by comparing their mass spectra with that available in MS libraries search (ADAMS, WILEY 275 and NIST 17). Furthermore, alkanes (C₈-C₃₀) were used to calculate the linear retention indice (LRI) of compounds by the same conditions described above. The VOCs percentage was obtained by peak area normalization. Data were processed by One-way ANOVA followed by post-hoc Tukey HSD test with $p < 0.05$ as significant.

2.12. Insecticidal assays

2.12.1. Acute toxicity on *C. quinquefasciatus* larvae

Mosquitoes were reared using the same protocol reported by Benelli et al. (2019b). Larvicidal assays were consistent with the WHO (1996) method with slight modifications (Pavela et al., 2020). The *A. oleracea*-borne products (EO, EO-NE, hexane extract and spilanthol) were diluted in dimethyl sulphoxide (DMSO), then in water (i.e. the EO and spilanthol), or directly in water (i.e. the EO-NE and the extract), preparing a serial dilution of test concentrations (a series of 5 concentrations ranging from 10 to 30 $\mu\text{L/L}$, from 300 to 900 $\mu\text{L/L}$, from 5 to 20 $\mu\text{L/L}$ and from 1 to 10 $\mu\text{L/L}$ for the EO, EO-NE, extract and spilanthol, respectively). Third instar larvae were transferred in distilled water (25 mL). A glass bowl (500 mL) containing a homogeneous solution of 224 mL of distilled water plus 1 mL of different *A. oleracea*-borne product concentrations was used; afterwards, larvae were transferred into the prepared test solution (25 larvae/becker, surface area of 125 cm²). Four replicates (25 larvae/replicate) were done testing the following LC₃₀, i.e. 12.4, 343.7, 7.3, and 2.3 $\mu\text{L/L}$ for EO, EO-NE, extract and spilanthol, respectively. A growth chamber (16:8 (L:D), 25°C) was used for experiments. The mortality was calculated 24 h post-exposure, with no food given to the larvae (Benelli et al. 2019b). Decis Forte containing 100 g/L (10.5%) of deltamethrin (Bayer S.A.S., France) was used as positive control (Rahman and Howlader, 2018).

2.12.2. Effect of lethal concentrations on larval development and fertility of new adults

According to the method by Benelli et al. (2017), early 3rd instar larvae of *C. quinquefasciatus* were transferred into a plastic bowl (20 × 20 × 20 cm) filled with water (3 L). After 1 h, the calculated LC₃₀ for the EO, EO-NE, extract and spilanthol (i.e. 12.4, 343.7, 7.3, and 2.3 $\mu\text{L/L}$, respectively) were added to the water. EO, extract and spilanthol were diluted in DMSO. The negative control was water containing the same amount of DMSO. Each trial, including 100 larvae, was conducted thrice. 24 h-post treatment, larvae were transferred into clean water containing dog biscuits and yeast powder (3:1, w:w) as larval food, till adult emergence. Mortality was calculated 24 and 48 h

post-treatment. In addition, final mortality, emerged adults (%), and their sex were noted (Benelli et al., 2017). For the evaluation of the exposure effect to LC₃₀ of *A. oleracea*-borne products on the mosquito fecundity, fertility and natality, the method by Benelli et al. (2017) was followed. The adults of both sexes were inserted in breeding cages (25 × 25 × 30 cm) and fed following Benelli et al. (2017). The oviposition was evaluated in a bowl (10 cm in diameter filled with water) placed in the cage, and the number of laid eggs was determined every day using a Leica light microscope. Each experiment was replicated 4 times in a growth chamber (16:8 (L:D), 25 ± 1 °C) (Benelli et al., 2017).

2.12.3. Statistical analysis

In mosquito experiments, the Abbott's formula (Abbott, 1925) was used to correct mosquito mortality data, whereas probit analysis (Finney, 1971) was used to calculate the LC₃₀, LC₅₀, LC₉₀, regression equation and the 95% confidence limit. In sublethal assays, the percentage of mortality, emergence of adults and fecundity indicators were determined and transformed to arcsine square root values for analysis of variance. The analysis of variance (ANOVA) followed by Tukey's HSD test was used to analyse significant differences among means ($p < 0.05$).

2.13. Acetylcholinesterase (AChE) Inhibitory Activity

AChE inhibitory activity of *A. oleracea* EO, spilanthol and *A. oleracea* EO-NE samples were measured by using the Ellman assay (Ellman et al., 1961) with slight modification (Benelli et al., 2019c). All samples were diluted in methanol and used in the assay.

2.14. Cytotoxicity and anti-inflammatory effects of *A. oleracea* derivatives on human cells

2.14.1. Cell viability assay

Human breast adenocarcinoma cell line (MDA-MB 231), human malignant melanoma cell line (A375), and normal human fibroblast cell line (NHF-A12) were maintained in DMEM, with the addition of 1 mM sodium pyruvate. Murine microglial cells (BV-2) were provided by Prof. Elisabetta Blasi, University of Modena and Reggio Emilia, Italy, and cultured in DMEM. All media were supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 100 IU/mL penicillin,

100 µg/mL streptomycin, and 2 mM L-glutamine. The cells were incubated at 37°C with 5% CO₂. A375 cells, MDA-MB 231 cells, and fibroblasts were treated with different concentrations of *A. oleracea* EO (1.95-500 µg/mL), spilanthol (3.125–800 µg/mL), and *A. oleracea* HE (0.78-200 µg/mL), solubilized in dimethylsulphoxide (DMSO) for 72 h, then diluted in medium, and *A. oleracea* EO-NE (7.8-2000 µg/mL), diluted directly in medium. The BV-2 cells were treated with different concentrations of *A. oleracea* EO (0.5-500 µg/mL), *A. oleracea* EO-NE (0.001-10 mg/mL), and spilanthol (10-200 µM) for 24 h and then activated with 100 ng/ml LPS for 24 h. At the end of each experiment cell viability was evaluated by the reduction of MTT to formazan by mitochondrial succinate dehydrogenase as previously reported (Nkuimi Wandjou et al. 2020; Quassinti et al., 2013). The absorbance was measured at 540 nm using a microplate spectrophotometer FLUOstar Omega (BMG Labtech). GraphPad Prism 5 software was used to calculate the cytotoxicity that was reported as the concentration of sample reducing cell growth by 50% (IC₅₀). Cell viability of BV-2 cells was express as % of control cells.

2.14.2. DCFH-DA assay

The fluorescent DCFH-DA probe was used to determine the intracellular Reactive Oxygen Species (ROS) (Marrazzo et al., 2019) in microglial BV-2 cells. After the different experiments, 10 µM DCFH-DA in DMEM 1% FBS without phenol red were added in each well and cells. After 30 min DCFH-DA solution was replaced with PBS. Cell fluorescence was quantified ($\lambda_{\text{excitation}} = 485 \text{ nm}$ and $\lambda_{\text{emission}} = 535 \text{ nm}$) on a microplate spectrofluorometer (VICTOR3 V Multilabel Counter, PerkinElmer).

2.14.3. Real-Time polymerase chain reaction (PCR)

RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) was used to extract total RNA from BV-2 cells. RNA quality was evaluated measuring A_{260}/A_{280} and A_{260}/A_{230} with NanoVue Spectrophotometer (GE Healthcare, Milano, Italy). iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA) was used to reverse-transcribed 1 µg of total RNA to cDNA. To carry out PCR in a total volume of 10 µL were added 12.5 ng of cDNA, 5 µL SsoAdvanced Universal SYBR

Green Supermix (BIO-RAD) and 500 nM of each primer. In [Table 1SM](#) are reported the different primers used (SIGMA-ALDRICH, Milan, Italy). GAPDH was considered as the reference gene. Real-time PCR was performed using SsoAdvanced Universal SYBR Green Supermix (BioRad). The polymerase was activated at 95°C for 30 s, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. Expression levels were normalized in respect to the reference gene and compared with control cells using the $2^{-\Delta\Delta CT}$ method.

3. Results and Discussion

3.1. Chemical analyses of *A. oleracea* EOs obtained by HD and MAE

MAE allowed us to obtain a higher EO yield than conventional HD, i.e. 0.47 vs 0.22%, respectively. The chemical compositions of these EOs are reported in Table 2. The major constituents of the EO obtained by HD were monoterpene hydrocarbons (48.8%), followed by sesquiterpene hydrocarbons (38.6%), oxygenated sesquiterpenes (2.9%), alkylamides (2.3%) and oxygenated monoterpenes (0.5%); the compounds identified accounted for the 95.3% of the total composition. On the other hand, the EO obtained by MAE was mainly dominated by sesquiterpene hydrocarbons (41.2%), monoterpene hydrocarbons (35.5%), alkylamides (11.7%), oxygenated sesquiterpenes (0.9%) and oxygenated monoterpenes in traces, with the 97.8% of identified compounds. The main constituents of both EOs were (*E*)-caryophyllene, myrcene, β -pinene, germacrene D, spilanthal, β -phellandrene and (*Z*)- β -ocimene. Comparing the two chemical compositions, significant differences were detected in the percentage of spilanthal, which resulted higher in MAE EO rather than in HD EO (11.7 vs 2.3%, respectively), as shown in Fig. 1SM. Other differences between HD and MAE EOs are represented by sabinene (0.7 and 1.5%, respectively), β -pinene (14.7 and 10.8%, respectively), myrcene (17.4 and 12.3%, respectively) and germacrene D (11.8 and 15.2%, respectively). For the other chemical constituents, no differences were detected. Noteworthy, acmellonate, a ketol ester [(7*Z*,9*E*)-2-oxo-undeca-7,9-dienyl 3-methylbut-2-enoate] similar to spilanthal but devoid of the amide group, was also identified according to the MS

fragmentation pattern reported by Ley et al. (2006); its percentage was 2.1 and 2.5% in HD and MAE EO, respectively. As described in sect. 1, MAE represents an innovative, green and advantageous extraction procedure assuring higher yield and high quality profile of the EOs obtained (Filly et al., 2014; Lucchesi et al., 2004). Also in this study, MAE led to an improvement in terms of EO yield, which was double compared with the HD one. The MAE EO yield obtained in this work resulted also higher than the HD EO yield of *A. oleracea* reported by Benelli et al. (2019a), leading to the conclusion that the MAE extraction process resulted the best technique for the achievement of higher yields. Another advantage brought by the use of MAE is the possibility to change the operative conditions for the modulation of the EO chemical composition and to increase the percentage of some components of biological importance (Fiorini et al., 2019). In this work, MAE led to higher amounts of spilanthal, but also of sabinene, β -pinene, myrcene and germacrene D. These results were also confirmed by comparing the data of Benelli et al. (2019a) work, in which the HD *A. oleracea* EO showed lower amounts of spilanthal, (*Z*)- β -ocimene, δ -elemene, germacrene D and (*Z,E*)- α -farnesene.

