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Repellent Activity against *Aedes aegypti* and Metabolomic Profiling of *Myrica gale* L. Essential Oils from Irish Boglands

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Abstract:

Bog myrtle (*Myrica gale* L.) is an ethnobotanically significant bogland plant with historic use as an insect repellent. Essential oils, hydro-distilled from bog myrtle leaves and fruits collected from four locations in Ireland, were analysed by GC-MS and tested for repellence against *Aedes aegypti*, the yellow fever mosquito. Contact (arm-in-cage) and spatial (Y-tube olfactometer) repellence assays were used. Commercial essential oils from *M. gale, Myrtus communis* and *Syzygium aromaticum* (clove oil) were also tested. The most effective bog myrtle essential oil in both assays was a Clevenger-hydro-distilled fruit oil, MG4C. Whilst exhibiting significant mean complete protection time in the arm-in-cage assay, it also exhibited strong spatial repellence in the Y-tube olfactometer. Repellence in *M. gale* samples was linked to a higher monoterpene content reflected by the monoterpene to sesquiterpene (M/S) ratio calculated using GC-MS data. Furthermore, metabolomic analysis linked spatial repellence to α-phellandrene and myrcene, whilst quantification of key terpenes alluded to delta-3-carene, β-pinene, γ-terpinene and camphene potentially contributing to the observed effects. This study establishes that specific bog myrtle essential oils are effective mosquito repellents.

Keywords**:** bog myrtle, essential oils, insect-repellent, multivariate data analysis, Y-tube olfactometer, arm-in-cage.

Introduction

Biting arthropods are considered a burden to both human health and agricultural livestock. On the continent of Africa they drain economic resources with an estimated 1.8 billion USD (€1.6 billion) spent on malaria control annually [1]. Finding sources of natural insect repellents that can be locally grown would be more environmentally and economically sustainable whilst offering alternatives to current products. *N,N*-diethyl-m-toluamide (DEET), since its conception in the mid-20th century, has been the most used repellent worldwide [1,2]. However, DEET's interaction with some environmental systems may be of concern as it does not readily degrade by hydrolysis and is regarded as a ubiquitous pollutant in aquatic ecosystems [3]. Thus, the question of DEET's environmental impact coupled with newfound insecticide resistant strains of mosquitoes showing reduced response to this repellent have resulted in a compelling need to search for alternatives [4]. Driven by rising consumer demand, the attention of commercial and academic research groups is turning towards the development and understanding of the mode of action of naturally derived repellents, such as essential oils (EOs), which can be described as effective pre-emptive tools in minimising the impact biting arthropods have as vectors of infectious diseases [5]. Plant species from families such as *Lamiaceae*, *Rutaceae*, *Myrtaceae* and *Annonaceae* have been shown to produce EOs that are effective repellents towards arthropods from orders such as *Diptera* (*Anopheles, Culex*), *Coleptera* (*Tribolium, Lasioderma*), *Lepidoptera* (*Cydia)* and *Isoptera* (*Coptotermes).* They contain chemicals that volatilise readily and these metabolites singularly, or in additive or synergistic ways, exhibit insect repellence. These volatiles can disable biting arthropods' abilities to detect and orientate towards a host [6] and repellence can be measured in terms of short and long distance. A biting arthropod can employ a range of factors to locate a host, and modes of host detection can vary between species. Furthermore, olfaction cues themselves differ at contrasting distances. For example, CO_2 a well-known mosquito kairomone, is a primary cue that primes the flight response and guides insects like mosquitoes toward a potential host from several meters away [7]. Conversely, lactic acid typically acts at a shorter distance as an attractant [8], with temperature and moisture being the final landing cues [9]. Effective deterrence of biting arthropods at both close range and at distance adds to the versatility and ultimate success of a repellent, and so it is important to establish what kind of repellence a candidate exhibits.

Myrica gale L. or bog myrtle, as it's more commonly known, is an aromatic shrub occurring on boglands across Ireland and the Northern Hemisphere. It has an extensive historical record relating to its pharmacological and cultural uses and it is evident from the literature that one of its primary ethnobotanical uses was as an insect repellent. Some Scottish accounts describe beds made from branches and plant stems left in cupboards to deter bed bugs, moths and other pests [10,11]. In Irish literature, farmers are said to have worn sprigs of the shrub in their jackets and crafted wreaths of the plant for their cattle's necks to discourage biting midges [12]. Recent investigations on *M. gale* have been directed towards elucidating the chemical profiles of the EOs present in the various plant structures [13-15]. A small harvesting scheme in the Scottish Highlands found variance in chemical constituents when bog myrtle was cultivated on a large-scale for cosmetic products [16]. They experienced difficulties in maintaining consistency between the plant stands, yearly harvests, and the resulting EO quality. These challenges limited the use of this plant commercially [16-18]. With regards to the repellent activity of *M. gale*, the literature is punctuated with references to it repelling midges [14] and ants [19]. In addition, *M. gale* has been shown to repel mosquitoes, however an intensive investigation on the repellence of different EOs from *M. gale* using multiple assays has not yet been done.

As an extension of our previous work [14] on how extraction techniques impact the chemical profiles of Irish *M. gale* EOs, the current study investigates the contact and spatial mosquito repellence activity of selected *M. gale* EOs and aims to delineate the chemical components responsible. In the current study all EOs were extracted from either the leaf or fruit, collected from different Irish locations, during autumn or summer of the same year. Furthermore, one commercial bog myrtle EO, two commercial *Myrtus communis* EOs (commonly known as myrtle oil), and a commercial *Syzygium aromaticum* (clove bud) EO were included in the study for comparison due to their reported insect repellence in the literature [5]. The insect repellent activities of the EOs were evaluated against *Aedes aegypti* mosquitoes, employing both arm-in-cage and Y-tube olfactometer assays. Chemical profiling of the EOs was performed by GC-MS analysis, followed by multivariate data analysis to correlate the chemical profiles to insect repellence activity and decipher the components present in the EOs that may be contributing to the activity.

Materials and Methods

Chemicals and reagents:

If not stated otherwise, all chemicals were of analytical grade, purchased from Merck. Ethylenediaminetetraacetic acid (BDH, Dubai, U.A.E), β-mercapto-ethanol (PanReac Applichem, ITW Reagents, Barcelona, Spain), chloroform (Fisher Chemicals, Dublin, Ireland), boric acid (Fisher Chemicals, Dublin, Ireland), agarose (Meridian Bioscience, Memphis, TN, United States) and organic lotion base (chemistrystore.com, Stephenson Organic Lotion Base, product code SKU: 82067). Detailed information regarding the composition of the organic lotion base has been provided in **supplemental Table S1**.

Plant material:

Leaves and fruit were collected from various *M. gale* stands in four locations in Ireland, Clonlisk (Co. Offaly), Clara (Co. Offaly), Inishbiggle (Co. Mayo) and Derrymore East (Co. Kerry). Materials were collected at various time points between June and November 2022 (**Table 1**). Plant material was stored at 4 – 8°C until extraction of DNA and EO. Plant voucher samples were prepared and deposited in the herbarium at the National Botanic Gardens of Ireland. Sophie E. Whyms was responsible for the formal identification of the plant material used in this study. All five plant specimens used in this study have been genetically characterised (accession numbers: PV150710, PV150709, PV150708, PV150707, PV150706). Representative voucher specimens were deposited in the herbarium at the National Botanic Gardens, Glasnevin, Dublin, Ireland (voucher numbers: DBN0007689, DBN0009190, DBN0009193).

