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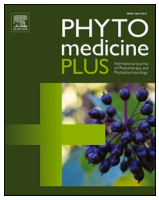

In vitro study on the inhibitory effect of various essential oils against murine coronavirus mouse hepatitis virus A-59 replication

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ABSTRACT

Background: The spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) worldwide has become one of the biggest health problems due to the lack of knowledge about effective therapy.
Purpose: Some scientific studies have shown that essential oils (EOs) have anti-inflammatory, immunomodulatory, and antiviral properties.
Study design: This study demonstrated the potential antiviral activity of EOs in emulsion and in vapor forms to reduce the replication of MHV-A59, a murine surrogate of SARS-CoV-2.
Methods: In the present study, 32 EOs were screened *in vitro* against MHV-A59 on DBT cells using plaque assay. EOs in emulsions were applied at their maximum noncytotoxic concentrations to MHV-A59 after penetration of the viruses into the host cells for 1 h during intracellular virus replication.
Results: Monarda didyma at 5000 μg/ml showed a reduction of 100% of viral plaque. *Tanacetum annuum* at 10000 μg/ml inhibited 5.03-log MHV-A59. Beyond 4-log, the drug can be qualified as an antiviral according to the guidelines of ICH. In vapor form, none of the EOs showed potential inhibitory effects against MHV-A59.
Conclusion: Results demonstrated that all 32 undiluted EOs, incubated with MHV-A59 for 30 min, had a ≤1.09-log inactivation compared to an untreated virus. The findings of this study may provide proof-of-concept and insight into related trials.

Abbreviations

DBT	Delayed brain tumor
DMSO	Dimethyl sulfoxide
EMEM	Eagle's minimal essential medium
EO	Essential oil
EOs	Essential oils
FBS	Fetal bovine serum
MHV-A59	Mouse Hepatitis Virus strain A59
MTT	Tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PFU	Plaque-forming units
SARS	Severe acute respiratory syndrome
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
WHO	World Health Organization

Introduction

The first pandemic of the 21st century was caused by Coronavirus, named severe acute respiratory syndrome (SARS) in 2003, and shocked the world with the speed at which the virus was transmitted from continent to continent (Peiris et al., 2003). Further, in December 2019, the Corona Virus Disease 2019 (COVID-19) caused by a new Coronavirus (SARS-CoV-2) started to rage in China. The virus began to spread worldwide, leading to a declaration as a pandemic by the World Health Organization (WHO) in March 2020 (Vaishnav et al., 2020). Through some lessons and experiences from the 2003 outbreak, SARS-CoV-2 could be quickly identified and characterized.

Coronaviruses are members of the Coronaviridae family and are enveloped viruses with single-stranded positive-sense RNA. The envelope of SARS-CoV-2 is composed of membrane lipids with several proteins, like envelope protein, membrane protein, and spike glycoprotein. The latter plays a decisive role in modifying the pathogenicity of the

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virus, in particular, due to its high mutagenicity (Grabherr et al., 2021). Indeed, the spike protein has hypervariable regions that allow the virus to modify its cellular tropism and prevent the cellular and humoral immune response (Vabret and Miszczak, 2010).

Because SARS-CoV-2 is not yet fully understood, and its infection can be dangerous for human health, more precautions are required to work with it. This may hinder studies on global understanding of the virus, the virus-host interaction, and even the development of new antivirals (Barranco et al., 2021). Therefore, murine coronavirus MHV-A59 (Mouse Hepatitis Virus strain A59) is an ideal surrogate virus for some studies (Grabherr et al., 2021). The genetic proximity of MHV-A59 to SARS-CoV-2 results have the same mode of viral infection and common clinical signs of disease with COVID-19 (Körner et al., 2020). MHV-A59 can be used as an animal model for a lung infection and severe pneumonia without a high level of containment, in addition to inducing an acute respiratory distress syndrome in mice similar to SARS-CoV-2 in humans (Yang et al. 2014)

This recent pandemic highlighted the importance of identifying effective new approaches to prevent and treat viral infections. Furthermore, the demand for natural products and suitable medicines is of great concern to people and scientists worldwide. Although essential oils (EOs) have been used for decades for their beneficial effects on health, they are not classified as therapeutic molecules and are, therefore, exempt from any regulation (Kubeczka. 2020). On the other hand, interest in EOs extracted from medicinal plants has motivated the studies of various secondary metabolites and chemical compounds produced that have notable antibacterial, antifungal, anti-inflammatory, and antiviral activities (Raut and Karuppayil, 2014). Terpenes, flavonoids, and polyphenols are plant-derived antiviral molecules found in EOs extracted from seeds, roots, flowers, bark leaves, peel, and so on (Tongnuanchan and Benjakul, 2014). In recent phytomedicine studies, the antiviral activities of peppermint oil and tea tree oil on the Herpes simplex virus (HSV) have been described (Schnitzler 2019). Another study has shown that some EOs obtained from plants have inhibitory effects on Yellow Fever Virus replication *in vitro* (Meneses et al. 2009). Despite the lack of consensus on the mechanisms of action of EOs on viruses, several studies suggest a potential interaction between the proteins of enveloped viruses and the molecules of EOs, pre-infection, which would impact the ability of the virus to penetrate the host cells to replicate (Ma and Yao, 2020; Meneses et al. 2009). Other studies indicated that antiviral inhibition could be caused by a synergy between secondary metabolites and chemical compounds in EOs (Silva et al., 2020).

In this study, the inhibitory potential of 32 pure EOs and 12 pre-made blends of EOs was tested on MHV-A59 replication *in vitro*. In addition, experiments were done with emulsions and vapors of the EOs and pre-made blends, and the variations between the effects of the two different phases on the replication of the viruses are described. The findings of this study do not provide proof of therapeutic effect against SARS-CoV-2 through the utilization of EOs, but results may provide proof-of-concept and insight into related trials.

Materials and methods

Materials

EOs were provided by Aliksir Huiles Essentielles (Quebec, Canada). To ensure good identification, quality, and purity of the products, Aliksir analyzed the chemical composition of each essential oil (EO) by GC/MS methods. They are also organic products certified OCQV, USDA, and Agriculture biologique. The delayed brain tumor cells (DBT cells) and MHV-A59; ATCC VR-764 were provided by Professor Alain Lamarre (Institut national de la recherche scientifique, Laval, Québec, Canada). All other chemicals were purchased from Sigma-Adrich (St. Louis, MO, USA) unless otherwise stated.