The objective of this study was also the quantification through GC-FID analysis of the *A. oleracea* marker compounds in the HD and MAE EOs, besides also the quantification of spilanthal in the extract. Figure 1 shows that the major differences between HD and MAE EOs consisted in the spilanthal content, which was 13.31% in MAE EO and 2.24% in HD EO. Other significative differences were found in the content of (*E*)-caryophyllene, α -humulene, germacrene D, β -pinene, and myrcene. The (*E*)-caryophyllene, α -humulene, and germacrene D content was higher in the MAE EO, with a concentration of 16.34, 10.42, and 7.60%, respectively. Conversely, β -pinene, and myrcene concentrations resulted higher in the HD EO, showing values of 13.30 and 16.50%, respectively. The results obtained seem to suggest that MAE, relying on a higher penetration of microwaves through the plant matrix, can boost the extraction of fatty acid derivatives such as spilanthal. On the other hand, the content of spilanthal was lower in the EO obtained by HD since this process normally produces a strong oxidation of the fatty matter from which spilanthal is formed (Barbosa et al., 2017).

In this study, we determined also the enantiomeric distribution and the enantiomeric excess (EE) of the chiral components of the EO of *A. oleracea*, obtained by MAE. The use of a chiral column (HP chiral 20 B) coupled with GC-MS led to the successful separation and identification of the enantiomers of the following compounds (Table 3): α -pinene, β -pinene, limonene, β -phellandrene, (*E*)-caryophyllene and caryophyllene oxide. In particular, The order of elution of the enantiomers of both α -pinene and β -pinene was in accordance with data reported by Dahmane et al. (2015) using the same stationary phase; while the elution order of the enantiomers of limonene, (*E*)-caryophyllene and caryophyllene oxide was linear with the data reported by Fiorini et al. (2020). The order of elution of β -phellandrene enantiomers followed the one reported by Yassaa and Williams (2005). The present analysis showed that the EO contained both enantiomers of the monoterpenes α -pinene, β -pinene, limonene and β -phellandrene. Conversely, for the sesquiterpene (*E*)-caryophyllene and for the oxygenated sesquiterpene caryophyllene oxide, both compounds were detected only in the (-)-form. For α -pinene the (-)-enantiomeric form was predominant with an enantiomeric excess (EE) of 90.5%, as also for β -pinene whose EE of the (-)-form was of 99.6%. This is also the case of limonene, whose (+)-enantiomeric form had an EE of 69.2%, and of β -phellandrene, with an EE for the (-)-form of 74.5%.

3.2. Nuclear Magnetic Resonance (NMR) and High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) analysis of *A. oleracea* hexane extract (HE)

¹H-NMR analysis of the *A. oleracea* HE showed the occurrence of signals ascribable to alkylamides and spilanthal resulted the most abundant member of this class of compounds. The signal associated to H-2 was chosen for the NMR quantification, being the only doublet common to almost all the possible derivatives of *A. oleracea*. The total amount of alkylamides expressed as spilanthal content in the extract was of 50.9% (Fig. 2).

The assignment of alkylamides in HE was achieved through a LC-MS method. Fragmentation pathway of each ion was compared with that reported by Boonen et al. (2010) and each peak was tentatively assigned as reported in Table 4 and Fig. 2SM. Spilanthal resulted the most abundant alkylamide (42.67%), followed by (2*E*,6*Z*,8*E*)-*N*-(2-methylbutyl)-2,6,8-decatrienamide (6.1%) and (2*E*,7*Z*)-*N*-isobutyl-2,7-decadienamide

(0.89%). The results are in accordance with literature data. Firstly, the alkylamides found in the *A. oleracea* HE were also reported by Boonen et al. (2010) who analyzed the ethanolic extract of *A. oleracea*. Besides the different extraction solvents used for the extraction, the main alkylamide found in the ethanolic extract resulted to be spilanthol, followed by (2*E*,6*Z*,8*E*)-*N*-(2-methylbutyl)-2,6,8-decatrienamide. Also, the study of Barbosa et al. (2016) on *A. oleracea* methanolic extract confirmed spilanthol and (2*E*,6*Z*,8*E*)-*N*-(2-methylbutyl)-2,6,8-decatrienamide as the main alkylamide.

3.3. Development and characterization of *A. oleracea* EO nanoemulsions

Acmella oleracea EO was encapsulated in NEs to obtain a nanoformulation with a potential application as biopesticide. For this purpose, a series of formulations was prepared by varying the total oil phase amount (0.6, 0.75 and 0.9% w/w; EO to ethyl oleate ratio of 0.5) and the percentage of the surfactant (P80 at 2, 3.5 and 5% w/w) for the optimization of the amount of components in order to achieve stable *A. oleracea* EO-based NEs. All the prepared NEs (NE_1–NE_5) appeared as isotropic samples, without any presence of oil droplets above 1000 nm upon observation through polarised optical microscope both after preparation and up to 1-year (Fig. 3A), suggesting the effective reduction of the droplet size by high-pressure homogenization and the formation of nano-emulsified systems. Moreover, all NEs showed a monomodal droplet size distribution below 1000 nm, as revealed by dynamic light scattering (DLS) technique, which is maintained over time (up to 1-year), confirming the achievement of real NEs (Fig. 3B). A more detailed comparison among all NEs is presented in terms of Z-average (d.nm) and polydispersity index (PDI) (Fig. 3C). Specifically, the droplet size was below 100 nm for all formulations after preparation (T0), increasing up to 120-200 nm after 15 days (T15) and remained in this range for all the observation time (up to 1 year, T360). PDI values were between 0.2-0.3 for all formulations up to 3 months (T0-T90), showing a reduction (0.1-0.2) for a longer time (up to 1-year, T360). All these results confirmed the physical stability of all the prepared formulations as nano-emulsified systems at least up to 1-year. On the base of these findings, the nanoemulsion NE_3 containing the highest amount of oil phase (9% w/w) and the lower amount of surfactant (2% w/w of P80) was selected for further investigations.

The chemical stability of NE_3 over time was evaluated by the characterization of the headspace chemical fingerprint by HS-GC/MS analysis. Using this method only the most volatile components were revealed in the chromatograms (Fig. 4), i.e. those with high vapour pressure such as monoterpenes. On the other hand, neither most sesquiterpenes nor spilanthol could be found in the NE headspace due to their higher boiling points. Overall, the GC-MS chemical fingerprints of the vapor phase of *A. oleracea* EO and its nanoencapsulated form were overlapping, suggesting that the nanoemulsion retained the whole EO chemical profile. The most abundant compound detected in the vapor phase was β -pinene (66.5 and 65.6%, respectively, semi-quantitative data) followed by β -phellandrene (9.3 and 10.0%, respectively), α -pinene (8.0 and 5.3%, respectively), limonene (6.0 and 4.8%, respectively), sabinene (4.2 and 3.4%, respectively), (*E*)-caryophyllene (3.1 and 3.0%, respectively) and other minor components. In both EOs (pure and formulated) there was a prevalence of monoterpene hydrocarbons (96.0 and 96.3%, respectively) rather than sesquiterpene hydrocarbons (4.0 % and 3.7%, respectively). The nanoencapsulated *A. oleracea* EO was also analysed four months after the preparation of the formulation to evaluate its stability over time. Statistical analysis showed the absence of significant differences between the means \pm SD of all compounds, suggesting that storage did not alter the chemical fingerprint of the nanoencapsulated EO.

3.4. Insecticidal activity

All tested *A. oleracea*-borne products showed relevant activity against *C. quinquefasciatus* 3rd instar larvae. The most effective product was the major constituent spilanthol, achieving a LC₅₀ value of 3.1 μ L/L, followed by HE (LC₅₀=9.5 μ L/L) and EO (LC₅₀=16.1 μ L/L). 95% confidence intervals failed to overlap, highlighting a significant difference among the tested products (Table 5). It is worth noting that spilanthol and, to a minor extent, the HE, showed lower LC₅₀ values than deltamethrin (15.3 μ L/L), while an overlapping value was displayed by EO. The latter, when formulated in an EO-NE, achieved a LC₅₀ of 407.5 μ L/L, while 90% of the exposed *C. quinquefasciatus* population was killed after exposure to 617.7 μ L/L (Table 5). However, if we recalculate the lethal concentrations of the nanoemulsion to the EO content (6%) as the active substance EO-NE, then we would conclude that the lethal concentrations do not differ significantly compared to LC₅₀₍₉₀₎ of

EO, as their confidence intervals overlap. From this point of view, EO-NE can therefore be considered to be approximately as effective as EO itself.

Furthermore, exposing *C. quinquefasciatus* larvae to the estimated LC₃₀ of the EO, EO-NE, HE and spilanthol (12.4, 343.7, 7.3, and 2.3 µL/L, respectively) led to significant changes on mosquito biology, causing further mortality as well as a significant decrease in adult emergence rates (Table 6). The exposure to the EO, EO-NE, HE and spilanthol LC₃₀ also reduced the adult fertility, in terms of egg hatchability (%) and the overall abundance of F₁ larvae if compared with the control (Table 6). The product causing the highest reduction in egg hatchability (%) was the *A. olearacea* EO-NE (i.e. 71.2 vs. 97.4% of the control). Both the exposure to EO-NE and the HE led to > 65% of natality inhibition over control mosquitoes (Table 6). Earlier research on *A. olearacea* showed that its EO has a rather broad insecticidal activity against mosquitoes, including *C. quinquefasciatus* (LC₅₀=42.2 mg/L), as well as on *Musca domestica* L. females (LD₅₀=44.3 µg/adult) and *Spodoptera littoralis* (Boisduval) 3rd instar larvae (LD₅₀ = 68.1 µg/larva) (Benelli et al., 2019a). Spilanthol was also studied for its insecticidal efficacy; this substance showed high biological efficacy in the tests done and can be considered as a very promising active substance for manufacturing botanical insecticides. For example, spilanthol demonstrated a very good insecticidal efficacy against some important field crop pests such as the larvae of diamondback moth, *Plutella xylostella* L. (LC₅₀ = 1.5 g/L) (Sharma et al., 2012), and the tomato leafminer, *Tuta absoluta* (Meyrick) (LD₅₀ = 0.13 µg/mg) (Moreno et al., 2012). Notably, spilanthol was selective to the stingless bee, *Tetragonisca angustula* (Latr.), which acts as a pollinator in Central and South America (Moreno et al., 2012).

Spilanthol was effective against *Aedes aegypti* L. larvae, with LC₁₀₀ of 12.5 µg/mL (Ramsewak et al., 1999). Also, spilanthol, at 7.5 ppm concentration, caused 100% motility of larvae of *Anopheles*, *Culex*, and *Aedes* mosquitoes; at lower concentrations it is effective on eggs and pupae (Saraf and Dixit, 2002). Spilanthol and derived amides isolated from the *Heliopsis longipes* (A.Gray) S.F.Blake roots were tested for toxicity against 3rd instar larvae of *Anopheles albimanus* C. R. G. Wiedemann and *Ae. aegypti*, showing LC₅₀ of 4.2 and 7.3 mg/L, respectively (Hernández-Morales et al., 2015).

Overall, further efforts to shed light on the toxicity of spilanthol are needed. Data reported in literature underline the chief contribution of spilanthol to the insecticidal properties of *A. oleracea* (Table 7). This molecule is endowed with an aliphatic chain with three conjugated double bonds at positions 2E, 6Z and 8E. These unsaturated moieties have been proposed as crucial for the insecticidal activity (Greger, 1984). A recent study showed also that these double bonds are essential for the maintenance of the insecticidal activities against *An. albimanus* and *Ae. aegypti* (Hernández-Morales et al., 2015). Even if the mechanism of action has not been determined yet, spilanthol seems to affect the central nervous system, because some abnormal movements, i.e. not coordinated muscular activity, have been observed in the tested insects. In addition, the rapid mortality of larvae after a short exposure to spilanthol and other alkylamides showed that they disrupt the processes of histolysis of larval tissues (Moreno et al., 2012). Saraf and Dixit (2002) described a high pupal mortality of *C. quinquefasciatus*, *An. culicifacies* and *Ae. aegypti* after spilanthol treatment. This supports the hypothesis that spilanthol affects histolysis and histogenesis processes.