Plant DNA extraction, PCR, and sequencing:

Method for DNA extraction and sequencing follows that used by Hodkinson et al [20]. For each sample, a weight in the range 0.01 – 0.1 g of dry plant material was ground using 5 mL CTAB in ultrapure H_2O (0.1 g hexadecyltrimethyl-ammonium, 0.1 M tris, 0.02 M EDTA, 1.4 M sodium chloride, H_2O) and 20 µL of mercapto-ethanol. Samples were then incubated in a water bath at 65°C for 10 min, before mixing with chloroform-isoamyl alcohol (24:1). Samples were centrifuged at 4000 RCF for 10 min before adding 5 mL of isopropanol. Samples were then stored at -20°C for 2 days to allow for further precipitation of the DNA. Following precipitation, the DNA pellet was centrifuged at 2000 RCF for 5 min and supernatant removed. The pellet was then washed using 3 mL of 70% ethanol three times and centrifuged again at 2000 RCF for 3 min. Supernatant was removed and tubes were left to dry for approximately 30 min. To dissolve the pellet, 0.5 mL of cold TE buffer in ultrapure H_2O (5 µL 10 mM Trizma base, 0.4 µL EDTA) was added. DNA was further purified using a PCR purification kit (Invitrogen, ThermoFisher Scientific, Dublin, Ireland) following the manufacturer's instructions. DNA was then quantified by agarose gel electrophoresis prior to PCR. PCR was performed using BioMix (Meridean Bioscience, Memphis, TN, United States) following the manufacturer's instructions. Primer sequences and PCR parameters can be found in supplementary material. Amplified products were sent to Source BioScience for Sanger sequencing.

Sequence analysis:

DNA sequences were received in .ABI format chromatogram files from Source BioScience. Sequences were individually assessed, reverse and forward scripts aligned, combined and cleaned in Geneious 11.0 (https://www.geneious.com). Final combined files for the six samples of bog myrtle were assessed using BLAST.ncbi[21,22] for species identification. Secondly, they were aligned in AliView (version 1.28)[23] and PhyML (version 3.1/3.0) was employed (https://phylogeny.fr) to statistically assess any variance between sequences.

Extraction of essential oils from M. gale:

The fresh plant material was segregated into leaf and fruit. Six EOs from five plant material collections of bog myrtle were extracted employing Clevenger and microwave-assisted hydro-distillations using established extraction methods [14]. Microwave-assisted hydro-distillation of two leaf samples yielded MG1M and MG2M and Clevenger hydro-distillation of two fruit samples produced MG4C and MG5C. The fruit collected from Clonlisk was subjected to microwave-assisted to yield sample MG3M. For Clevenger hydro-distillation, fresh (undried) plant material was suspended in Milli-Q® purified water (6 mL/g) and heated to reflux for 3 h using a conventional Clevenger apparatus with a heating mantle. In microwave-assisted hydro-distillation, fresh plant material was pre-soaked in Milli-Q® purified water (2–3 mL/g) for 30 min, then heated to reflux using microwave-assisted extraction at 600 W for 15 min, followed by a 500 W holding step for 40 min. After refluxing and cooling at room temperature for 30 min, the EO was collected, extracted into diethyl ether, and dried over anhydrous sodium sulfate. The extracted EOs were stored at 4–8°C. The plant material collection details and extraction details are provided in **Table 1**.

Table 1: Details on isolated and commercial EOs used in the study and their corresponding sample codes. The suffix 'M', 'C', 'S' and 'D' in sample codes denote extraction methods-

Microwave assisted hydrodistillation, Clevenger hydrodistillation, steam distillation and distillation respectively.

Collection ID	Species	Sample Code	Isolated/ Commercial, brand	Plant source	Collection Date	Plant Part	Extraction Type	DNA Barcoding (Yes/No)
NTP0356	*M. gale*	MG1M	Isolated	Inishbiggle, Ireland	03-05-2022	Leaf	MAH^1	Yes
NTP0371	*M. gale*	MG2M	Isolated	Clonlisk, Ireland	27-06-2022	Leaf	MAH^1	Yes
NTP0380	*M. gale*	MG3M	Isolated	Clonlisk, Ireland	21-10-2022	Fruit	MAH^1	Yes
NTP0381	*M. gale*	MG4C	Isolated	Derrymore East, Ireland	02-11-2022	Fruit	CH^2	Yes
NTP0385	*M. gale*	MG5C	Isolated	Clara, Ireland	09-11-2022	Fruit	CH^2	Yes
NTP0298	*M. gale*	MG6S	Commercial, Zayat Aroma, Canada	Canada	-	Leaf	SD^3	NA
MCMM	*M. communis*	MC1S	Commercial, Mystic moments, United Kingdom	Morocco	-	Flower	SD^3	NA
MCPR	*M. communis*	MC2D	Commercial, Huiles Essentielles, France	Morocco	-	Leaf	Distillation*	NA
Clove Bud	*S. aromaticum*	SA1S	Commercial, Irish apothecary, Ireland	Sri Lanka	-	Bud	SD^3	NA

*^{1}MAH- Microwave assisted hydro-distillation; ^{2}CH- Clevenger hydro-distillation; ^{3}SD – Steam distillation; *No information available on type of distillation.*

Gas Chromatography–Mass Spectrometry (GC-MS) analysis:

A Shimadzu GC2010 gas chromatograph with an autosampler AOC-5000 hyphenated to a Shimadzu QP2010SE mass spectrometer was used for data acquisition. The chromatographic separation of EO components was performed on a ZB-5Plus (5% phenylmethylsiloxane) capillary column (length: 30 m, internal diameter: 0.25 mm, film thickness: 0.25 µm) using a method established in our previous study[14]. The column temperature program consisted of four sequential ramps: the oven temperature was first increased from 40 °C to 85 °C at a rate of 7 °C per min and held isothermally for 2 min. This was followed by a second ramp from 85 °C to 95 °C at 2 °C per min held for 1 min. The third ramp raised the temperature from 95 °C to 200 °C at 4 °C per min, with a 4-min hold. Finally, the oven temperature was increased from 200 °C to 300 °C at a rate of 15 °C per min and held for 3 min. The injector temperature was set at 250 °C, with helium used as the carrier gas at a flow rate of 1.0 mL/min and a split ratio of 5:1. The interface temperature was maintained at 310 °C, and mass spectra were acquired over a scan range of 50–550 amu. All samples were prepared at 1% v/v in hexane and filtered through a 0.22 µm filter prior to GC-MS analysis. The samples were run in quadruplicate.