Preparation of EOs emulsion

The EOs were chosen according to the literature on antiviral and antimicrobial properties (Table 1). EOs were stored at 4 °C until used. The water-in-oil microemulsions of EOs and EOs-blends were prepared with water and dimethyl sulfoxide (DMSO). EOs and DMSO were mixed in a 1:1 ratio, and water was added to adjust the concentration. The mixture was homogenized for 1 min at 15,000 rpm using UltraTurrax (TP18/1059 homogenizer) and then sterilized by passing through a 0.2 µm membrane filter. DMSO was selected as the emulsifier since it acted as a co-surfactant for stabilizing the microemulsions, and at low concentrations, it does not induce cell toxicity (Jaiswal et al., 2015).

DBT cells culture

DBT cells were used as host for viral infection and replication, and they were stored at -80 °C until used. The DBT cells were grown in Eagle's minimal essential medium (EMEM 1X) containing 10 % heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific Inc., Waltham, MA, USA) and 1 % sodium pyruvate. Penicillin/Streptomycin 100X solution was used only if needed. The cells are incubated at 37 °C in an atmosphere of 5 % CO_2. When cell confluence reached 90 %, the monolayers were detached from the plastic surface by trypsinization with Trypsin-EDTA 0.25 % for sequential dilutions. Cells were plated in 96-well culture plates for cytotoxicity assay, 6-well culture plates for viral production, and 24-well culture plates for plaque assay.

MHV-A59 preparation

MHV-A59 is an enveloped single-strand RNA virus and an accepted surrogate of the SAR-CoV-2 virus (Kalaiselvan et al., 2022). MHV-A59 stocks were stored at -80 °C until use. MHV-A59 were prepared from supernatants of infected DBT cells at an appropriate multiplicity of infection. Viral titers in per mL (PFU/ml) of virus stocks were determined by a standard plaque assay on DBT cells. Viruses are kept in EMEM media (1X) containing 1 % heat-inactivated FBS and 1 % sodium pyruvate.

Cytotoxicity assay of EOs emulsions

The cell proliferation assay of DBT cells was performed to evaluate the dose-dependent cytotoxicity of EOs by the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay described elsewhere with slight modification (Shankar et al., 2018; Jaiswal et al., 2020). DBT cells were seeded in a 96-well plate at a density of 5×10^5 cells per well in 100 µl of EMEM media containing 10 % FBS and then incubated at 37 °C and 5 % CO_2 for 24 h. After 24 h, the media were removed and replaced with the fresh media containing microemulsion of different concentrations (20000, 10000, 5000, 2500, 1250 µg/ml) of EOs, and incubated the cell for a further 24 h at 37 °C and 5 % CO_2. Each plate included controls, microemulsions with water, DMSO, and fresh medium without any EOs, at the same concentrations as the tests. The medium was removed, and 20 µl of MTT solution was added and incubated for 1 to 3 h at 37 °C in the dark. Then the solution was removed, and 150 µl of DMSO containing 2 % glycine was added to each well to dissolve the crystals. Within 30 min, the absorbance was measured at a wavelength of 595 nm in a spectrophotometer, and the percentage cell viability was calculated by comparing the values of treated cells with those of untreated cells.

$$\text{Cell viability } (\%) = \frac{Ac - At}{Ac} x\ 100$$

where Ac is the absorbance of the control sample without any treatment and At is the absorbance of the treated sample.

The cytotoxic concentrations of EOs in the vapor phase were not

Table 1
List of selected EOs and pre-made blends by Aliksir.