In this scenario, the present study further supported a major contribution of spilanthol to the mosquitocidal activity of *A. oleracea*-related botanicals. Indeed, the present larvicidal assays carried out on *C. quinquefasciatus* 3rd instar larvae showed that spilanthol was the most toxic tested product (LC₅₀=3.1 µL/L), followed by the HE (LC₅₀=9.5 µL/L) and the EO (LC₅₀=16.1 µL/L). In any case, it is worth mentioning that all tested *A. oleracea*-related botanical products showed promising effectiveness for further development of larvicidal formulations, according to the criteria established by Pavela (2015) and Pavela et al. (2019a).

Besides basic toxicological tests, we performed tests focusing on the effect of LC₃₀ concentrations of botanicals extracted from *A. oleracea* on further development of the exposed mosquitoes, in terms of subsequent larval development, fecundity, fertility and potential natality. Our results can be considered as very important for the implementation of mosquito larvicidal tools. Indeed, the application of botanical larvicides in natural breeding sites cannot be expected to achieve uniform levels, and thus the impact of low concentrations and short exposure times also need to be monitored.

Exposing *C. quinquefasciatus* larvae to the estimated LC₃₀ of the *A. oleracea* EO, EO-NE, extract and spilanthol (i.e. 12.4, 343.7, 7.3, and 2.3 µL L⁻¹, respectively) significantly reduced the adult emergence rates.

LC₃₀ exposure also reduced the adult fertility, in terms of egg hatchability rates and natality. The *A. oleracea* EO-NE was the most effective in reducing the egg hatchability (71.2 vs. 97.4% in the control). Both the exposure to EO-NE and HE caused more than 65% natality inhibition compared with control mosquitoes. As follows from the results obtained, the extract as well as the EO-NE provided higher efficacy at LC₃₀ than spilanthol alone. It is likely that the extract contains other compounds that may increase the biological efficacy, similarly as the EO. In fact, besides the well-recognised role of spilanthol for the insecticidal properties of *A. oleracea*, also terpenoids found in this plant have shown a great importance for this biological activity. The data available in literature suggest that the toxic and sublethal behavioural effects caused in insects by terpenoids are caused by neurotoxicity or interaction with receptors in the insect nervous system (Isman, 2020b). Some of these compounds can inhibit the AChE enzyme, which is one of the most important enzymes in neuro-neuronal and neuromuscular junctions in both insects and mammals (Isman and Tak, 2017; Jankowska et al., 2018). In this regard, we tested the *A. oleracea* EO, HE, and spilanthol against AChE using galantamine as positive control. As shown in Table 8 the *A. oleracea* EO showed an IC₅₀ of 1.2 mg/mL and was 13-fold more active than the isolated spilanthol (IC₅₀=15.54 mg/ml). In both cases the inhibitory concentrations were far distant from that of galantamine (IC₅₀= 0.6 10⁻³) used as reference. The chemical composition revealed the presence of monoterpene hydrocarbons (α - and β -pinene and β -phellandrene) and sesquiterpene hydrocarbons (germacrene D and (*E*)-caryophyllene) that may contribute to the AChE inhibition (Bonesi et al., 2010; Politeo et al., 2011). For instance, (*E*)-caryophyllene showed an AChE inhibitory activity with IC₅₀ of 20 μ g/mL (Borrero et al., 2019). Moreover, docking modelling of this molecule on sterol carrier protein-2 (SCP-2) of *Ae. aegypti* revealed high affinity with the active site of the enzyme, and this could be another indication for the mechanism of action (Franca et al., 2021). When compared with the insecticidal EO from *Carlina acaulis* L. (Benelli et al., 2019c), the *A. oleracea* EO displayed a two-fold higher IC₅₀ value. Thus, we assume that other modes of action may be responsible for the overall effect of *A. oleracea* EO; they are the result of a complex interaction of terpenoids with spilanthol.

A better efficacy of the *A. oleracea* HE when compared with that of spilanthol has been confirmed by Hernández-Morales (2015), who found significantly lower LC₅₀ for the extract from *H. longipes* compared

with spilanthol, both for the larvae of *An. albimanus* and *Ae. aegypti*. Thus, it is important to focus more on the study of synergistic relationships among substances contained in the extract (see also Benelli et al., 2017). Although no synergism was observed in terms of acute toxicity (Table 5) in the present case, it may be noted when testing the LC₃₀ (Table 6).

As indicated above, the tested NE-EO showed a more significant effect at the LC₃₀ compared with the EO itself. This has been confirmed also by other studies which reported that EO-loaded NEs gave a higher larvicidal activity than that of the whole EO (Bai and McClements 2016; Oliveira et al. 2016). Compared with the whole EO, EO-loaded NEs show some advantages (Pavoni et al., 2019). One of them is the hydrophilicity enhancement, supporting the use for different applications (Pavela et al., 2019b,c). Better utilization of various mechanisms of action is yet another advantage. The larvicidal mechanisms include denaturation of proteins essential for the insect's metabolism (Ryan and Byrne 1988), increase permeability of cuticle (Lomonaco et al. 2009), and disruption of larval morphogenesis (Rattan, 2010; Benelli, 2018). Other described mechanisms include acetylcholinesterase, chitin synthesis, and GABA inhibitions (Braga and Valle 2007; Lopez and Pascual-Villalobos, 2010).

The larvicidal effect of NE has been attributed to the particle sizes in the nanoscale (Oliveira et al. 2016) and a long-term release of active substances. However, it is also likely that the larvicidal effects may be the result of several mechanisms occurring contemporaneously (Dias and Moraes, 2014; Pavela et al., 2019a). On the other hand, the improved bioavailability warranted by nanoparticles might potentiate the NE larvicidal effect (Pavela et al., 2019b,c).

3.5. Cytotoxic and anti-inflammatory activity of *A. oleracea* derivatives on human cells

One of the hallmarks of botanical insecticides is the safety to humans. Thus, in this work the cytotoxic effect of *A. oleracea* EO, EO-NE, HE, and spilanthol has been evaluated on normal human fibroblast cell line (NHF-A12) and two human tumor cell lines (A375 and MDA-MB 231) by the MTT assay. Data showed a moderate cytotoxic activity of *A. oleracea* EO against all cell lines with an accentuated activity on MDA-MB 231 cells (IC₅₀ value of 87.80 µg/mL) (Table 9). Noteworthy, *A. oleracea* EO showed a lower activity on normal human

fibroblasts than on the treated cancerous cell lines, with an IC₅₀ value of 266.3 µg/mL indicating that the EO is not cytotoxic to normal cells according to the guidelines of the International Organization for Standardization (ISO, 20093–5, 2009). Spilanthol was not very toxic in our experimental model as demonstrated by the low IC₅₀ values ranging from 166.0 to 526.2 µg/mL for NHF-A12 and MDA-MB 231, respectively. Our results are in agreement with data reported in literature where spilanthol presents a IC₅₀ value of 260 µg/mL in HEK293 cell line (Gerbino et al., 2016). A slight increase of activity on human fibroblast has been shown for the HE where the IC₅₀ value was 120.6 µg/mL. Referring to the spilanthol content present in the HE (50.9%), this does not justify the increase in activity exerted by the latter. Probably other compounds present in the extract could be responsible for the effect. The cytotoxic result of EO-NE was evaluated in the same cell pattern. As reported in Table 9 the EO-NE showed a moderate activity on all cell lines, with IC₅₀ values ranging from 245.3 to 291.9 µg/mL. Noteworthy, *A. olearacea* EO was more active on breast adenocarcinoma than malignant melanoma cell lines. The major constituents of the EO obtained by MAE were β-pinene, myrcene, (*E*)-caryophyllene, germacrene D, and spilanthol. It has been reported that these compounds showed cytotoxicity on tumour cell lines (Venditti et al., 2013; Woguem et al., 2014; Setzer, et al. 2006, Willig, et al., 2019). In conclusion, as regards the safety of the preparations obtained from *A. olearacea*, these show low cytotoxicity, however further investigations must be extended to other toxicity tests (Jennings, 2015).

Acmella olearacea EO, EO-NE, and spilanthol anti-inflammatory activity was investigated using murine microglial BV-2 cells. BV-2 cells are the counterpart of macrophages in the brain and in normal physiological conditions protect the central nervous system against exogenous threats (Wake et al., 2019). However, their excessive activation can trigger neurotoxicity because of the overproduction of pro-inflammatory agents like TNF-α, IL-1β, nitric oxide, IL-6, and ROS (Block and Hong, 2005). In the last years, a key role of microglia in the advance of neurodegenerative disorders such as Parkinson's and Alzheimer's diseases has been established (Bartels et al., 2020; Dansokho and Heneka, 2018; Kaminska et al., 2016).

Initially, the anti-inflammatory activity of *A. olearacea* EO and spilanthol was studied. To assess their potential cytotoxic effect, BV-2 cells were treated with different concentrations of EO and spilanthol for 24

h and cell viability was measured by MTT assay (Fig. 3SM). *Acmella olearacea* EO was not cytotoxic up to 50 $\mu\text{g}/\text{mL}$ (Fig. 3SMA), meanwhile spilanthol was not cytotoxic at the tested concentrations (Fig. 3SMB).

The ability of EO and spilanthol to counteract inflammation was investigated treating BV-2 cells with different concentrations of EO and spilanthol for 24 h before inducing inflammation by activating cells with LPS (Fig. 5). *In vitro*, LPS is commonly used as an inflammatory agent as it triggers the pro-inflammatory pathway in microglial cells (Ding et al., 2018; Lee et al., 2020). As expected, LPS was able to significantly reduce cell viability compared to control cells. EO treatment at 0.5-50 $\mu\text{g}/\text{mL}$ significantly improved the cell viability in respect to LPS treated cells. Interestingly 50 $\mu\text{g}/\text{mL}$ EO showed the highest ability to counteract LPS induced damage (Fig. 5A). Similarly, spilanthol protected cells against LPS induced inflammation (Fig. 5B). In particular 10, 50 and 100 μM spilanthol significantly increased cell viability in respect to LPS activated cells. On the base of these results, for the next experiments we chose 50 $\mu\text{g}/\text{mL}$ EO and 10 μM spilanthol. It has been reported that LPS not only increases pro-inflammatory agents, but also causes oxidative stress by increasing the reactive oxygen species (ROS) production (He et al., 2019; Yang et al., 2018). To investigate the potential antioxidant effects of EO and spilanthol against LPS-triggered oxidative stress, BV-2 cells were treated with *A. olearacea* EO (50 $\mu\text{g}/\text{mL}$) and spilanthol (10 μM) and after 24 h were activated with 100 ng/mL LPS for 24 h. ROS levels were measured by DCFH-DA assay (Fig. 6). Interestingly, only EO was able to significantly decrease ROS levels when compared with LPS exposed cells. In this respect, spilanthol did not influence this parameter.