Compound identification:

The compounds were identified based on the mass spectral fragmentation patterns and Kovat's retention indices (RI). For robust peak annotation, the GC-MS data was analysed in parallel, employing Shimadzu GCMSsolution software Ver. 4 containing the NIST 17 library and utilising the Global Natural Products Social Molecular Networking (GNPS) online server via the 'GC-MS EI Data Analysis' module [https://gnps. ucsd.edu (accessed on 17 July 2024 and thereafter)] [24] and the results from the two independent analyses were compared to confirm each peak annotation. In our first approach, raw data files were processed in .qgd format in the Shimadzu GCMSsolution software and compounds were annotated by NIST 17 and the RI was calculated based on an n-alkanes series (C7–C40, (Supelco (49452-U), Merck, Germany) run on the same GC-MS method as the samples. In the second approach, the raw data files were converted to .cdf format by Shimadzu GCMSsolution software and submitted to the online server for data processing and deconvolution. The deconvoluted data, along with other input files (carbon marker file, metadata, available online libraries), were then fed to the server for library search and RI calculation. The integral table generated after data deconvolution was used for chemical profiling of the EOs as well as for multivariate data analysis. Relative chemical compositions of EOs were determined by averaged peak area normalisation approach [25], where the samples were run in quadruples and peak area was normalised and averaged over four injections for each compound per sample [14,25]. The major constituents in the EOs were quantified by GC-MS using calibration curves generated by authentic standards (range 0.5 µg/mL to 500 µg/mL dilutions in hexane). Multivariate data analysis was performed in SIMCA 18.0, wherein, the partial least square–discriminant analysis (PLS-DA) model was applied to compare the chemical profiles of EOs across three species, *M. gale*, *M. communis* and *S. aromaticum*, while principal component analysis, orthogonal partial least square–discriminant analysis (OPLS-DA) and the S-plot generated from OPLS modelling were applied to correlate the chemical profiles with short distance repellence activity determined in the arm-in-cage assay (see below).

Mosquito culture:

For all experiments conducted in this study, female yellow fever mosquitos, *Ae. aegypti,* from the UGAL strain (University of Georgia Laboratory strain) were used. Batches of approximately 500 eggs were hatched in 33 x 51 x 5 cm pans which contained 3 L of deionised (DI) water. Cat pellets were fed to the larvae *ad libitum*. Following metamorphosis, the pupae were then sorted into 200 mL plastic cups filled with DI water and transferred into BugDorm-1 Insect Rearing Cages (30 x 30 x 30 cm, Bug dorm Company, Taichung, Taiwan). Weekly, an Erlenmeyer flask containing a 20% sucrose solution with a cotton wick, was placed in the cages. Cages were kept inside an insectary room that maintained a constant temperature of 27°C and humidity of 80% with a light dark cycle of 14 h/10 h, respectively.

Formulations:

A 10% v/v EO emulsion in an organic lotion base (OLB) was formulated for each sample. Commercial *M. gale* oil (MG6S) was procured from Zayat Aroma, Canada (source: leaf from Canada, extraction: steam distillation). The myrtle oils from *M. communis* (MC1S and MC2D) were purchased from Mystic Moments, United Kingdom (source: flower from Morocco, extraction: steam distillation) and Huiles Essentielles, France (source: leaf from Morocco, extraction: distillation), respectively. The commercial clove bud oil (SA1S) was sourced from Irish Apothecary, Ireland (source: bud from Sri Lanka, extraction: steam distillation).

Arm-in-Cage Assay (Contact Repellence):

All assays were conducted with modifications following the published guidelines of the U.S. Environmental Protection Agency for testing insect repellents that are applied to human skin [5,26]. A sample size of 25 female *Ae. aegypti* mosquitoes, ranging from 1 – 2 weeks old were selected for this study. Each volunteer was instructed to wash their forearm with unscented soap, before drying and wiping with an ethanol-soaked paper towel. An untreated control arm was introduced into a mosquito-filled BugDorm cage to assess mosquito host-seeking behaviour. A reported bite within 1 min qualified the cage viable for further experimental testing. A bite was defined as the insertion of a mosquito's proboscis into a volunteer's skin while probing for blood. The 10% v/v EO lotion emulsion was vortexed for 1 min. A positive control of a 10% v/v DEET emulsion in the OLB was sampled as well as an OLB control. A 170 μL volume of the emulsion was pipetted onto the exposed area of the volunteer's forearm and was spread evenly and thoroughly using the pipette tip. Upon treatment application, a timer was started, and complete protection time (CPT) was recorded. The CPT is defined as the time from the application of a treatment up to when the volunteer receives a first bite. A variety of 16 volunteers, both male and female, were used in this study. For each sample, 4 arm-in-cage tests were carried out, with two male and two female volunteers being used for each sampled oil.

Y-Tube Olfactometer Assay (Spatial Repellence):

Four experimental replicates were performed for each EO, with a control performed prior to each replicate. Each replicate and control were carried out using approximately 25 mosquitoes, ranging from 1 – 2 weeks old. Airflow in the tube was set to 0.4 m/s in the holding chamber and 0.2 m/s in both arms. Mosquitoes were placed in the holding chamber and the volunteer placed their hand at the end of one port. The mosquitoes were then left to acclimatise to the odour in the holding chamber for 30 s, after which the doors were opened. Mosquitoes flew for 2 min before all chamber doors were closed and the number of mosquitoes per chamber was calculated as a percentage, referred to as the mean percentage attraction (MPA). MPA describes the percentage of mosquitoes in the Y-tube attracted towards the sample. Effective repellents will have a low MPA. For experiments, the volunteer held a piece of 10 x 10 cm Kimtech paper soaked in 100 μL of the undiluted EO. The same treated paper was re-tested every 15 min for the first hour or until loss of efficacy. Loss of efficacy was declared when mosquitoes flew towards the volunteer at a similar rate as the control. If repellence continued past 1 h, testing was conducted every 30 min until 3 h had elapsed at which point 1 more hour without testing was allowed to pass before finally testing at the 4 h mark following which testing was ceased. A total of 6 EOs*,* were tested in the Y-Tube model*.*

Ethics declaration:

Experimental protocols conducted in this study have been reviewed and approved by New Mexico State University Institutional Review Board and follow – IRB protocol number 227006850R003 (#22010). All participants were both verbally informed about the assay and informed consent was obtained by providing them a participant information leaflet complete with consent form to be signed. Vulnerable persons i.e., minors, prisoners, immunocompromised people, those sensitive to mosquito bites and pregnant/nursing women, were excluded from this study. Participants were advised to avoid alcohol, tobacco and any scented products at least 12 h prior to testing.

Statistical analysis of repellence assays:

Statistical significance was determined using a one-way analysis of variance (ANOVA) and treatments were compared using Tukey's multiple pairwise-comparison tests. A p-value of < 0.05 was considered significant.