EOs number	Botanical name	Family	Common name	Major constituents
1	Achillea millefolium L.	Asteraceae	Yarrow	1,8-cineole (10.1%), camphor (9.2%), germacrene D (7.8%), piperitone (6,2%), α-pinene (5.9%) and artemisia ketone (5.7%)
2	Ambrosia artemisiifolia L.	Asteraceae	Ragweed	germacrene D (32.92%), β-pinene (15.14%), limonene (9.90%), and caryophyllene (4.49%)
3	Angelica archangelica L.	Apiaceae	Angelica or Wild celery	α-pinene (21.3%), δ-3-carene (16.5%), limonene (16.4%) and α-phellandrene (8.7%)
4	Artemisia annua L.	Asteraceae	Sweet wormwood	camphor (up to 48%), germacrene D (up to 18.9%), artemisia ketone (up to 68%), and 1,8 cineole (up to 51.5%)
5	Artemisia dracunculus L.	Asteraceae	Tarragon	essential oils (0.15-3.1%), coumarins (>1%), flavonoids, and phenolcarbonic acids
6	Cinnamomum camphora (L.) J. Presl	Lauraceae	Camphor	D-camphor (51.3%), 1,8-cineole (4.3%), α-terpineol (3.8%), and 3-methyl-2-butenoic acid, oct-3-en-2-yl ester (3.1%)
7	Cinnamomum cassia	Lauraceae	Chinese cinnamon	Cinnamaldehyde (69.15%), methoxycinnamic acid (21.18%), benzyl alcohol (6.14%), and benzyl benzoate (3.53%)
8	Cinnamosma fragrans Baill.	Canellaceae	Saro	1,8-cineole (51.0%) and sabinene (10.6%)
9	Citrus limon (L.) Osbeck	Rutaceae	Lime	monoterpene hydrocarbon (57.2%), namely limonene (31.5%) and sabinene (15.9%), and oxygenated monoterpenoids citronellal (11.6%), linalool (4.6 %) neral (4.5%), geranial (4.5%) and geranyl acetate (3.4 %)
10	Commiphora myrrha (T.Nees) Engl.	Burseraceae	Myrrh	furanoeudesma-1,3-dione (31.1%) followed by curzerene (23.1%), germacra-1 (10),7,11-trien-15-oic acid, 8,12-epoxy-6-hydroxy-gammalactone (14.4%), lindestrene (11.9%)
11	Cymbopogon martini var. sofia B.K.Gupta	Poaceae	Palmarosa (Palm rose)	myrcene (0.1–-0.2%), cis–ocimene (0.2–0.3%),trans--ocimene (1.3–-1.4%), linalool (2.1—2.5%), neral (0.1%), geraniol (66.2–76.9%), geranial (0.1–-0.4%), geranylacetate (14.9–-24.6%), caryophyllene (0.3-–0.5%), geranyl isobuterate (0.1%), and farnesol (0.9–-1.3%)
12	Cymbopogon winterianus Jowitt ex Bor	Poaceae	Java citronella	citronellal (23,59%), geraniol (18,81%) and citronellol (11,74%)
13	Eucalyptus citriodora (Hook.) K.D.Hill & L.A.S. Johnson	Myrtaceae	Lemon-scented gum	β-Citronellal (71.8%), (−) Isopulegol (7.3%), Isopulego (4.3%), β-Citronellol (2.9%), α-Pinene (1.1%), and 1,8-Cineole (0.8%)
14	Eucalyptus globulus Labill.	Myrtaceae	Blue gum	1,8-Cineole (83.9%), (+) Limonene (8.2%), α-Pinene (4.2%), o-Cymene (2.9%)
15	Eucalyptus polybractea R. T.Baker	Myrtaceae	Blue-leaved mallee	1, 8-cineole (91.7-94.2 %), α-pinene (0-1.2 %), β-pinene (0.4-2.3 %), limonene (0.2-1.3 %), p-cymene (1.23-2.75 %), and terpinene-4-ol (0.6-0.92 %)
16	Eucalyptus radiata Sieber ex DC.	Myrtaceae	Narrow-leaved peppermint	1,8-cineole (58.7-74.2%), α-terpineol (8.4 to 17.2%) and limonene (4.1 -8.9%)
17	Eugenia caryophyllus (Spreng.) Bullock & S.G. Harrison	Myrtaceae	Clove	eugenol (76.8%), β-caryophyllene (17.4%), α-humulene (2.1%), and eugenyl acetate (1.2%)
18	Euthamia graminifolia (L.) Nutt.	Asteraceae	Grass-leaved goldenrod	sabinene (18%), β-pinene (10%) and β-phellandrene (23%)
19	Laurus nobilis L.	Lauraceae	Bay laurel	1,8-Cineole (31.9%), sabinene (12.2%), and linalool (10.2%)
20	Melaleuca alternifolia (Maiden & Betche) Cheel	Myrtaceae	Tea tree	α-pinene (21.64 %), γ-terpinene (21.09 %), terpinen-4-ol (17.31 %), limonene (9.37 %), and o-cymene (6.54 %)
21	Melaleuca quinquenervia (Cav.) S.T.Blake	Myrtaceae	Broad-leaved paperbark or Niaouli	1,8-cineole (21.60%), α-pinene (15.93%), viridiflorol (14.55%), and α-terpineol (13.73%)

(*continued on next page*)

Table 1 (*continued*)

EOs number	Botanical name	Family	Common name	Major constituents
22	Melissa officinalis L.	Lamiaceae	Lemon balm	geranial (43.96%-54.93%), neral (29.95%-34.66%), geraniol (3.11%-12.85%), and (ε)-caryophyllene (2.62%-6.66%)
23	Monarda didyma L.	Lamiaceae	Crimson beebalm	thymol (59.3%), p-cymene (10.3%), terpinolene (9.2%), δ-3-carene (4.4%), myrcene (3.7%), and camphene (3.4%)
24	Ocimum basilicum L.	Lamiaceae	Sweet Basil	methyl eugenol (78.02%), α-cubebene (6.17%), nerol (0.83%) and ε-muurolene (0.74%)
25	Picea mariana Britton, Sterns & Poggenburg	Pinaceae	Black spruce	Bornyl acetate (34.2 %) α-pinene (12.9 %) and camphene (16.4 %)
26	Picea glauca (Moench) Voss	Pinaceae	White spruce	camphor (65 %), borneol (10 %)
27	Pimenta dioica (L.) Merr	Myrtaceae	Allspice	eugenol (48.67%), β-pinene (18.52%) and (1E)-Phenol-2-propenyl (7.61%)
28	Rosmarinus officinalis L.	Lamiaceae	Camphor Rosemary	cineole (28.5%), camphor (27.7%), and alpha-pinene (21.3%)
29	Rosmarinus officinalis L.	Lamiaceae	Cineol Rosemary	cineole (28.5%), camphor (27.7%), and alpha-pinene (21.3%)
30	Salvia officinalis L.	Lamiaceae	Sage	α-thujone (26.68%), (E)-β-caryophyllene (7.47%), 1,8-cineole (7.19%), α-humulene (6.11%), β-pinene (5.44%), β-thujone (5.35%), camphor (4.84%), allo-aromadendrene (4.55%), borneol (3.69%), and α-pinene (3.58%)
31	Tanacetum annuum L.	Asteraceae	Blue tansy	camphor (16.69%), α-pinene (12.37%), bornyl acetate (11.97%), limonene (11.10%), borneol (6.33%), α-terpinyl acetate (4.62%) and chamazulene (3.49%)
32	Thymus vulgaris L.	Lamiaceae	Thyme	p-cymene (8.41%), γ-terpinene (30.90%) and thymol (47.59%)

EOs 22 was used only in EOs in vapor phase experiment.

determined because they were not in contact with any cells when the virus was pretreated with EOs.

Antiviral activity of EOs

MHV-A59 production for antiviral activity of EOs in emulsion phase

DBT cells were seeded in a 6-well culture plate at a concentration of 5×10^5 cells/ml in 2 ml/well of EMEM containing 10 % FBS. After incubating the plate for 24 h at 37 °C and 5 % CO_2, the medium was removed, and cells were infected with MHV-A59 for 2 h at 37 °C and 5 % CO_2 while stirring the plate frequently in 1 ml/well of EMEM with 1 % FBS. The medium was then removed, and fresh medium containing the appropriate dilution of EOs emulsions (based on cell toxicity assay result) was added to each well. As a control, viruses were also produced without any EOs (incubated with microemulsions of water, DMSO, and medium) by the same procedure as the tests. After 24 h, the medium was centrifuged and supernatants containing viruses produced (which replicated on contact with the different concentrations of EOs) were kept.