To better clarify the mechanisms underpinned by EO and spilanthol anti-inflammatory activities, we investigated the expression of pro-inflammatory genes such as IL-1 β , TNF- α , iNOS and COX-2 by RT-PCR analysis (Fig. 7).

The expression of IL-1 β , TNF- α , iNOS, and COX-2 were significantly up-regulated in LPS-activated BV-2 cells. EO treatment significantly decreased the expression of all the tested genes showing a very strong effect on

the two anti-inflammatory enzymes iNOS and COX-2. On the contrary, spilanthol did not modulate mRNA levels of TNF- α , iNOS, and COX-2 and significantly down-regulated the expression of only IL-1 β .

The anti-inflammatory activity of *A. oleracea* is mainly attributed to spilanthol, its major *N*-alkamide present in the aerial parts (Bakondi et al., 2019; C. H. Huang et al., 2018; W. C. Huang et al., 2018; Rahim et al., 2021; Stein et al., 2021; Wu et al., 2008). Our data demonstrated that EO was able to counteract neuroinflammation with a higher degree in respect to spilanthol suggesting that other compounds besides spilanthol could be involved in the observed anti-inflammatory effect. Moreover, it is the first time that *A. oleracea* is investigated in microglia cells, suggesting that its EO could be a potential anti-neuroinflammatory agent. In particular the pre-treatment with EO significantly down-regulated the expression of IL-1 β , TNF- α , COX-2 and iNOS in respect to LPS activated cells, meanwhile spilanthol significantly reduced the expression of only TNF- α . Bakondi et al. (Bakondi et al., 2019) showed that spilanthol exerts its anti-inflammatory activity by inhibiting the iNOS expression and NO production in RAW macrophages. Using the same cell model, Wu et al. (Wu et al., 2008) demonstrated that spilanthol inhibited the NF- κ B activation through downregulation of iNOS, COX-2, IL-1 β , IL-6, and TNF- α . Spilanthol (50-200 μ M) was also tested on vascular smooth muscle cells (VSMC) after stimulation with a high glucose medium to induce inflammation. Spilanthol treatment reduced the production of NO and the expression of chynase and up-regulated the antioxidant enzyme catalase (Stein et al., 2021). In HaCaT keratinocytes, spilanthol exerted anti-inflammatory activity through different mechanisms: inhibiting the formation of pro-inflammatory IL-6, IL-8, MCP-1, ICAM-1 and COX-2; enhancing heme oxygenase 1 expression levels; inhibiting the pJNK-MAPK signaling pathway (Huang et al., 2018). All these studies seem to disagree with our results that did not evidence a strong ability of spilanthol in reducing the anti-inflammatory mediators in BV-2 cells. Indeed, these differences could be ascribed to the experimental design as we used a different cell model and, to our knowledge, this is the first time that spilanthol is studied in microglial cells. Moreover, we treated cells with lower spilanthol concentrations compared to the concentrations used in the studies reported above. In particular, the lower spilanthol concentration with the highest anti-inflammatory activity was 10 μ M that is ten times lower than the concentrations tested in the other

studies. On the other hand, our data evidenced a strongest activity of *A. oleracea* EO in respect to spilanthol suggesting that EO could possess other compounds able to counteract inflammation. The main components of *A. oleracea* EO, besides spilanthol, are β -pinene, myrcene, (*E*)-caryophyllene, and germacrene D. Of note, these compounds are present in different EOs that exert anti-inflammatory activity. In particular, three different *Pinus* EOs rich in β -pinene, (*E*)-caryophyllene, and germacrene D showed anti-inflammatory activity in LPS-stimulated macrophages reducing IL-6 secretion (Basholli-Salihu et al., 2017). Different EOs obtained by leaves and fruits of *Schinus polygama* (Cav.) Cabrera, whose main component is β -pinene, were able to reduce inflammation in guinea pigs (Erazo et al., 2006). The EO from *Eremanthus erythropappus* (DC.) MacLeish leaves, whose main compounds were β -pinene, (*E*)-caryophyllene, myrcene, and germacrene D, was able to counteract inflammation in rats inhibiting carrageenan-induced paw oedema (Sousa et al., 2008). The EO from *Liquidambar formosana* Hance leaves rich in β -pinene exhibited anti-inflammatory activity in mouse macrophages stimulated by LPS (Hua et al., 2014). (*E*)-Caryophyllene showed anti-inflammatory action in a mouse model of inflammation related to the inhibition of cytokines with the involvement of the arachidonic acid and histamine pathways (Oliveira-Tintino et al., 2018).

To further explore the use of *A. oleracea* EO to counteract inflammation, we investigated the effect of EO-NE treatment in LPS activated microglial cells. First of all, we studied the potential EO-NE cytotoxic effect treating cells with 0.001–10 mg/mL EO-NE for 24 h (Fig. 8A). EO-NE maintained cell viability to values comparable to control cells up to 0.1 mg/mL, meanwhile it significantly and strongly reduced cell viability at 1 and 10 mg/mL in respect to controls. EO-NE anti-inflammatory activity was investigated pre-treating cells with different concentrations of EO-NE before activating BV-2 cells with LPS (Fig. 8B). Interestingly, EO-NE significantly increased cell viability compared to LPS exposed cells by nearly 30% at 0.25 mg/mL and by nearly 40% at 0.5 mg/mL. It must be noted that these EO-NE concentrations contain 15 and 30 μ g/mL EO, respectively, suggesting that the formulation EO-NE is able to boost the bioactivity of EO likely promoting its availability at a cellular level. This hypothesis is also supported by the fact that 1 mg/mL EO-NE contains 60 μ g mL EO and in the previous experiments with EO, this concentration was not cytotoxic.

4. Conclusions

Acmella oleracea is an economically important plant crop whose cultivation is spreading all over the world due to its several industrial applications in cosmetics, pharmaceuticals, and food industry. The plant uses are mostly related to the presence of alkylamides among which spilanthol is the most representative constituent. However, the plant biomass resulting from the large-scale cultivation can function as a source of an EO which deserves further consideration in pest and vector management science. This product combines the pharmacological effects of terpenes, such as β -pinene, myrcene, (*E*)-caryophyllene, and germacrene D, with that of spilanthol. In this respect, this study showed that the MAE of the plant biomass is more effective than traditional HD to yield spilanthol-rich EO. The latter, after proper encapsulation revealed to be quite effective and promising for IVM strategies. Notably, this work shed light on the effectiveness of *A. oleracea* NE on larval development, fecundity, fertility and natality of *C. quinquefasciatus* when applied at LC₃₀ concentrations and short exposure times. Noteworthy, the *A. oleracea* EO and its active ingredient spilanthol reduce vitality of mosquito larvae, but they are also safe to mammal cells, and protect them against LPS-induced inflammation. Overall, the presented results can be considered as important for implementation of mosquito larvicidal tools based on *A. oleracea* derivatives. Further studies should be carried out on the enhancement of spilanthol extraction and formulation, and to widen its insecticidal spectrum and shed lights on ecotoxicological safety.

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Conflicts of Interest

The authors declare no conflict of interest.

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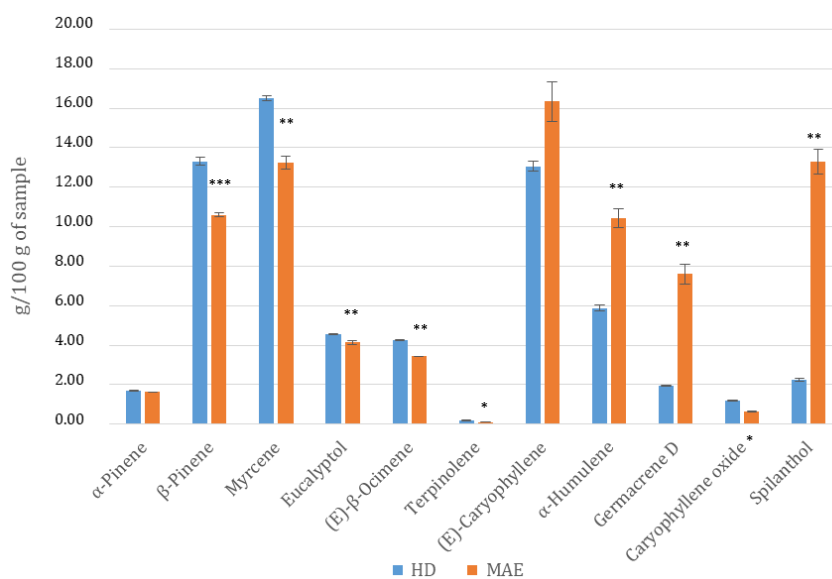


Fig. 1. Quantification by GC-FID analysis of the marker compounds of *Acemella oleracea* essential oil obtained by hydrodistillation (HD, blue) and microwave-assisted extraction (MAE, orange). Data were analysed by one-way ANOVA, highlighting various degrees of significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) between HD and MAE.

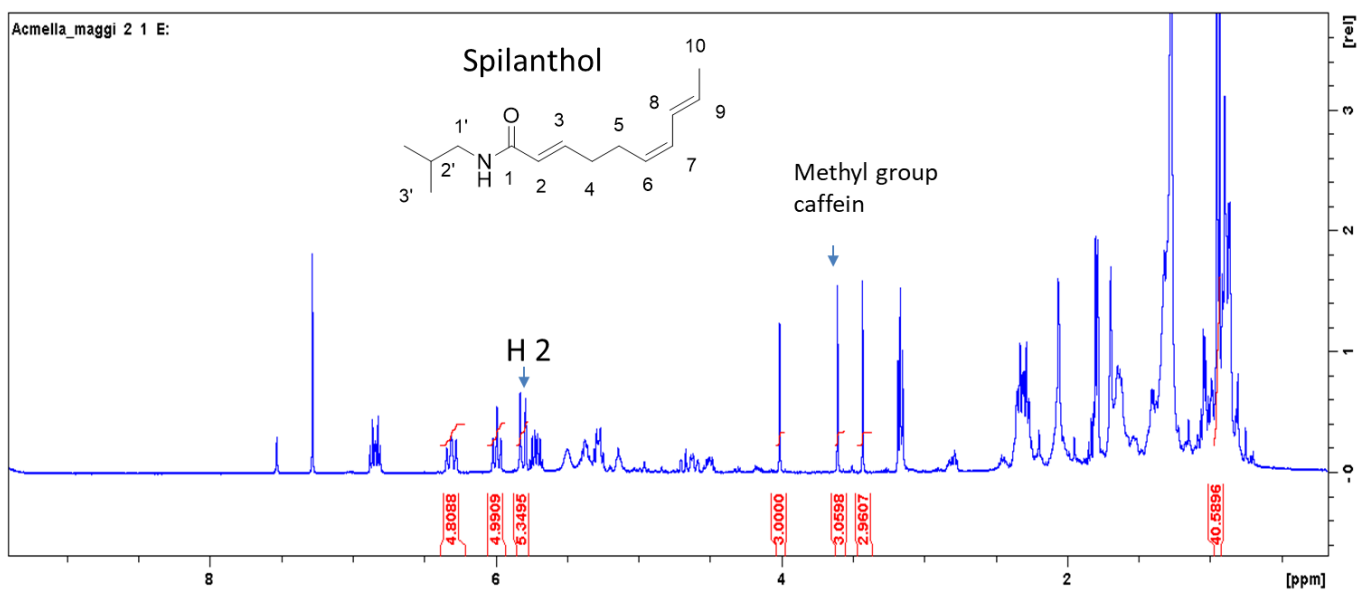


Fig. 2. ^1H NMR of the of *Acmella oleracea* hexane extract and caffeine; integral of signal H-2 of spilanthol and methyl group of caffeine are reported.