Results

Plant collection and DNA sequencing:

The results of Sanger sequencing of PCR products obtained from isolated DNA of five collections (collection IDs: NTP0356, NTP0371, NTP0380, NTP0381, NTP0385)[27-31] confirm that all five plant samples belong to the species, *M. gale* L. (**supplemental Fig. S1**). This was confirmed using BLAST.ncbi [21,22] and by aligning and comparing the sequences in Aliview (version 1.28) and PhyML (version 3.1/3.0). No significant variation was observed between sequences. A single nucleotide polymorphism (**Table 2**) was acknowledged for sample NTP0385 at position 2 as a substitution and for NTP0371 at position 895 as a deletion of a base pair (**supplemental Fig. S1**). The gamma shape parameter is reported to be 61.818 suggesting that the sequence data is highly conserved and/or homogenous thus confirming that they are all sequences from the same species. Determined by BLAST search, this species is *M. gale* L. No varieties are present (e.g., *M. gale* var. *tomentosa*) even for the cultivated monoculture stand (NTP0381)

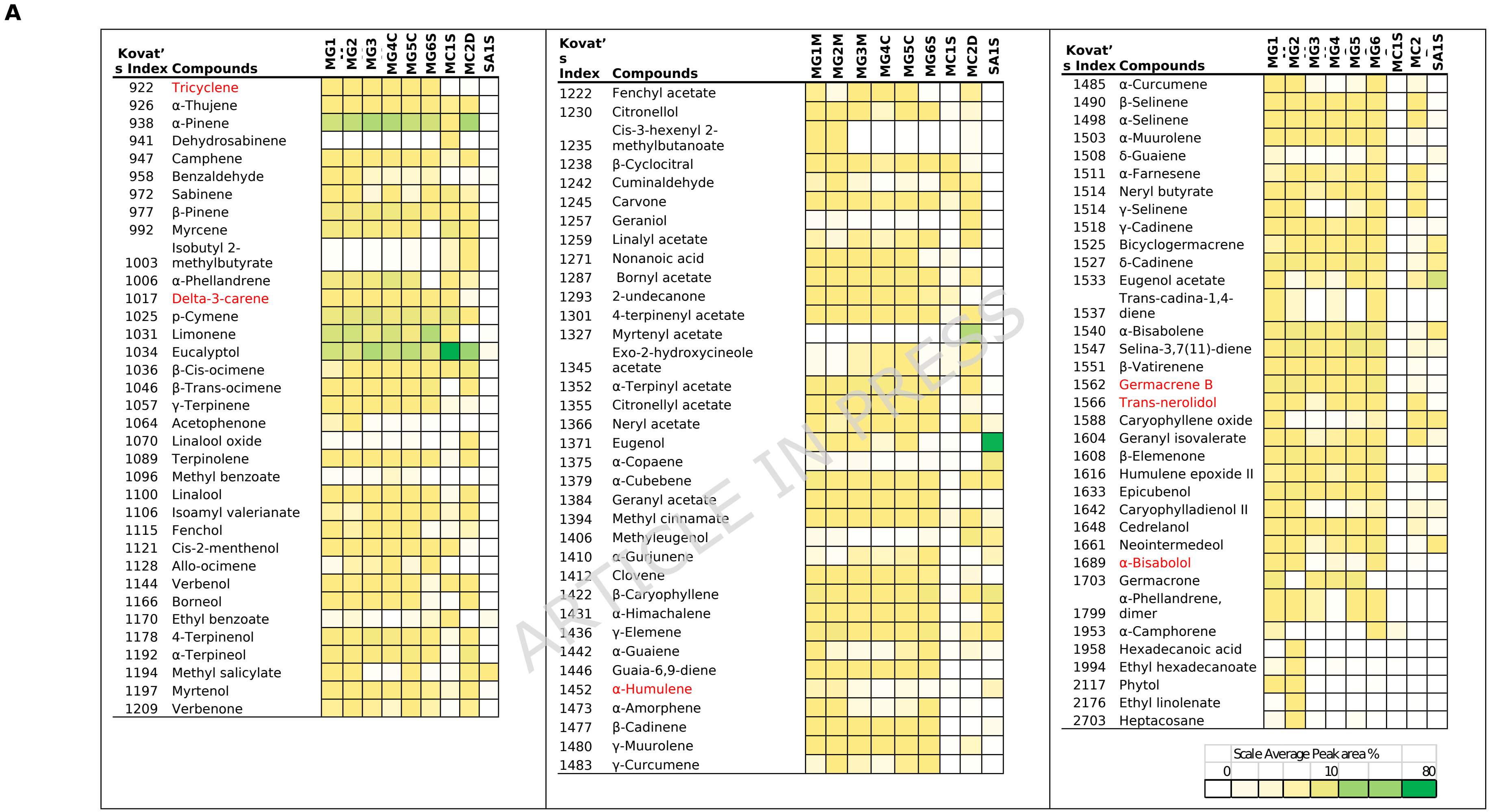
Table 2. DNA sequencing sample results and information for 5 DNA sequences (NTP0356, NTP0380, NTP0381, NTP0385, NTP0371) derived from 5 plant collections of native Irish plant *M. gale,* from a total of 4 sites; Clonlisk, Clara, Inishbiggle and Derrymore East, during the period of May 2022 – November 2022. bps = base pairs.

Sample ID	Sequence Identifier on Genbank Database	Sequence Length (bps)	Substitutions/Deletions	Genbank Accession Code
MG1M	NTP0356	884	N.A.	PV150710
MG3M	NTP0380	884	N.A.	PV150709
MG4C	NTP0381	928	N.A.	PV150708
MG5C	NTP0385	884	1 bp substitution at position 2	PV150707

MG2M	NTP0371	818	1 bp deletion at position 895	PV150706

Chemical profiles of essential oils:

A total of 105 compounds were annotated in the EOs from three species *M. gale, M. communis* and clove bud oil with α-pinene (0.0 –194.69 mg/mL), limonene (0.0 – 219.81 mg/mL), eucalyptol (0.0% – 582.00 mg/mL), myrtenyl acetate (0.0 – 327.64 mg/mL), eugenol (0.0 – 727.73 mg/mL) and eugenol acetate (0.0 – 13.57 mg/mL) as major components (**Fig. 1A**). Eight out of nine EOs (except clove oil) were found to contain α-pinene (1.58 – 194.69 mg/mL), β-pinene (4.06 – 31.17 mg/mL), p-cymene (3.08 – 43.97 mg/mL) and eucalyptol (32.41 – 582.00 mg/mL) as common constituents. Eugenol (727.73 mg/mL) and eugenol acetate (13.57 mg/mL) were the principal components of clove bud oil SA1S*,* together constituting approximately 89% of the oil by peak area percentage*.* A detailed list of EO components with their averaged peak area percentages for nine EOs has been provided in **supplemental Table S2**, followed by a quantification table of major 24 components in nine EOs in **supplemental Table S3**. Along with individual components, a trend was observed in the proportions of monoterpenes to sesquiterpenes (M/S ratio) in the EOs. As shown in the pie charts in **Figure 1B**, the M/S ratio was calculated for each oil by dividing the sum of normalised mean peak area of monoterpene hydrocarbons and oxygenated monoterpenes by the sum of normalised mean peak area of sesquiterpene hydrocarbon and oxygenated sesquiterpenes. All *M. gale* samples exhibited similar qualitative chemical profiles with M/S ratios ranging from 1.94 to 9.89. Conversely, the commercial oils from *M. communis* and clove oil were found to have completely different profiles. The flower oil MC1S from *M. communis* did not contain any sesquiterpenes while the leaf oil MC2D had an M/S ratio of 22.50. The main components of clove bud oil SA1S were eugenol and eugenol acetate of the phenylpropanoid class and hence the M/S ratio was zero.