MHV-A59 production for antiviral activity of EOs in vapor phase

Filter papers were glued inside the lid of a Petri dish, and the assembly was sterilized under UV radiation overnight. Filter papers were soaked with 150 µl of each EO. Besides, 300 µl of MHV-A59 were spread in Petri disc with sterile glass beads in order to distribute them over the entire surface. Petri dishes with the virus (without EOs on the filter paper) were also produced as controls. After incubating the virus for 30 min at room temperature, viruses were recovered by washing the Petri dish with fresh medium (EMEM 1 % FBS). The supernatants containing viruses were kept. Virus production was conducted in duplicate plates.

Plaque assay

Plaque assay was performed by the method of Freppel et al. (2018) with slight modification. Briefly, healthy DBT cells were seeded at a concentration of 5×10^5 cells/mL in a 24-well culture plate in 500 µL/well of EMEM containing 10 % FBS. After incubating the plate for 24 h at 37 °C and 5 % CO_2, the medium was removed, and 200 µl/well of MHV-A59 previously produced with EOs in emulsions or in vapor phase with their respective controls were added to infect the host cells. Serial dilutions of the viruses were made, and the plate was incubated for 1 h at 37 °C and 5 % CO_2. The viral inoculum was aspirated, and infected cells were overlaid with MEM-CMC solution (carboxymethylcellulose sodium salt, medium viscosity in MEM with L-Glutamine) for 48 h at 37 °C and 5 % CO_2. After removing the overlay solution and washing with D-PBS, cells were fixed and stained with 500 µl/well of a fixation/coloration solution containing 0.50 % crystal violet, 0.80 % sodium chloride, 50 % anhydrous ethanol, and 1.85 % formaldehyde for 3 h at room temperature. After rinsing the plates, the number of plaques in wells was counted to determine viral titers expressed in PFU/ml of tests (EOs--produced viruses) and viral titers of controls. The reduction of plaques was counted and compared with untreated control and then expressed in a percentage (%) reduction. Results were also expressed in log inactivation (log10 of the sample treated with EOs from the log10 of the control sample incubated without EOs).

Statistical analysis

All the experiments were performed in triplicate, and results are presented as mean±standard deviation. The cytotoxic concentrations of the EOs were determined by Student's t-test. If the cell viability results with an EO were significantly different from the cell viability of the untreated control ($p\text{-value} \leq 0.05$), the EO was discarded and was not used for further testing with EOs in emulsion.

Results

Cytotoxicity assay

MHV-A59 viruses were treated with EOs in emulsions during their replication. At first, cytotoxicity assay of all EOs was carried out to identify the highest concentration of EOs, which should not be toxic to the cells. For this, five different concentrations (20000, 10000, 5000, 2500, and 1250 μg/ml) of all EOs were tested against DBT cells using MTT assay and compared to control (DBT cell without any EOs treatment). Table 2 shows the percentage of cell viability compared to the controls (cell viability of 100 %). As shown in Table 2, EOs 1, 3, 13, 29, and 30 did not induce significant cell inhibition on DBT cells at the concentration of 20000 μg/ml. EOs 4, 5, 6, 8, 9, 14, 18, 20, 24, 25, and 31 did not show significant cell inhibition up to the concentration of 10000 μg/ml, EOs 2, 16, 19, 23, and 26, upto concentration of 5000 μg/ml, EOs 12 upto concentration of 2500 μg/ml, and EOs 11 upto concentration of 1250 μg/ml. The results of EOs 7, 10,15, 17, 22, 27, 28, and 32 exhibited less than 80 % of cell viability even at the lowest concentration tested; therefore, they were not selected for the antiviral activity in emulsions. Based on the results of cell toxicity assay, some EOs (7, 10, 12, 15, 17, 20, 21, 22, 24, 27, 28, and 32) were not chosen for further studies.

Antiviral activity of EOs in emulsions on MHV-A59

The inhibitory potential of each EO on viral replication was determined by plaque assay. To know the antiviral activity of EOs on the viral replication, EOs emulsion was applied to MHV-A59 infected DBT cells after penetration of the viruses into host cells. Virus stocks were produced with the non-cytotoxic concentration of EOs emulsions (Table 1). Viruses produced in these emulsions were harvested and preserved at -80 °C until tested for plaque assay. Antiviral activity is expressed in a log reduction of plaque related to % inactivation of MHV-A59.

Table 3 represents the antiviral activity of EOs in emulsions at the highest concentration tested expressed in plaque reduction and log inactivation of MHV-A59. Among all EOs tested, EOs 2, 3, 4, 5, 6, 18, 23, and 31 showed more than 3 log reduction of MHV-A59. A reduction of 100 % in plaque formation can not be calculated in the logarithm scale because there was not any plaque in the cellular layer. EOs 23 (*Monarda didyma*) exhibited the best antiviral activity, as there was no plaque formation. EOs 19, 29, and 30 did not reduce the viral plaque formation significantly, which is expressed by their result below 90 % and, therefore, an inactivation of 0 to 1-log against MHV-A59. EOs 1, 9, and 16 demonstrated 1 to 2-log inactivation (≥90 % to <99 % of reduction). EOs 8, 13, 14, 25, and 26 had a better potential of inactivation against MHV-A59 with a maximum of 2-log inactivation (≥99 % to <99.9 % of reduction). All EOs that induced a reduction of more than 3-log (≥99.9 % to 100 % of reduction) were then tested at a lower concentration to determine the lower concentration of EOs that have an inhibitory effect against MHV-A59. Therefore, EOs 2, 3, 4, 5, 6, 18, and 31 were then tested at 5000 μg/ml, EO 23 (*Monarda didyma* L.) was tested at 2500 μg/ml.

Results in Table 4 are expressed as log reduction of MHV-A59 at the 2-fold diluted concentration of EOs. The concentrations tested were more dilute than those that had worked to have a 3-log or more inactivation (Table 3). Results showed that none of the tested EOs exhibited more than a 3-log inhibitory effect against MHV-A59 because EOs were not potent enough to induce antiviral activity during intracellular virus penetration at this diluted concentration. Only EO 2 (*Ambrosia artemisiifolia*) had a 2-log inactivation against MHV-A59, and EOs 3, 4, 5, 6, 18, 23, and 31 had 1-2-log inactivation.

Antiviral activity of EOs in vapor on MHV-A59

Unlike EOs in emulsion, EOs in vapor form did not come into contact

Table 2
Results of the cellular viability test (%) of DBT cells after being in contact with EOs in emulsions at concentrations 1250–20000 μg/ml.