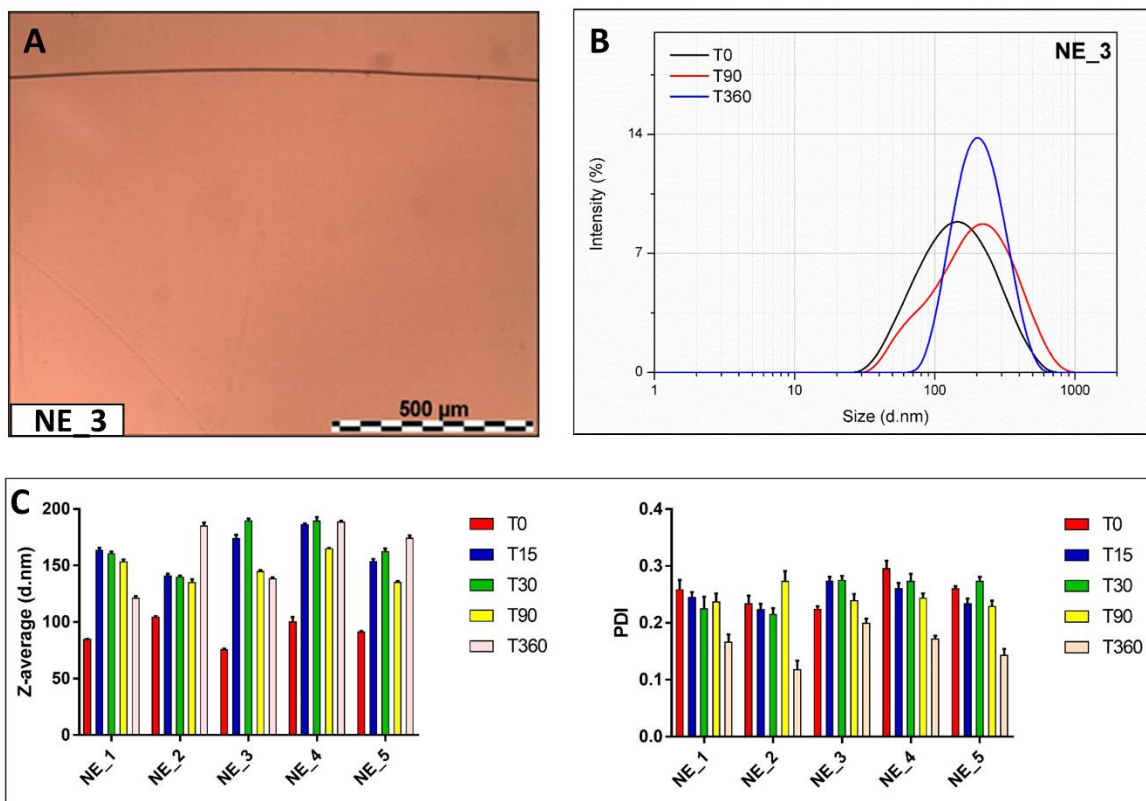


Fig. 3. Polarised optical microscope image (10x of magnification) of the *Acmella oleracea* essential oil-based nanoemulsion NE_3 stored for an year at room temperature (A). Droplet size distribution (intensity %) from DLS for the nanoemulsion NE_3 recorded at different stability time points (0, 90 and 360 days, i.e. T0, T90 and T360 days, respectively) (B). Z-average (d.nm) and polydispersity index (PDI) from DLS for all prepared nanoemulsions (NE_1–NE_5) at the different stability time points (0, 15, 30, 90 and 360 days, i.e. T0, T15, T30, T90 and T360, respectively) (C).

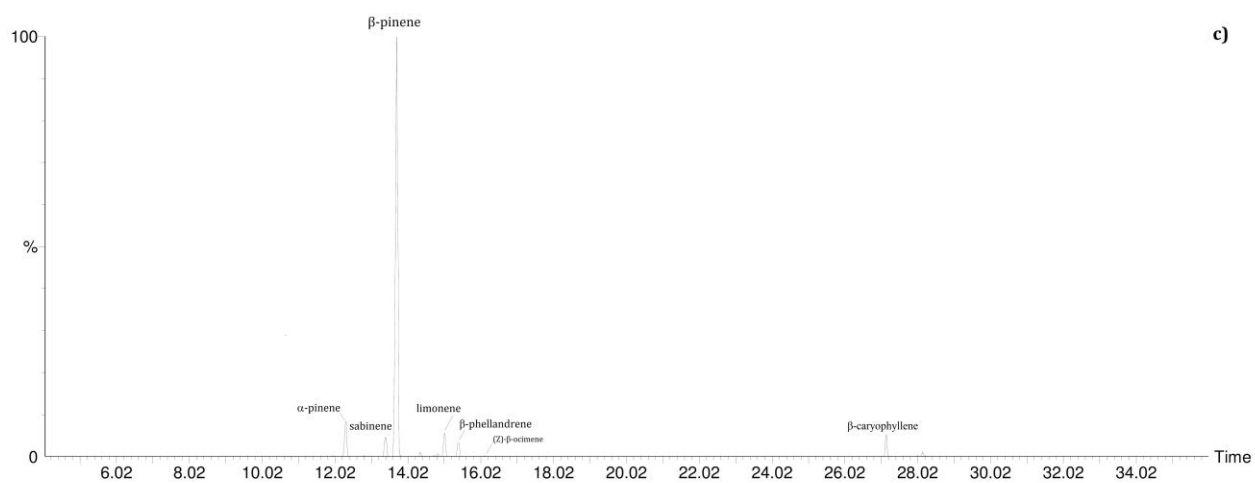
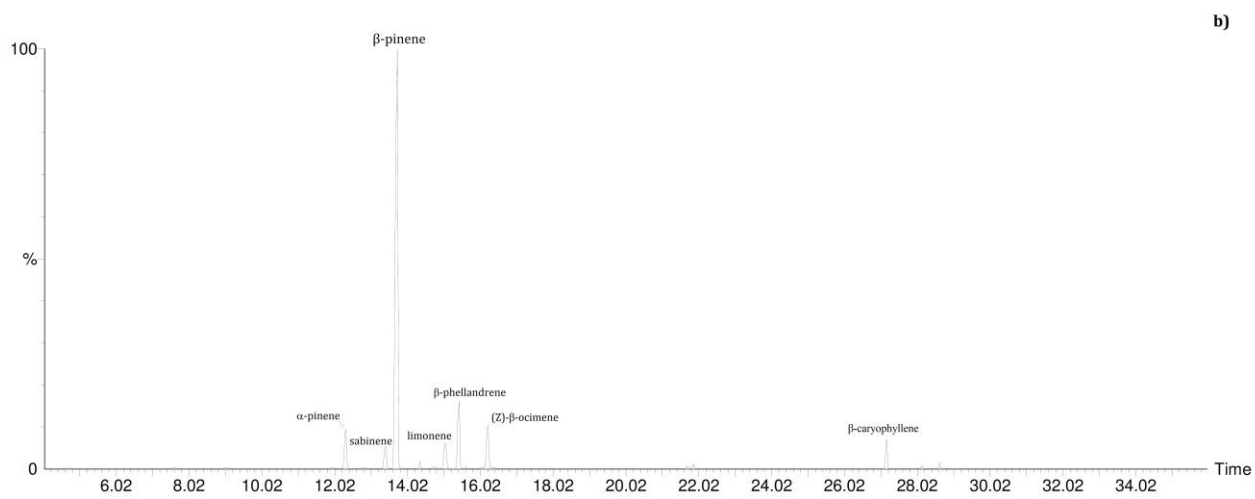
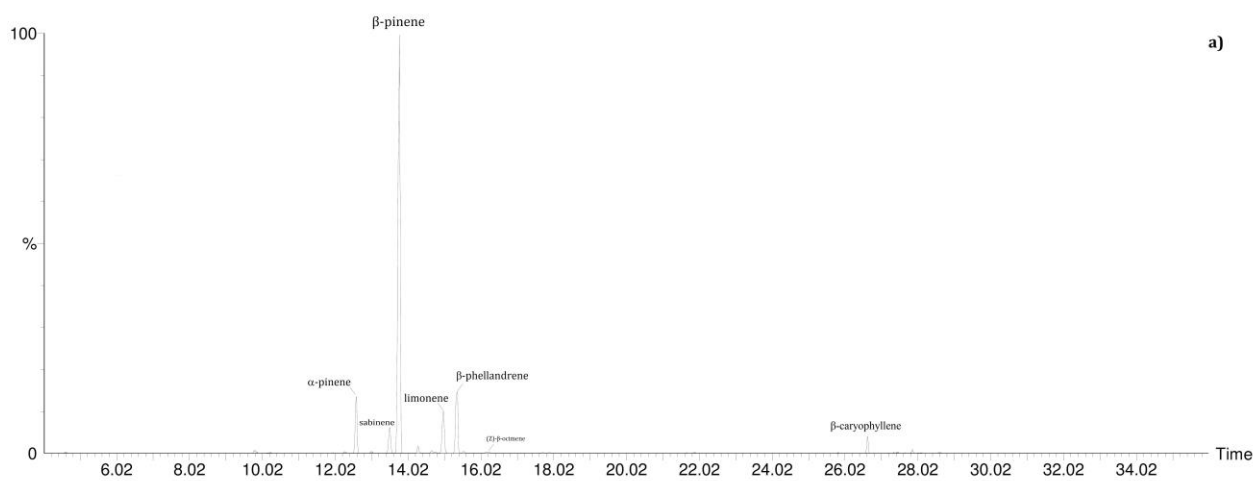


Fig. 4. GC-MS chemical fingerprint of the vapour phase of *Acemella oleracea* essential oil (a), its nanoemulsion at time 0 (b), and after 4 months of storage (c).

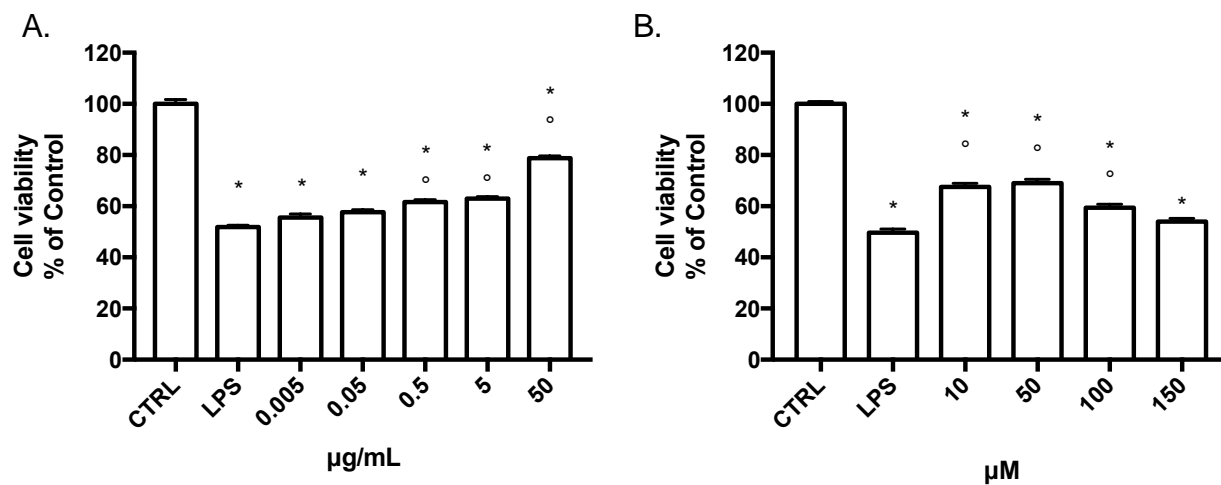


Fig. 5. Anti-inflammatory activity of *Acmella oleracea* essential oil (EO) and spilanthol against LPS in BV-2 cells. Cells were treated with (A) 0.005–50 µg/mL EO and (B) 10–150 µM spilanthol for 24 h, exposed to 100 ng/mL LPS for further 24 h and MTT test was used to evaluate cell viability. Each bar represents means ± SEM of at least 4 independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test. * $p < 0.05$ compared to control (CTRL); ° $p < 0.05$ compared to LPS.