A
Kovat's Index
Compounds
MG1
MG2
MG3
MG4C
MG5C
MG6S
MC1S
MC2D
SA1S
922 Tricyclene
926 α-Thujene
938 α-Pinene
941 Dehydrosabinene
947 Camphene
958 Benzaldehyde
972 Sabinene
977 β-Pinene
992 Myrcene
1003 Isobutyl 2-methylbutyrate
1006 α-Phellandrene
1017 Delta-3-carene
1025 p-Cymene
1031 Limonene
1034 Eucalyptol
1036 β-Cis-ocimene
1046 β-Trans-ocimene
1057 γ-Terpinene
1064 Acetophenone
1070 Linalool oxide
1089 Terpinolene
1096 Methyl benzoate
1100 Linalool
1106 Isoamyl valerianate
1115 Fenchol
1121 Cis-2-menthenol
1128 Allo-ocimene
1144 Verbenol
1166 Borneol
1170 Ethyl benzoate
1178 4-Terpinenol
1192 α-Terpineol
1194 Methyl salicylate
1197 Myrtenol
1209 Verbenone
Kovat's Index
Compounds
MG1M
MG2M
MG3M
MG4C
MG5C
MG6S
MC1S
MC2D
SA1S
1222 Fenchyl acetate
1230 Citronellol
1235 Cis-3-hexenyl 2-methylbutanoate
1238 β-Cyclocitral
1242 Cuminaldehyde
1245 Carvone
1257 Geraniol
1259 Linalyl acetate
1271 Nonanoic acid
1287 Bornyl acetate
1293 2-undecanone
1301 4-terpinenyl acetate
1327 Myrtenyl acetate
1345 Exo-2-hydroxycineole acetate
1352 α-Terpinyl acetate
1355 Citronellyl acetate
1366 Neryl acetate
1371 Eugenol
1375 α-Copaene
1379 α-Cubebene
1384 Geranyl acetate
1394 Methyl cinnamate
1406 Methyleugenol
1410 α-Gurjunene
1412 Clovene
1422 β-Caryophyllene
1431 α-Himachalene
1436 γ-Elemene
1442 α-Guaiene
1446 Guaia-6,9-diene
1452 α-Humulene
1473 α-Amorphene
1477 β-Cadinene
1480 γ-Muurolene
1483 γ-Curcumene
Kovat's Index
Compounds
MG1
MG2
MG3
MG4
MG5
MG6
MC1S
MC2
SA1S
1485 α-Curcumene
1490 β-Selinene
1498 α-Selinene
1503 α-Muurolene
1508 δ-Guaiene
1511 α-Farnesene
1514 Neryl butyrate
1514 γ-Selinene
1518 γ-Cadinene
1525 Bicyclogermacrene
1527 δ-Cadinene
1533 Eugenol acetate
1537 Trans-cadina-1,4-diene
1540 α-Bisabolene
1547 Selina-3,7(11)-diene
1551 β-Vatirenene
1562 Germacrene B
1566 Trans-nerolidol
1588 Caryophyllene oxide
1604 Geranyl isovalerate
1608 β-Elemenone
1616 Humulene epoxide II
1633 Epicubenol
1642 Caryophylladienol II
1648 Cedrelanol
1661 Neointermedeol
1689 α-Bisabolol
1703 Germacrone
1799 α-Phellandrene, dimer
1953 α-Camphorene
1958 Hexadecanoic acid
1994 Ethyl hexadecanoate
2117 Phytol
2176 Ethyl linolenate
2703 Heptacosane
Scale Average Peak area %
0
10
80

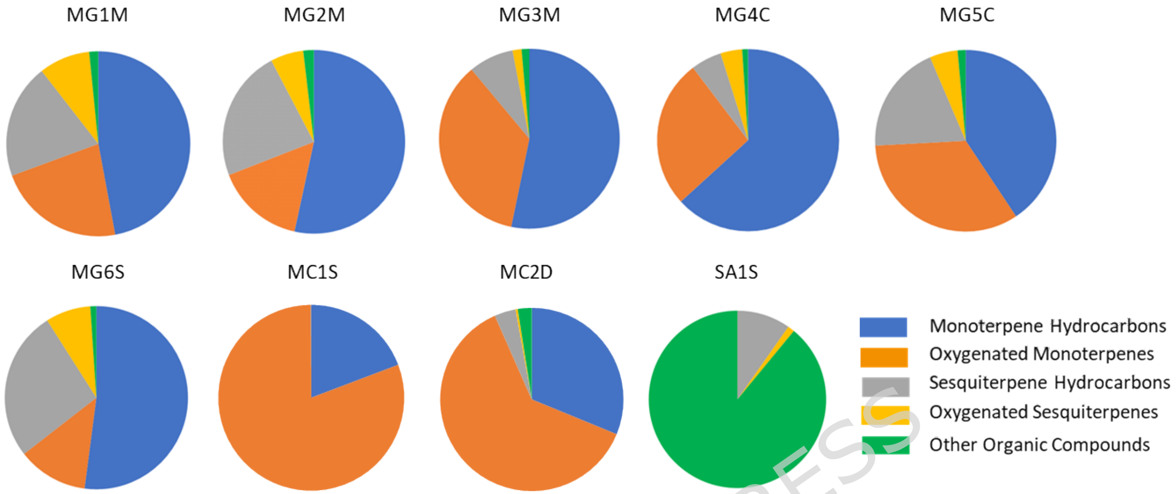


Figure 1. Chemical composition of nine EOs from *M. gale* (MG1M, MG2M, MG3M, MG4C, MG5C, MG6S), *M. communis* (MC1S, MC2D) and *S. aromaticum* (SA1S)—Shown are the results of GC-MS analysis. (**A**) Heatmap of identified compounds in each EO, where each cell colour represents the averaged peak area % calculated from four injections per sample (n = 4). Cells with a low or non-detectable component are represented in white while deep green represents components with relatively large peaks across all components and sample types. (**B**) Pie charts showing distribution of monoterpene hydrocarbons (blue), oxygenated monoterpenes (orange), sesquiterpene hydrocarbons (grey), oxygenated sesquiterpenes (yellow) and other organic compounds (green) within each EO sample.