EO	20000 μg/ml	10000 μg/ml	5000 μg/ml	2500 μg/ml	1250 μg/ml
Control	100.00[a]	100.00[a]	100.0[a]	100.00[a]	100.00[a]
1	95.18 ± 0.13[a]	87.49 ± 0.11[a]	86.55 ± 1.02[a]	94.42 ± 3.02[a]	98.88 ± 1.15[a]
2	1.45 ± 0.01[b]	101.35 ± 1.18[a]	97.86 ± 2.67[a]	103.40 ± 1.85[a]	103.66 ± 0.42[a]
3	108.64 ± 0.47[a]	101.81 ± 0.09[a]	106.89 ± 0.75[a]	99.71 ± 3.37[a]	102.33 ± 1.14[a]
4	33.12 ± 0.29[b]	100.74 ± 0.65[a]	107.67 ± 0.52[a]	96.01 ± 1.18[a]	99.02 ± 0.50[a]
5	55.52 ± 0.86[b]	95.02 ± 0.46[a]	93.16 ± 1.33[a]	91.06 ± 2.96[a]	93.65 ± 1.20[a]
6	1.26 ± 0.09[b]	86.58 ± 3.20[a]	88.86 ± 2.36[a]	95.83 ± 0.39[a]	93.54 ± 0.14[a]
7	3.43 ± 0.05[b]	2.36 ± 0.06[b]	2.33 ± 0.02[b]	4.23 ± 0.05[b]	12.86 ± 0.34[b]
8	2.93 ± 0.08[b]	102.84 ± 0.65[a]	96.58 ± 0.92[a]	97.63 ± 1.74[a]	96.85 ± 0.48[a]
9	2.91 ± 0.01[b]	80.89 ± 0.46[a]	108.00 ± 0.34[a]	101.74 ± 2.24[a]	104.26 ± 1.01[a]
10	4.77 ± 0.08[b]	13.08 ± 0.37[b]	16.69 ± 0.27[b]	37.97 ± 0.10[b]	62.23 ± 1.29[b]
11	1.45 ± 0.01[b]	0.88 ± 0.01[b]	39.27 ± 0.45[b]	56.25 ± 2.89[b]	89.61 ± 1.27[b]
12	0.24 ± 0.04[b]	42.09 ± 3.24[b]	76.66 ± 0.31[b]	87.60 ± 0.49[a]	84.20 ± 0.28[a]
13	127.29 ± 3.01	107.83 ± 0.53	99.91 ± 0.37[a]	96.19 ± 1.35[a]	97.87 ± 0.31[a]
14	2.66 ± 0.12[b]	88.49 ± 5.38	93.84 ± 3.06[a]	91.38 ± 1.88[a]	99.40 ± 0.26[a]
15	4.29 ± 0.12[b]	32.14 ± 0.31[b]	76.98 ± 1.92[b]	78.14 ± 0.46[b]	74.76 ± 0.22[b]
16	0.66 ± 0.02[b]	74.03 ± 0.31[b]	82.18 ± 2.72[a]	98.41 ± 1.52[a]	95.59 ± 1.17[a]
17	3.17 ± 0.04[b]	1.63 ± 0.03[b]	3.11 ± 0.19[b]	3.92 ± 0.43[b]	9.99 ± 0.18[b]
18	8.63 ± 0.99[b]	95.81 ± 1.71[a]	99.94 ± 1.79[a]	97.74 ± 1.37[a]	97.95 ± 0.48[a]
19	0.95 ± 0.02[b]	56.18 ± 2.06[b]	81.25 ± 0.38[a]	86.91 ± 0.35[a]	91.98 ± 1.51[a]
20	0.13 ± 0.03[b]	90.20 ± 0.29[a]	89.8 ± 0.69[a]	80.60 ± 0.78[a]	80.7 ± 0.88[a]
21	n.d	n.d	n.d	n.d	n.d
22	2.75 ± 0.02[b]	1.69 ± 0.02[b]	1.60 ± 0.01[b]	22.98 ± 2.62[b]	53.75 ± 1.53[b]
23	3.99 ± 0.02[b]	1.40 ± 0.02[b]	104.77 ± 1.31[a]	104.88 ± 2.59[a]	101.93 ± 1.88[a]
24	5.12 ± 0.12[b]	85.08 ± 0.15[a]	81.08 ± 0.23[a]	82.20 ± 1.40[a]	88.14 ± 0.58[a]
25	35.92 ± 0.22[b]	84.48 ± 1.95[a]	102.19 ± 1.66[a]	91.88 ± 2.71[a]	102.51 ± 0.72[a]
26	2.35 ± 0.10[b]	73.16 ± 1.74[b]	94.77 ± 1.59[a]	95.59 ± 1.12[a]	94.17 ± 1.07[a]
27	2.72 ± 0.02[b]	1.61 ± 0.01[b]	1.68 ± 0.03[b]	3.02 ± 0.14[b]	10.48 ± 0.29[b]
28	28.31 ± 3.12[b]	80.14 ± 0.46[b]	79.93 ± 1.09[b]	75.50 ± 0.84[b]	75.18 ± 1.39[b]
29	92.67 ± 4.40[a]	99.51 ± 1.92	97.83 ± 0.41[a]	97.12 ± 1.48[a]	93.66 ± 0.98[a]
30	84.18 ± 1.21[a]	102.03 ± 0.88	98.01 ± 1.63[a]	96.66 ± 3.89[a]	101.47 ± 1.51[a]
31	4.28 ± 0.03[b]	86.57 ± 0.73	102.02 ± 0.55[a]	96.48 ± 1.94[a]	101.43 ± 0.45[a]
32	9.62 ± 0.03[b]	6.14 ± 0.04[b]	5.79 ± 0.06[b]	5.25 ± 0.00[b]	5.51 ± 0.03[b]

Data are presented as mean (%) ± SD.
n.d.: not determined, EOs not used in this part of the experiment.
[a] : *p-value* > 0.05 means that no statistically significant effect was observed on cell viability and therefore results are in the same group as control of each concentration
[b] : *p-value* ≤ 0.05, results are statistically significantly different from the control of each concentration.

Table 3
Antiviral activity of EOs in emulsions at the highest concentration tested expressed in log inactivation against MHV-A59.