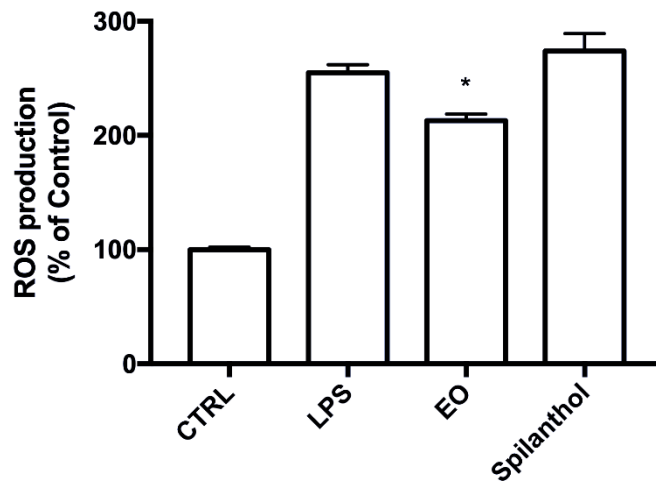


Fig. 6. Effect of *Acmella oleracea* essential oil (EO) and spilanthol on intracellular reactive oxygen species (ROS) production in LPS-activated BV-2 cells. Cells were pre-treated with 50 $\mu\text{g}/\text{mL}$ or 10 μM spilanthol for 24 h before activation with 100 ng/mL LPS. After 24 h intracellular ROS were measured using the peroxide-sensitive fluorescent probe DCHF-DA. Each column represents the mean \pm SD of three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test. * $p < 0.05$ compared to control (CTRL); $^{\circ}p < 0.05$ compared to LPS.

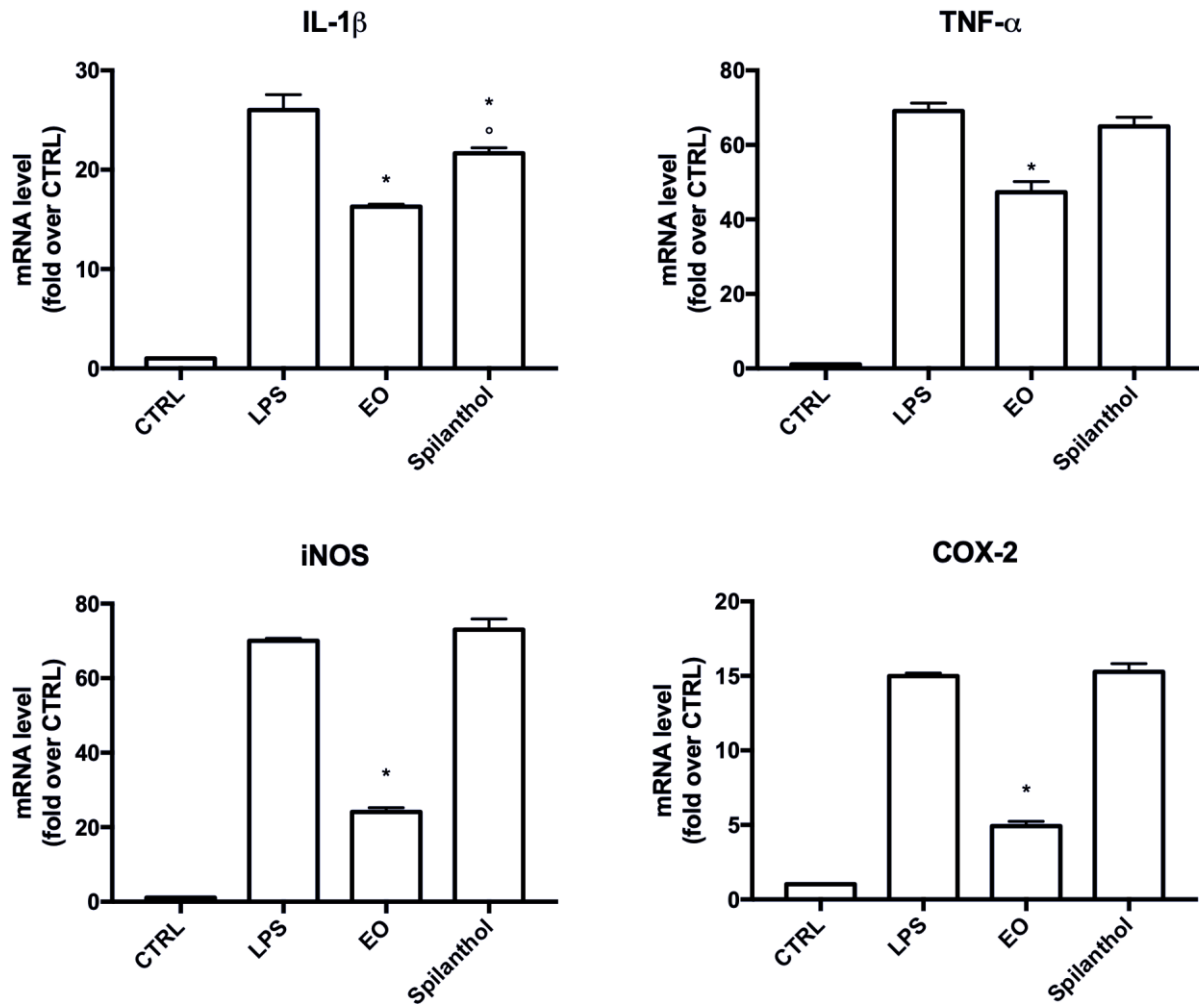


Fig. 7. Expression of IL-1, TNF-, iNOS and COX-2 in BV-2 cells treated with *Acmella oleracea* essential oil (EO) and spilanthol. Cells were treated with EO 50 $\mu\text{g}/\text{mL}$ or spilanthol 10 μM for 24 h, exposed to 100 ng/mL LPS for 24 h and real time-PCR was performed. Data are expressed as relative abundance compared to untreated cells. Each bar represents the mean \pm SEM of three independent experiments. Data were analysed by a one-way ANOVA followed by Bonferroni's test. $^{\circ} p < 0.05$ vs. control (CTRL), $* p < 0.05$ vs. LPS.

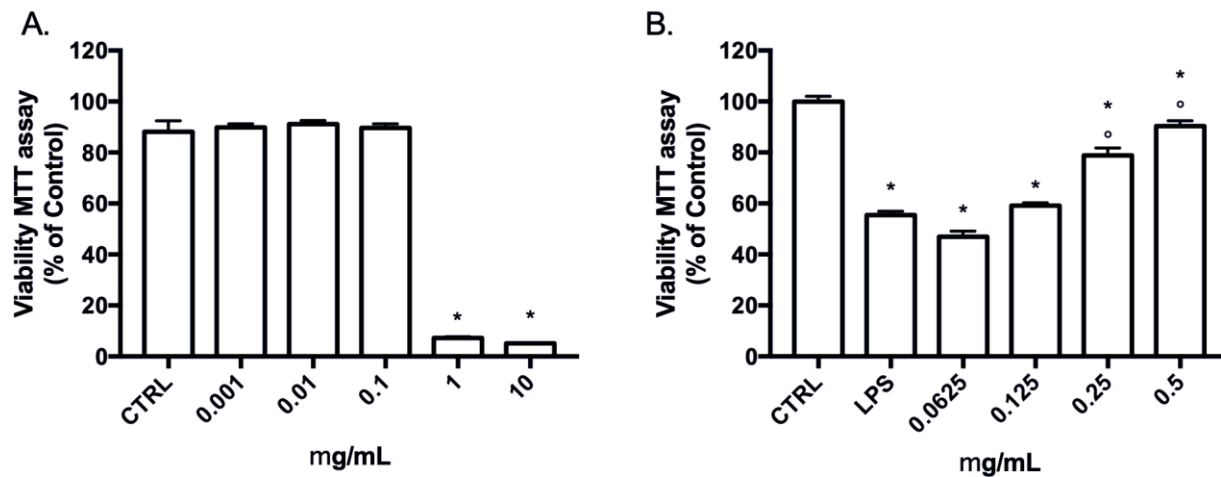


Fig. 8. Effect of *Acmella oleracea* essential oil nanoemulsion (EO-NE) on BV-2 cells. Cells were treated with (A) 0.001–10 mg/mL EO-NE for 24 h and cell viability was evaluated by MTT assay, (B) 0.0625–0.5 mg/mL EO-NE, activated with 100 ng/mL LPS for 24 h and cell viability was evaluated by MTT assay. Each bar represents means \pm SEM of at least four independent experiments. Data were analysed by one-way ANOVA followed by (A) Dunnett's test. (B) Bonferroni's test * p <0.05 compared to CTRL, [°] p <0.05 compared to LPS.

Table 1. Composition of the *Acmella oleracea* essential oil based-nanoemulsions (NEs).

Nanoemulsion	<i>A. oleracea</i>		Total oil phase (OP)	Polysorbate 80 (P80)	EO/Ethyl oleate ratio	P80/OP ratio
	essential oil (EO)	Ethyl oleate				
NE_1	0.4	0.2	0.6	0.2	0.5	0.33
NE_2	0.4	0.2	0.6	0.5	0.5	0.83
NE_3	0.6	0.3	0.9	0.2	0.5	0.22
NE_4	0.6	0.3	0.9	0.5	0.5	0.55
NE_5	0.5	0.25	0.75	0.35	0.5	0.46

Table 2. Chemical composition of the essential oil of *Acmella oleracea* obtained by hydrodistillation (HD) and microwave-assisted extraction (MAE).