Mosquito contact repellence of M. gale EOs:

Arm-in-cage assay was performed as a contact repellence assay to determine short-range repellence of isolated and commercial EOs against *Ae. aegypti* mosquitoes (**Fig. 2A**). **Figure 2B** illustrates the mean CPTs for all samples formulated and tested in this assay. 'OLB' or organic lotion base was used as a vehicle control whilst DEET in the same 10% v/v formulation in the OLB was used as a positive control. The OLB (CPT = 2.775 min) elicited no significant activity when compared against an untreated control ($p = 0.9899$), whereas the positive control DEET in the OLB presented the most significant activity with a CPT lasting > 6 h ($p < 0.0001$). The CPTs of the individual EOs ranged from 3.39 (MC1S) – 67.93 (SA1S) min, with some EOs showing significant repellent activity. Two Irish bog myrtle EOs showed significant activity, MG4C (18.45 min, $p < 0.0001$) and MG3M (11.45 min, $p < 0.01$). Fruit EOs (MG3M and MG4C) proved to be better performing than leaf EOs (MG1M and MG2M), which showed no activity at all ($p > 0.05$). This also is true for the commercial *M. gale* leaf EO, MG6S which had a CPT = 5.12 min ($p = 0.9994$). Even within the fruit EOs tested, further variation in activity was observed. MG5C showed no significant activity when compared to the vehicle control, OLB (CPT = 7.96 min and $p = 0.0883$) whereas MG4C and MG3M displayed significant activity, as discussed. Clove oil, SA1S, demonstrated a CPT value of 67.93 min ($p < 0.0001$). Lastly, the two commercial *M. communis* oils, MC1S (CPT = 3.39 min) and MC2D (CPT = 4.05 min) showed no significant activity in the arm-in-cage assay ($p > 0.05$).

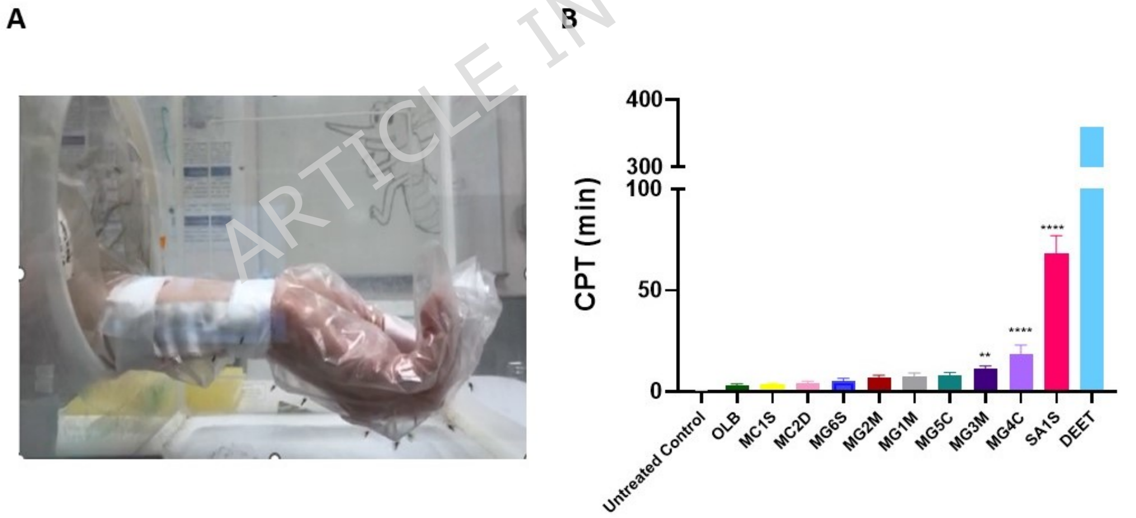


Figure 2. Arm-in-cage assay and complete protection time (CPT) results for nine EOs from *M. gale* (MG1M, MG2M, MG3M, MG4C, MG5C, MG6S), *M. communis* (MC1S, MC2D) and *S. aromaticum* (SA1S). (**A**) Volunteer illustrating the arm-in-cage method. (**B**) Bar chart describing the CPT results for arm-in-cage assay. The OLB was sampled as a vehicle control. DEET was sampled as a positive control. Significant results are denoted by '****' – $p \leq 0.0001$ and '**' - $p \leq 0.01$. For all samples tested, n = 4.

Mosquito spatial repellence of M. gale EOs:

In **Figure 3,** six EOs were tested for spatial repellence, MG1M, MG2M, MG3M, MG4C, MG6S, and SA1S (**Table 1**). The results for the Y-tube olfactometer assays display a similar trend to the arm-in-cage assays for the isolated oils i.e., those oils showing significant activity in arm-in-cage (MG3M and MG4C) also showed the most significant activity in the Y-tube olfactometer assay, when compared against the bare hand control. MG4C had the greatest CPT in arm-in-cage (18.45 min) and also maintained a significantly low MPA for the longest period of time in the Y-tube olfactometer (150 min). This was then followed by MG3M (CPT in arm-in-cage = 11.45 min, Y-tube = 90 min). MG1M and MG2M exhibited no significant activity in either assay. MG6S and SA1S varied in their activity between assays. MG6S showed no activity in the arm-in-cage assay but significant activity for up to 30 min in the Y-tube olfactometer. SA1S showed activity for only up to 15 min in the Y-tube olfactometer but had the longest CPT for arm-in-cage assays of all EOs sampled.