EO	Concentration (μg/ml)	Log inactivation
1	20000	1.14[b]
2	5000	3.92[a]
3	10000	3.95[a]
4	10000	3.63[a]
5	10000	3.13[a]
6	10000	3.40[a]
8	10000	2.18[b]
9	5000	1.10[b]
11	1250	0.83[b]
13	20000	2.86[b]
14	10000	2.05[b]
16	5000	1.33[b]
18	10000	3.37[a]
19	5000	0.07[b]
23	5000	n.a.[a]
25	10000	2.42[b]
26	10000	2.51[b]
29	20000	0.96[b]
30	20000	0.03[b]
31	10000	5.03[a]

n.a.: not applicable, log inactivation was not calculated because 100 % of MHV-A59 were reduced when counting the number of plaques (100 % reduction compared to the untreated control).
[a] : Inactivation of MHV-A59 of 100 % or ≥ 3.00
[b] : log inactivation of < 3.00

Table 4
Antiviral activity of EOs in emulsions at 50 % more diluted concentration expressed in log inactivation against MHV-A59.

EO	Concentration (μg/ml)	Log inactivation
2	5000	2.29[a]
3	5000	1.26[a]
4	5000	1.19[a]
5	5000	1.05[a]
6	5000	1.26[a]
18	5000	1.18[a]
23	2500	1.10[a]
31	5000	1.13[a]

[a] : log inactivation of < 3.00

with host cells when viruses were treated. The potential inhibitory effect against MHV-A59 of 44 EOs was determined by pretreatment of the viruses for 30 min at room temperature and then with the infection of DBT cells. A plaque assay was done to observe the percentage of plaque reduction and the log inactivation of the tested EOs compared with the untreated control. Because the host cells were not in contact with EOs, the first concentration tested to treat MHV-A59 was the highest concentration of EOs.

Table 5 shows the log inactivation of pre-treated MHV-A59 with all EOs undiluted in vapor form for 30 min. As seen from the table, none of the EO had a potential antiviral activity. *Cinnamomum camphora* (EO 7) exhibited 1.09-log inactivation. Whereas, other EOs had not demonstrated strong enough antiviral potential against MHV-A59 (>1-log inactivation). Since none of the EOs showed any potential inhibitory effect of interest (≥3-log inactivation), other concentrations of these EOs were not tested further in the vapor phase. There might be a better antiviral activity with higher contact time (more than 30 min), but MHV-A59 can not stay on a surface very long, or its potential for infection and replication in host cells might be compromised (Bueckert et al. 2020). Since *M. didyma* EO exhibited the best antiviral activity, the chemical composition of EO wre determined by gas chromatography and the results are presented in Fig. 1 and Table 6.

Table 5
Antiviral activity of EOs in vapor form at the highest concentration against MHV-A59.

EO	Log inactivation	EO	Log inactivation
1	0.08[a]	17	0.25[a]
2	0.31[a]	18	0.17[a]
3	0.31[a]	19	0.52[a]
4	0.79[a]	20	0.31[a]
5	0.22[a]	21	0.21[a]
6	1.09[a]	22	0.26[a]
7	0.47[a]	23	0.77[a]
8	0.31[a]	24	0.13[a]
9	0.35[a]	25	0.10[a]
10	0.30[a]	26	0.09[a]
11	0.15[a]	27	0.23[a]
12	0.97[a]	28	0.27[a]
13	0.58[a]	29	0.31[a]
14	0.33[a]	30	0.16[a]
15	0.20[a]	31	0.23[a]
16	0.31[a]	32	0.29[a]

[a] : log inactivation of < 3.00

Discussion

Cytotoxicity assay is the first step in choosing the concentration of antiviral compounds to be used. The cytotoxicity assay results suggest that the cytotoxic effects of selected EOs were concentration-dependent. Similar results of concentration-dependent cytotoxic effect of EOs are reported in the literature (Correa et al., 2023; Wani et al., 2021). The results also suggested that the cell toxicity of EOs depends on the types of EOs and their sources due to the presence of different chemical constituents. The antiviral activity of EO can be determined by a plaque assay to know the effect of EO on viral replication. Log inactivation is a way to express the number of viruses killed or unable to replicate by antiviral agents during intracellular replication. Log inactivation measures the effectiveness of antiviral compounds according to pre-established parameters (concentration, time, and temperature). According to the requirements referenced in ICH Q5 (quality guidelines), the claim “antiviral” can be attributed to a compound if it has an inhibitory effect greater or equal to 4-log (ICH Guideline 2022). In the present study, two EOs had a plaque reduction of more than 4-log against MHV-A59, only when they were prepared in emulsions. As seen in Table 3, EO numbers 23 (*M. didyma*) inhibited 100 % viral replication at the concentration of 5000 μg/ml. Another EOs that met the criteria of antiviral was EO 31 (*Tanacetum annuum*) at 10000 μg/ml (5.03-log inactivation).

Previous work on the antiviral action of some disinfectants against MHV-A59 revealed that common household disinfectants showed a log reduction of 3.0 to 4.5, which are similar to EOs number 23 (*M. didyma*) and 31 (*Tanacetum annuum*S. The household disinfectant such as 0.12 % parachlorometaxylenol, 0.05 % triclosan, 0.23 % pine oil, 0.21 % sodium hypochlorite, and 0.10 % alkyl dimethyl benzyl ammonium saccharinate with 79 % ethanol effectively inactivated MHV-A59; therefore, these disinfectants have potential against surrogates for SARS coronavirus (Dellanno et al., 2009). In the present work, since EOs 23 and 31 displayed similar values of log reduction to those of household disinfectants, they could be potential disinfectants against viruses. The results showed that EOs in emulsions are in direct contact with MHV-A59 during intracellular virus replication seems to be the best way to significantly reduce viral load compared to EOs in vapor forms. Usachev et al. (2013) demonstrated antiviral activity of tea tree and eucalyptus oil in aerosol and vapor forms against Influenza A virus and E. coli phage M13 and found that aerosol forms are more effective than vapor form. The EO 23 (*M. didyma*) showed the best antiviral activity among all the EO selected for the study.