No.	Component ^a	RI ^b	RI Lit. ^c	Relative peak area (%)		ID ^d
				HD	MAE	
1	(2 <i>E</i>)-hexenal	847	846	-	0.1	RI,MS
2	<i>n</i> -hexanol	864	863	-	tr ^e	RI,MS
3	α -thujene	921	924	0.1	tr	RI,MS
4	α -pinene	926	932	2.1	1.7	Std,RI,MS
5	Camphene	939	946	Tr	tr	Std,RI,MS
6	sabinene	965	969	0.7	1.5	Std,RI,MS
7	β -pinene	967	974	14.7	10.8	Std,RI,MS
8	myrcene	989	988	17.4	12.3	Std,RI,MS
9	<i>p</i> -mentha-1(7),8-diene	1001	1004	0.3	0.2	RI,MS
10	α -terpinene	1013	1014	0.2	tr	Std,RI,MS
11	<i>p</i> -cymene	1021	1020	0.2	tr	Std,RI,MS
12	β -phellandrene	1024	1025	6.5	4.9	RI,MS
13	(<i>Z</i>)- β -ocimene	1036	1032	6.0	3.8	Std,RI,MS
14	(<i>E</i>)- β -ocimene	1046	1044	0.1	tr	Std,RI,MS
15	γ -terpinene	1054	1054	0.3	tr	Std,RI,MS
16	terpinolene	1084	1086	Tr	0.1	Std,RI,MS
17	allo-ocimene	1128	1128	0.3	0.1	RI,MS
18	terpinen-4-ol	1171	1174	0.5	tr	Std, RI,MS
19	δ -elemene	1330	1335	0.3	0.8	RI,MS
20	α -copaene	1366	1374	0.1	0.2	RI,MS
21	β -elemene	1384	1389	0.3	0.2	RI,MS

22	cyperene	1386	1398	Tr	0.2	RI,MS
23	(<i>Z</i>)-caryophyllene	1396	1408	0.4	-	RI,MS
24	(<i>E</i>)-caryophyllene	1408	1417	19.4	19.4	Std,RI,MS
25	β -copaene	1418	1430	0.1	0.1	RI,MS
26	γ -elemene	1426	1434	0.3	0.1	RI,MS
27	α -humulene	1441	1452	1.3	1.1	Std, RI,MS
28	germacrene D	1470	1484	11.8	15.2	RI,MS
29	bicyclogermacrene	1486	1500	-	0.3	RI,MS
30	1-pentadecene	1492	1493	-	5.5	RI,MS
31	(<i>Z,E</i>)- α -farnesene	1493	1491	2.1	2.7	RI,MS
32	δ -amorphene	1498	1511	0.1	-	RI,MS
33	γ -cadinene	1503	1513	0.3	tr	RI,MS
34	(<i>E,E</i>)- α -farnesene	1505	1505	0.6	0.4	RI,MS
35	δ -cadinene	1515	1522	1.1	0.2	RI,MS
36	kessane	1520	1529	1.4	0.5	RI,MS
37	germacrene B	1543	1559	0.4	0.2	RI,MS
38	(<i>E</i>)-nerolidol	1560	1561	0.7	0.2	Std,RI,MS
39	caryophyllene oxide	1569	1583	0.9	0.3	Std,RI,MS
40	(<i>Z,Z</i>)-1,8,11-heptadecatriene	1660	1664	-	0.2	RIMS
41	spilanthol	1887	1888 ^f	2.3	11.7	MS ^g
42	acmellonate	1997	-	2.1	2.5	MS ^h
43	<i>n</i> -tricosane	2297	2300	Tr	0.2	RI,MS
44	<i>n</i> -pentacosane	2497	2500	Tr	0.1	RI,MS
	Oil yield (% w/w)			0.22	0.47	
	Total identified (%)			95.3	97.8	
	Grouped compounds (%)					

Monoterpene hydrocarbons	48.8	35.5
Oxygenated monoterpenes	0.5	tr
Sesquiterpene hydrocarbons	38.6	41.2
Oxygenated sesquiterpenes	2.9	0.9
Alkylamides	2.3	11.7
Others	2.2	8.6

^a The order of elution derives from an HP-5MS column (30 m x 0.25 mm, 0.1 μ m). ^b The linear retention index is in accordance with Van den Dool and Kratz (1963). ^c RI has been taken from ADAMS and/or NIST17 and FFNSC3 libraries. ^d Identification method: Std, comparison with analytical standards, RI, coherence of the calculated RI with those reported in ADAMS, FFNSC3 and NIST17 libraries. MS, mass spectrum overlapping with those recorded in ADAMS, NIST17, FFNSC3 and WILEY275 libraries. ^e Traces, relative % < 0.1. ^f RI according to Benelli et al. (2019). ^g MS fragmentation pattern is in accordance with that reported by Ramsewak et al. (1999) and Barbosa et al. (2017). ^h MS fragmentation pattern is consistent with that reported by Ley et al. (2006).

Table 3. Enantiomeric distribution of the chiral components of *Acmella oleracea* essential oil obtained from microwave-assisted extraction (MAE).

Enantiomer compound	RT ^a	RI ^b	% in EO ^c	Enant. % ^d	EE % ^e
(-)- α -pinene	15.604	1022	0.99	95.2	90.5
(+)- α -pinene	15.949	1027	0.05	4.8	
(+)- β -pinene	19.238	1081	0.02	0.2	
(-)- β -pinene	19.395	1083	12.05	99.8	99.6
(+)-limonene	20.220	1097	0.57	84.6	69.2
(-)-limonene	20.517	1101	0.10	15.4	
(+)- β -phellandrene	21.297	1114	0.34	12.7	
(-)- β -phellandrene	21.541	1117	2.30	87.3	74.5
(+)-(<i>E</i>)-caryophyllene	-	-	-	-	-
(-)-(<i>E</i>)-caryophyllene	44.688	1498	14.71	100	100
(-)-caryophyllene oxide	57.472	1741	0.05	100	100
(+)-caryophyllene oxide	-	-	-	-	-

^a Retention time of the different enantiomers from the chiral column (HP chiral 20B). ^b Linear retention index calculated using a *n*-alkanes mixture (C₇-C₃₀). ^c Absolute content of enantiomer in the essential oil determined by GC-MS analysis. ^d Relative content of enantiomeric pairs. ^e Enantiomeric excess.

Table 4. Identification and quantification of alkylamide derivatives in *Acmella oleracea* hexane extract. Retention time (RT) and [M+H]⁺ for each compound were reported.

[M+H] ⁺	RT	Identification	%
204	21	(2Z)-N-isobutyl-2-nonene-6,8-diynamide	0.18
232	23.9	(2E)-N-isobutyl-2-undecene-8,10-diynamide	0.43
222	25	(2E,6Z,8E)-N-isobutyl-2,6,8-decatrienamide (spilanthol)	42.67
246	25.8	(2E)-N-(2-methylbutyl)-2-undecene-8,10-diynamide	0.11
258	26.1	(2E,7Z)-N-isobutyl-2,7-tridecadiene-10,12-diynamide	0.43
224	27.1	(2E,7Z)-N-isobutyl-2,7-decadienamide	0.89
236	27.1	(2E,6Z,8E)-N-(2-methylbutyl)-2,6,8-decatrienamide	6.10
248	28	(2E,4E,8Z,10Z)-N-isobutyl-dodeca-2,4,8,10-tetraenamide	0.09

Table 5. Insecticidal activity of *Acmella oleracea*-borne botanicals against *Culex quinquefasciatus* 3rd instar larvae.

Tested product ^a	LC ₃₀ ^b (μL/L)	CI ₉₅ ^c	LC ₅₀ (μL/L)	CI ₉₅	LC ₉₀ (μL/L)	CI ₉₅	χ^2	df ^d	p-value
<i>A. oleracea</i> EO	12.4	6.7-15.7	16.1	15.9-24.1	29.8	25.7-36.5	3.038	3	0.385 ns ^e
<i>A. oleracea</i> EO- NE	343.7	271.5-358.7	407.5	392.8-466.9	617.7	582.6-712.3	3.957	4	0.412 ns
<i>A. oleracea</i> HE	7.3	3.8-8.1	9.5	8.9-12.7	17.7	15.5-28.9	4.074	3	0.253 ns
Spilanthol	2.3	2.1-2.5	3.1	2.8-3.4	6.3	5.6-7.3	4.721	4	0.317 ns
Positive control									
deltamethrin	3.8	3.2-5.6	15.3	10.1-17.5	56.5	48.9-67.2	2.859	3	0.523 ns

^a EO = essential oil; NE = nanoemulsion; HE = hexane extract. ^b LC = lethal concentration killing 50% (LC₅₀) or 90% (LC₉₀) of the exposed mosquito larvae. ^c CI₉₅ = 95% confidence interval; ^d df = degrees of freedom. ^e ns = not significant ($p > 0.05$)

Table 6. Sublethal effect of *Acmella oleracea*-borne botanicals formulated at their LC₃₀ on *Culex quinquefasciatus* larval mortality as well as on emergence, fecundity, fertility and natality of the new generation adults.

Treatment ^a	Tested concentration (µL/L)	Larval mortality (%) ^b			Emergence of adults (%) ^b			Fecundity and fertility indicators ^b		Natality ^b	
		24 h	48 h	Total	Female	Male	Total	Fecundity (no. eggs/female)	Fertility (egg hatchability %)	F ₁ generation larvae (no) out of 100 treated larvae	Natality inhibition over the control (%)
<i>A. oleracea</i> EO	12.4	5.6±1.2 ^a	8.0±1.4 ^b	18.1±3.6 ^b	45.9±1.8 ^b	34.2±1.7 ^b	80.2±4.5 ^{cd}	81.9±12.7	95.5±1.3 ^{bc}	3.230.7±239.7 ^b	24.1±8.5
<i>A. oleracea</i> EO-NE	343.7	20.6±2.7 ^b	23.5±1.5 ^c	33.3±2.9 ^c	31.5±1.2 ^a	32.2±1.5 ^b	63.8±1.5 ^b	103.7±17.2	71.2±5.4 ^a	1.483.8±258.8 ^a	65.1±7.9
<i>A. oleracea</i> HE	7.3	31.7±3.5 ^c	33.1±3.8 ^d	41.5±3.3 ^d	35.4±1.7 ^a	20.1±2.1 ^a	55.5±3.2 ^a	78.9±12.1	92.0±0.3 ^b	1.444.2±138.5 ^a	66.1±9.4
Spilanthol	2.3	3.7±0.5 ^a	6.2±0.2 ^b	19.5±2.2 ^b	45.8±1.1 ^b	31.8±2.5 ^b	77.7±1.6 ^c	101.2±15.9	90.0±1.1 ^b	3.241.2±202.5 ^b	23.9±3.9
Control	-	0.0±0.0 ^a	0.0±0.0 ^a	8.8±5.5 ^a	45.1±2.1 ^b	43.9±2.3 ^c	89.0±5.5 ^d	109.0±12.2	97.4±1.8 ^c	4.243.4±189.5 ^c	-
ANOVA <i>F</i> _{4,15} and <i>P</i> -value	-	122.2, <0.0001	148.5, <0.0001	95.0, <0.0001	147.5, <0.0001	185.7, <0.0001	227.8, <0.0001	ns	128.7, <0.0001	162.5, <0.0001	-

^a *C. quinquefasciatus* 3rd instar larvae were exposed to concentrations corresponding to the estimated LC₃₀ for each *A. oleracea*-borne product (i.e. 12.4, 343.7, 7.3, and 2.3 µL/L for EO, EO-NE, HE and spilanthol, respectively); EO = essential oil; NE = nanoemulsion; HE = hexane extract. ^b Mean% (±SE) within a column followed by the same letter do not differ significantly according to Tukey's HSD test at *p* < 0.05 (% = arcsine transformed data); ns= not significant (*p* > 0.05).