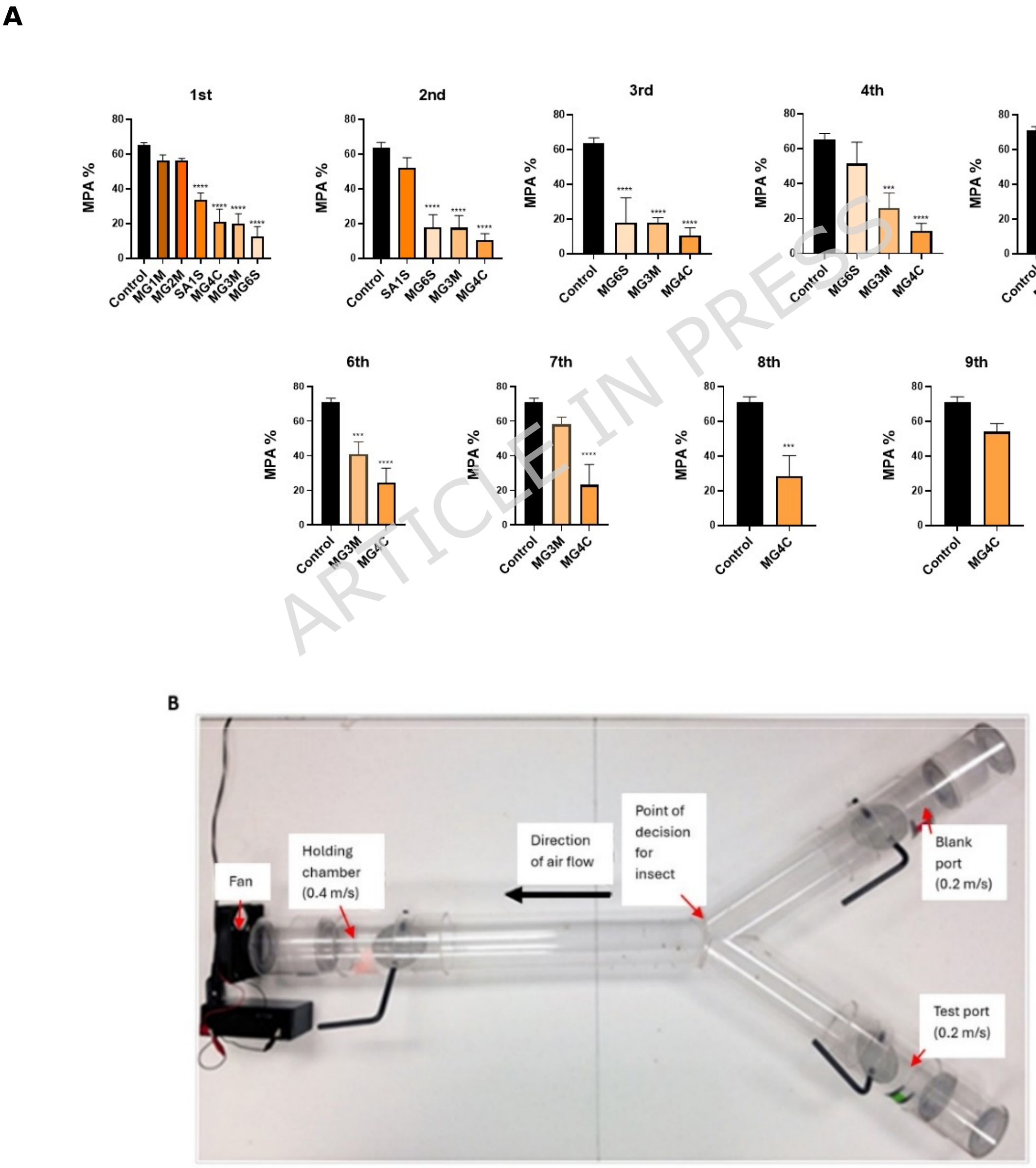
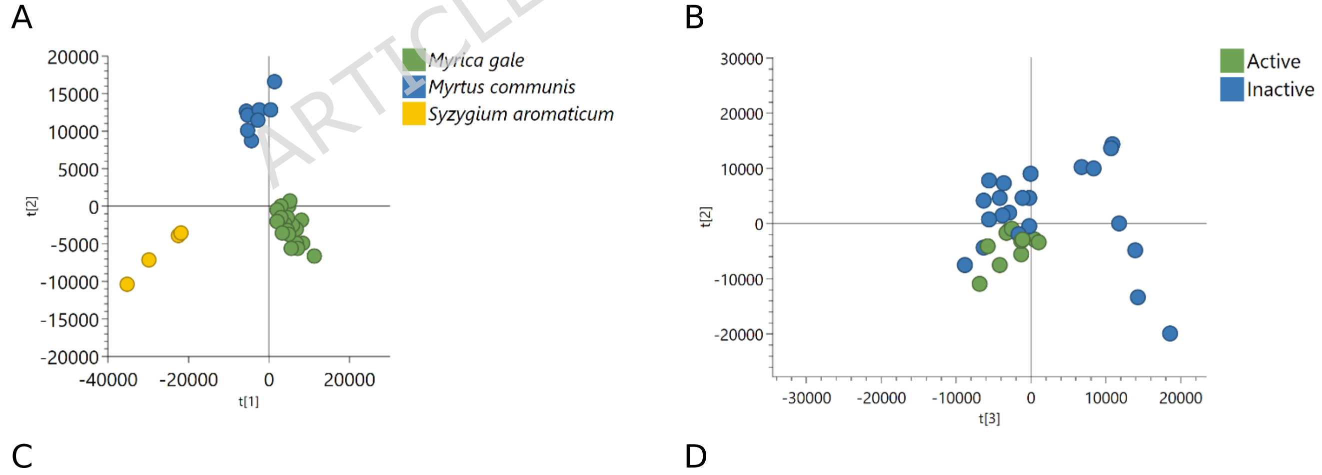


Figure 3. Y-tube olfactometer results for EOs from *M. gale* (MG1M, MG2M, MG3M, MG4C, MG6S) and clove bud oil (SA1S). (**A**) Bar charts showing MPA at various time points (1st – 0 min, 2nd – 15 min, 3rd – 30 min, 4th – 45 min, 5th – 60 min, 6th – 90 min, 7th – 120 min, 8th – 150 min, 9th – 180 min) in Y-tube olfactometer assays. Testing of samples ceased if no significant activity was shown following a certain time point. Significant results are denoted by '****' (P ≤ 0.0001) and '***' (P≤ 0.001). (**B**) Diagram of Y-tube set-up.

Correlation analysis between EO chemical constituents and repellence activity

EO constituents and contact repellence activity:

PLS-DA score plot of GCMS data for the EOs demonstrated a clear distinction between the chemical profiles of the oils from the three species, *M. gale, M. communis* and *S. aromaticum* (**Fig. 4A**). The marker compounds that differentiated clove bud oil, SA1S from *M. gale* and *M. communis* oils were eugenol and eugenol acetate, whereas a higher content of eucalyptol (582.00 mg/mL and 156.40 mg/mL) and myrtenyl acetate (not determined and 327.64 mg/mL) separated *M. communis* flower oil MC1S and leaf oil MC2D respectively from *M. gale* EOs. The major components in the latter were α-pinene (56.60 mg/mL – 176.88 mg/mL), limonene (69.10 mg/mL – 122.04 mg/mL) and eucalyptol (32.41 mg/mL – 115.31 mg/mL). To find correlation between chemical constituents and contact repellency, principal component analysis (PCA) was applied. After observing some clustering for active and inactive samples in PCA analysis (**Fig. 4B**), the OPLS-DA model was applied as a targeted approach. The model was validated by a high Q^2 value (0.745) and demonstrated a clear distinction between inactive samples and active samples, further separating clove oil active samples (black ring) from *M. gale* ones (green ring) (**Fig. 4C**). In addition, an S-plot was generated to correlate the EO components to the contact repellence activity (**Fig. 4D**), which depicted positively correlated green datapoint 1 as eugenol.