With regard to the mechanism of antiviral action, in most cases where antiviral properties have been evaluated before and after host-cell

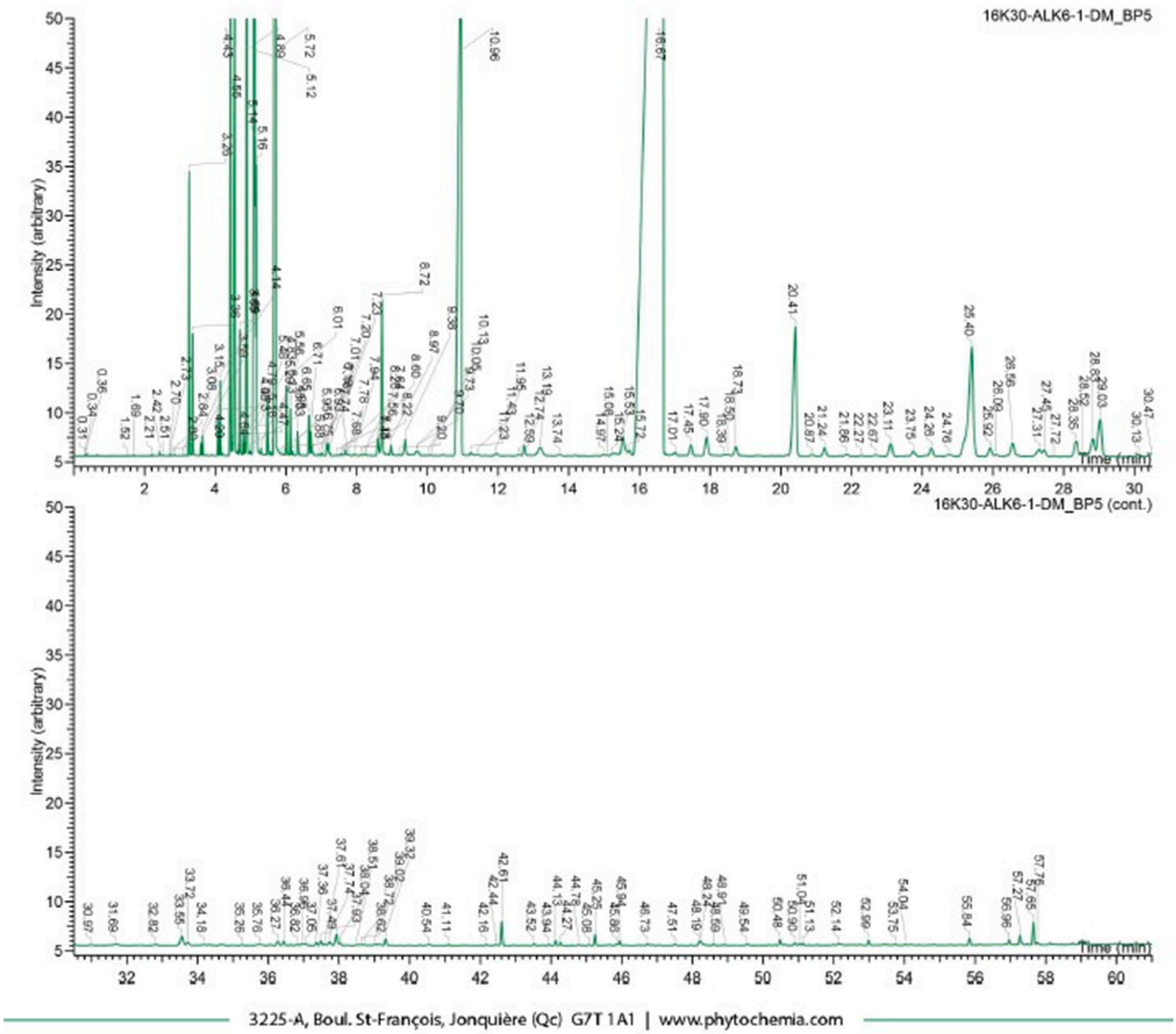

Fig. 1. Gas-chromatography of *Monarda didyma* L. essential oil.

adsorption, the antiviral action has happened mainly upon treatment of virus particles with EO prior to their addition or adsorption to cell monolayers (Gilling et al., 2014). This recommends a direct effect of EO on free virus particles rather than an intracellular virucidal activity. The antiviral effect of EOs, which are lipophilic by nature, likely act to disrupt or interfere with viral membrane proteins involved in host cell attachment (Schuhmacher et al., 2003). Virucidal activity of essential oils, which are lipophilic by nature, is probably due to disruption of the viral membrane or interference with viral envelope proteins involved in host cell attachment (Schnitzler et al., 2007).

Conclusion

The recent pandemic of COVID-19 highlighted the importance of identifying effective new approaches to prevent and treat viral infections. The present study evaluated the potential antiviral activity of EOs in emulsions and in vapor form to reduce MHV-A59, a murine surrogate of SARS-CoV-2. EOs in emulsions were applied to MHV-A59 after penetration of the viruses into the host cells during intracellular virus replication. *Monarda didyma* at 5000 µg/ml and pre-blend 095-blue complex at 20000 µg/ml showed a 100 % reduction of viral plaque in plaque assay. *Tanacetum annuum* and pre-blend Guardian complex at 10000 µg/ml inhibited MHV-A59 by 5.03-log and 4.88-log, respectively. In vapor form, none of the EOs showed potential inhibitory effects against MHV-A59. The results demonstrated that all 44 undiluted EOs, incubated with MHV-A59 for 30 min, had a $\leq$1.09-log inactivation compared to an untreated virus. The findings of this study show that the EOs or pre-blend of EOs exhibited more than 4 log reduction of MHV-A59 and have the potential to be used as disinfection for SARS-CoV-2.

CRediT authorship contribution statement

Karelle Contant: Writing – original draft, Methodology, Formal analysis, Data curation. **Shiv Shankar:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Conceptualization. **Monique Lacroix:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Table 6
The compounds identified in *Monarda didyma* L. using gas chromatography.