Table 7. Literature data on the toxicity of spilanthol against arthropod pests and vectors.

Species	Instar	Efficacy	Reference
<i>Aedes aegypti</i>	Larvae	LD ₅₀ = 6.25 µg/mL	Ramsewak et al. (1999)
		LD ₁₀₀ = 12.5 µg/mL	
<i>Anopheles albimanus</i>	Larvae	LC ₅₀ = 7.38 mg/L	Hernández-Morales et al. (2015)
		LC ₅₀ = 4.24 mg/L	
<i>Dermacentor nitens</i>	Larvae	100 % mortality at 12.5 mg/mL	Cruz et al (2016)
<i>Lipaphis erysimi</i>	-	mortality of 83 %	Gouvêa et al. (2019)
<i>Periplaneta americana</i>	Adults	LD ₅₀ = 2.46 µg/g	Kadir et al. (1989)
<i>Plutella xylostella</i>	Larvae	LC ₅₀ = 1.49 µg/L	Sharma et al. (2012)
		LC ₉₀ = 1.99 µg/L	
<i>Rhipicephalus microplus</i>	Engorged females	100 % mortality at 1.6 mg/mL	Cruz et al. (2016)
		92.9% mortality 20.0 mg/mL	
<i>Solenopsis saevissima</i>	Adults	LD ₅₀ = 0.18 µg/mg	Moreno et al. (2012)
		LD ₈₀ = 1.12 µg/mg	
<i>Tetragonisca angustula</i>	Adults	LD ₅₀ = 0.35 µg/mg	Moreno et al. (2012)
		LD ₈₀ = 0.81 µg/mg	
<i>Tuta absoluta</i>	Adults	LD ₅₀ = 0.13 µg/mg	Moreno et al. (2012)

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Table 8. Acetylcholinesterase inhibitory properties of *Acmella oleracea* essential oil (EO) and spilanthol.

Treatment ^a	IC ₅₀ (mg/ml)	mgGEIC/g ^b
<i>A. oleracea</i> EO	1.20±0.08	0.5±0.01
Spilanthol	15.54±1.8	0.038±0.01
<i>A. oleracea</i> EO-NE	NA ^c	NA ^c
<i>A. oleracea</i> HE	NA ^c	NA ^c
Positive control		
Galantamine	0.6(±0.02) 10 ⁻³	

^a EO = essential oil; NE = nanoemulsion; HE = hexane extract. ^bGEIC=galantamine-equivalent inhibition capacity. ^c Not active.

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Table 9. *In vitro* growth inhibition of cells by *Acmella oleracea* essential oil (EO), nanoemulsion (NE), spilanthol, and hexane extract.

Treatment ^a	Cell line (IC ₅₀ µg/mL) ^b			^a EO =
	NHF-A12 ^c	MDA-MB 231 ^d	A375 ^e	
<i>A. oleracea</i> EO	266.3	87.80	130.9	=
95% CI ^f	256.2–276.7	72.5–106.3	126.1–136.0	
<i>A. oleracea</i> EO-NE	246.1	291.9	245.3	
95% CI	229.2–264.3	274.9–309.8	226–266.3	
Spilanthol	166.0	526.2	287.2	
95% CI	124.3–182.7	475.6–582.4	243.7–332.3	
<i>A. oleracea</i> HE	120.6	> 200	> 200	
95% CI	98.14–154.05	-	-	
Positive control				
cisplatin	1.21	2.23	0.46	
95% CI	1.05–1.53	1.99–2.37	0.33–0.54	

essential oil; NE = nanoemulsion; HE = hexane extract. ^b IC₅₀ = The concentration of compound that affords a 50% reduction in cell growth (after 72 h of incubation). ^c Normal human fibroblast. ^d Human breast adenocarcinoma. ^e Human malignant melanoma. ^f CI Confidence interval.

SUPPLEMENTARY MATERIAL

Table 1SM

List of primers for real-time PCR.

Gene	Primer
GAPDH forward	5'ACCACAGTCCATGCCATCAC3'
GAPDH reverse	5'TCCACCACCCTGTTGCTGTA3'
IL-1β forward	5'GTTCCCATTAGACAACCTGCACTACAG3'
IL-1β reverse	5'GTCGTTGCTTGGTTCTCCTTGTA3'
TNFα forward	5'CCCCAAAGGGATGAGAAGTTC3'
TNFα reverse	5'CCTCCACTTGGTGGTTTGCT3'
iNOS forward	5'CCTCCTCCACCCTACCAAGT3'
iNOS reverse	5'CACCCAAAGTGCTTCAGTCA3'

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COX2 forward 5'TGGGGTGATGAGCAACTATT3'
COX2 reverse 5'AAGGAGCTCTGGGTCAAAC3'

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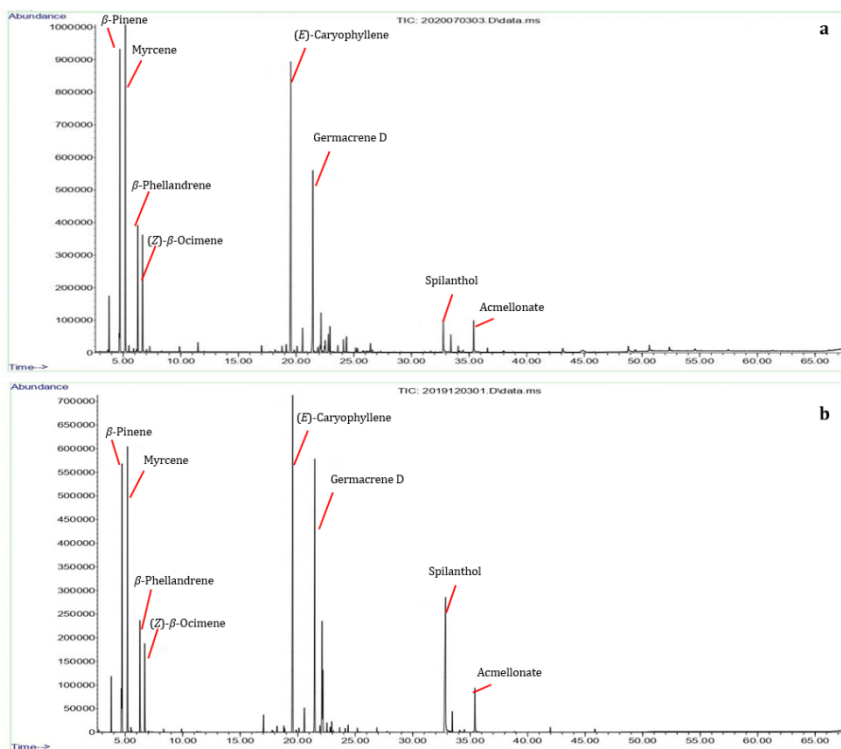


Fig. 1SM. GC-MS chromatograms of *Acemella oleracea* essential oil obtained by hydrodistillation (HD, a) and microwave-assisted extraction (MAE, b).

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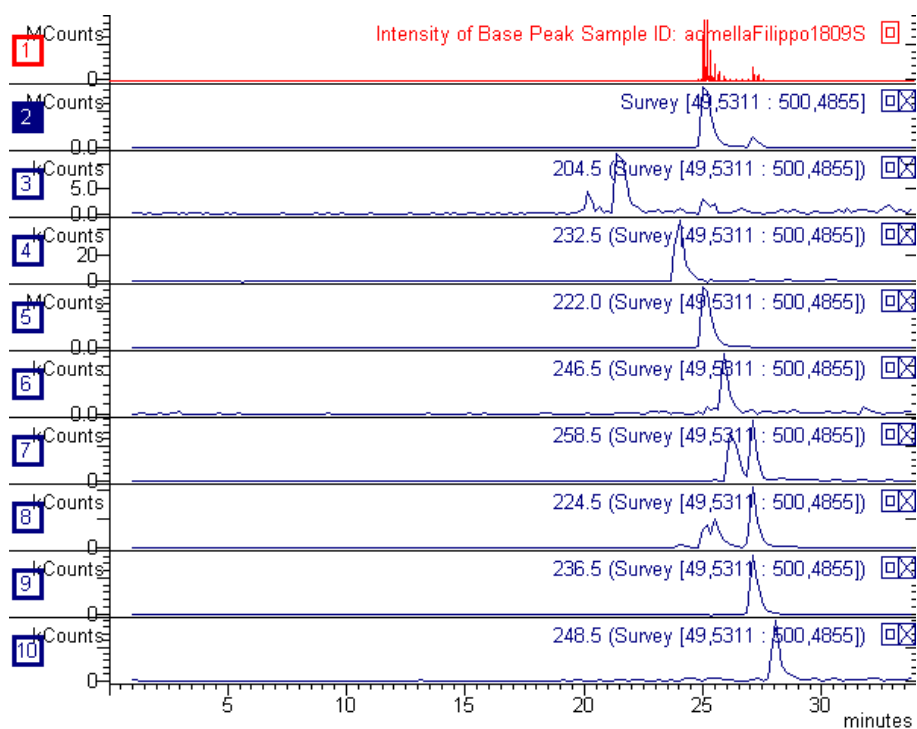


Fig. 2SM. Chromatographic peaks obtained by HPLC-MS of the main alkylamides in the hexane extract of *Acmella oleracea*.

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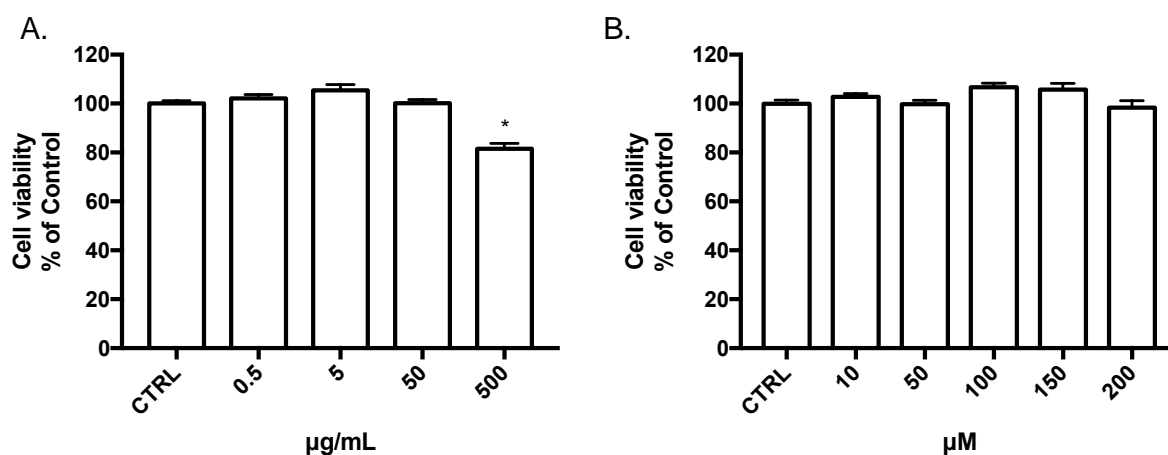


Fig. 3SM. Cytotoxicity of *Acmella oleracea* EO and spilanthol in BV-2 cells. Cells were treated with (A) 0.5–500 µg/mL essential oil and (B) 10–200 µM spilanthol for 24 h, and MTT test was used to evaluate cell viability. Each bar represents means ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test. * $p < 0.05$ compared to control (CTRL).