Identifiction	Column: BPS			Column: WAX			Molecular class
	R.T.	R.I.	%	%	R.I.	R.T.	
α-Thujene	3.26	918	0.64	0.66	947	0.87	Monoterpene
α-Pinene	3.36	925	0.26	0.23	940	0.84	Monoterpene
Unknown (m/z = 91, 92 (49), 65 (12)... 134? (2))	3.59	939	0.03	0.10	1082	1.70*	Monoterpene
Camphene	3.65	942	0.05	0.04	989	1.04	Monoterpene
Sabinene	4.08	968	0.09	0.08	1049	1.45	Monoterpene
β-Pinene	4.14	972	0.18	0.17	1033	1.32	Monoterpene
Myrcene	4.43	990	1.59	1.54	1110	1.93	Monoterpene
Octan-3-one	4.47	992	0.04	12.44	1189	2.84*	Aliphatic ketone
Octen-3-ol	4.55	997	2.41	2.42	1391	5.63	Aliphatic alcohol
α-Phellandrene	4.69*	1005	0.33	0.20	1102	1.86	Monoterpene
Δ3-Carene	4.69*	1005	0.33	0.10	1082	1.70*	Monoterpene
Octan-3-ol	4.79	1010	0.06	0.06	1339	4.88	Aliphatic alcohol
α-Terpinene	4.89	1016	1.84	1.85	1118	2.01	Monoterpene
para-Cymene	5.12	1028	7.69	7.79	1205	3.03*	Monoterpene
Limonene	5.14	1029	0.51	0.66	1134	2.21	Monoterpene
β-Phellandrene	5.16	1031	0.57	0.67	1140	2.27*	Monoterpene
1,8-Cineole	5.18	1032	0.04	0.67	1140	2.27*	Monoterp. ether
cis-β-Ocimene	5.29	1038	0.01	12.44	1189	2.84*	Monoterpene
trans-β-Ocimene	5.48	1048	0.18	7.79	1205	3.03*	Monoterpene
γ-Terpinene	5.72	1062	12.60	[12.44]	1189	2.84*	Monoterpene
cis-Sabinene hydrate	6.01	1077	0.18	0.18	1399	5.74	Monoterp. alcohol
Terpinolene	6.13	1083	0.12	0.13	1218	3.19	Monoterpene
para-Cymenene	6.33*	1094	0.10	0.04	1379	5.46	Monoterpene
Nonen-3-ol	6.33*	1094	[0.10]	0.09	1482	7.20	Aliphatic alcohol
Linalool	6.65*	1107	0.13	0.07	1485	7.27*	Monoterp. alcohol
trans-Sabinene hydrate	6.65*	1107	[0.13]	0.06	1469	6.99	Monoterp. alcohol
Nonanal	6.71	1109	0.08	0.06	1332	4.77	Aliphatic aldehyde
cis-para-Menth-2-en-1-ol	7.16	1126	0.04	0.03	1493	7.42	Monoterp. alcohol
Camphor	7.68	1146	0.02	0.02	1427	6.18	Monoterp. ketone
Borneol	8.60*	1180	0.10	0.14	1616	10.77*	Monoterp. alcohol
Terpinen-4-ol	8.72	1184	0.80	2.19	1525	8.05*	Monoterp. alcohol
Decen-3-ol	8.97	1194	0.05	0.04	1589	9.96	Aliphatic alcohol
α-Terpineol	9.38	1204	0.12	0.15	1630	11.26	Monoterp. alcohol
Carvacrol methyl ether	10.96	1239	7.04	7.46	1529	8.19	Monoterp. ether
Thymol	15.53	1327	0.25	0.23	2099	36.87	Monoterp. alcohol
Carvacrol	16.67*	1344	53.19	54.52	2122	37.63	Monoterp. alcohol
α-Cubebene	16.67*	1344	[53.19]	0.02	1410	5.91	Sesquiterpene
α-Copaene	17.45	1356	0.11	0.10	1434	6.32	Sesquiterpene
β-Bourbonene	17.90*	1363	0.22	0.13	1453	6.67	Sesquiterpene
Eugenol	17.90*	1363	[0.22]	0.05	2075	36.07	Phenylpropanoid
β-Elemene	18.73	1376	0.10	[2.19]	1525	8.05*	Sesquiterpene
β-Caryophyllene	20.41	1400	1.63	[2.19]	1525	8.05*	Sesquiterpene
β-Copaene	21.24	1410	0.10	0.24	1501	7.59	Sesquiterpene
α-Humulene	23.11	1433	0.16	0.14	1578	9.66	Sesquiterpene
Coumarin	23.75	1441	0.06				Coumarin
trans-β-Farnesene	24.26	1447	0.10	0.11	1627	11.11	Sesquiterpene
Germacrene D	25.40*	1461	2.09	1.72	1624	11.01	Sesquiterpene
γ-Muurolene	25.40*	1461	[2.09]	0.39	1607	10.53	Sesquiterpene
β-Selinene	25.92	1467	0.10	0.10	1633	11.42	Sesquiterpene
α-Muurolene	27.31	1483	0.12	0.08	1645	11.91	Sesquiterpene
4-epi-Cubebol	27.45	1484	0.09				Sesquiterp. alcohol
(*E,E*)-α-Farnesene	28.35	1495	0.22	0.28	1689	13.84*	Sesquiterpene
γ-Cadinene	28.83	1502	0.30	0.20	1666	12.84	Sesquiterpene
δ-Cadinene	29.03*	1505	0.55	0.53	1672	13.08	Sesquiterpene
Cubebol	29.03*	1505	[0.55]	0.04	1848	22.60*	Sesquiterp. alcohol
trans-Cadina-1,4-diene	30.13	1520	0.03	[0.28]	1689	13.84*	Sesquiterpene
α-Cadinene	30.47	1524	0.05	0.04	1699	14.31	Sesquiterpene
Caryophyllene oxide	33.55	1567	0.11	[0.04]	1848	22.60*	Sesquiterp. ether
(*E*)-Nerolidol	33.72	1569	0.04	0.03	1995	32.11	Sesquiterp. alcohol
Germacra-4(15),5,10(14)- trien-1-α-ol	39.32	1684	0.05				Sesquiterp. alcohol
Linalool	6.65*	1107	[0.13]	[0.07]	1485	7.27*	Monoterp. alcohol
Borneol	8.60*	1180	[0.10]	[0.14]	1616	10.77*	Monoterp. alcohol
Total identified			97.54 %	98.42 %			

[xx]: Duplicate percentage due to coelutions, not taken account in the identified total.
* : Two or more compounds are coeluting on this column

Declaration of competing interest

All authors state that there is no declation of interest.

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