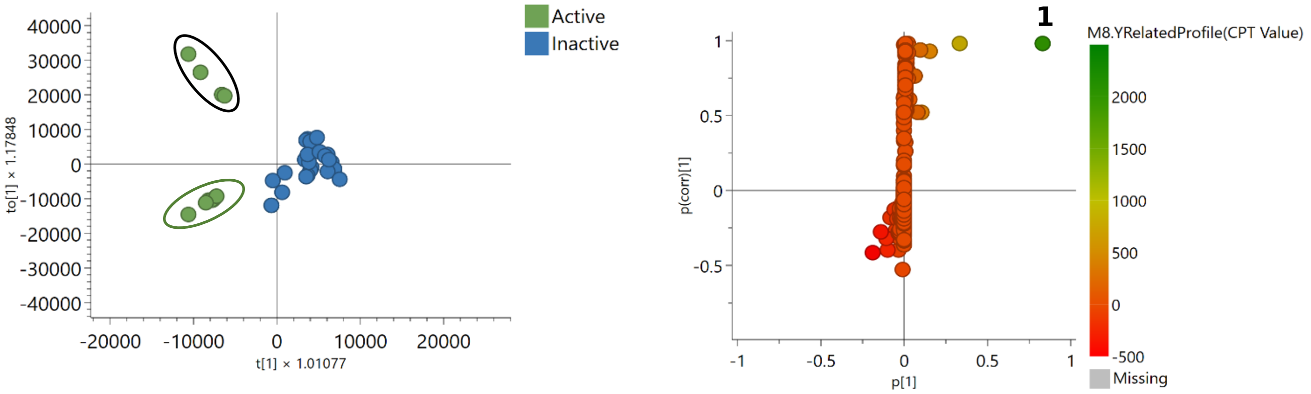
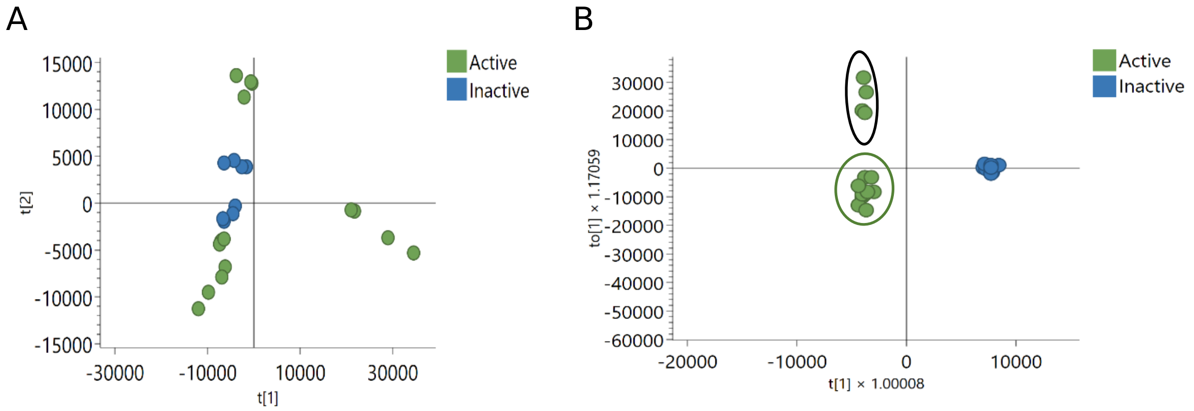


Figure 4. Metabolomics score plots generated based on GC-MS data. (**A**) PLS-DA modelling using three classes — *M. gale* (green), *M. communis* (blue) and *S. aromaticum* (yellow), R^2x (cum) = 0.444, R^2y(cum) = 0.914, Q^2(cum) = 0.854. (**B**) PCA showing partial clustering of active (green) and inactive (blue) samples in arm-in-cage assay, R^2x (cum) = 0.661, Q^2(cum) = 0.201. (**C**) OPLS-DA modelling showing clustering of active (green) and inactive (blue) samples in arm in cage assay, the cluster of active samples in the black ring represents clove bud oil, while the cluster in the green ring denotes *M. gale,* R^2x (cum) = 0.493, R^2y(cum) = 0.862 Q^2(cum) = 0.745. (**D**) S-plot generated from OPLS modelling correlating the insect repellent activity (quantitative) with chemical components of EOs from *M. gale, M. communis* and clove bud oil, R^2x (cum) = 0.448, R^2y(cum) = 0.835, Q^2(cum) = 0.753. Datapoint 1 corresponds to eugenol.

EO constituents and special repellence activity:

The Y-tube olfactometer data collected from 6 samples in quadruplicate for the first timepoint was correlated with GC-MS data using PCA, OPLS-DA and S-plot models. After observing some clustering in PCA (**Fig. 5A**), the OPLS-DA model (Q^2 = 0.977) gave a distinct clustering of active (green) EO samples from clove oil in the black ring and *M. gale* (MG4C, MG3M, MG6S) in the green ring and inactive samples (blue) (**Fig. 5B**). The S-plot revealed datapoints 2 and 3 as myrcene and α-phellandrene, respectively, correlating negatively with MPA (**Fig. 5C**).



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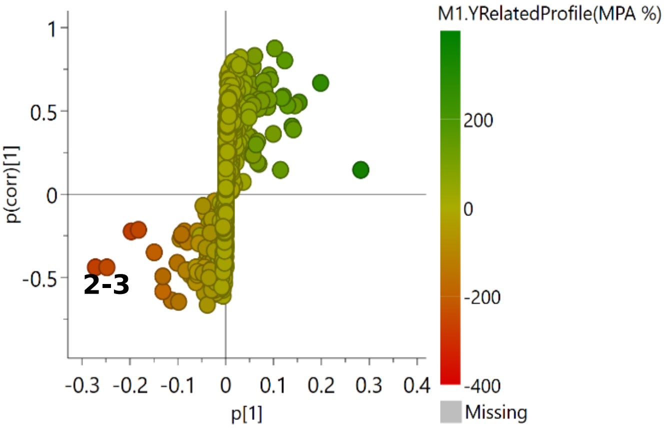

Figure 5. Metabolomics score plots generated based on GC-MS data and spatial repellence. (**A**) PCA showing partial clustering of active (green) and inactive (blue) samples in Y-tube assay at the first timepoint, R^2x (cum) = 0.737, Q^2(cum) = 0.467. (**B**) OPLS-DA modelling showing clustering of active (green) and inactive (blue) samples in the Y-tube assay at the first timepoint, the cluster of active samples under the black ring represents clove bud oil, while the cluster in the green ring denotes *M. gale,* R^2x (cum) = 0.841, R^2y(cum) = 0.995 Q^2(cum) = 0.977. (**C**) S-plot generated from OPLS modelling correlating the insect repellent activity (quantitative) with chemical components of EOs from *M. gale* and clove bud oil, R^2x (cum) = 0.572, R^2y(cum) = 0.795, Q^2(cum) = 0.585*.* The datapoints 3 and 4 correspond to myrcene and α-phellandrene respectively.

Discussion

Molecular species confirmation remains underused in the natural products industry, often due to cost and limited access to technology. However, advances in DNA sequencing highlight its potential in improving species identification and quality control. Currently, the phylogenetic information on *M. gale* is scant, with only 5 entries for *M. gale* L. resulting from a BLAST search on the National Center for Biotechnology Information (NCBI) website [21,22]. In the current study, five DNA samples correlating to the collected plant samples from Inishbiggle (NTP0356), Clonlisk (NTP0371 and NTP0380), Clara (NTP0385) and Derrymore East (NTP0381) were sequenced and analysed for potential variation. The limited base pair differences observed between NTP0371 and NTP0385 and the other three sequences coupled with the results obtained from BLAST.ncbi, confirm that all sequences are from *M. gale*. All 5 sequences shown in **Table 2** have been submitted to GenBank and accession codes have been generated and published [27-31].

In the arm-in-cage assay, differences in activity of the oils from *M. gale* samples were anticipated, given noticeable variation in colour (clear – yellow), viscosity and especially scent. Volunteers described *M. gale* oils as ranging from fusty and earthy to sweet and floral (unpublished data). Since mosquito repellent activity of EOs largely relies on olfactory cues [32], the odour and volatility of individual compounds and their combinations is likely play a key role in determining each sample's CPT and furthermore their effectiveness in such a model as arm-in-cage, which enlists a multitude of mosquito-host detection and landing cues. The most effective *M. gale* oil against *Ae. aegypti* mosquitoes was MG4C, a fruit-derived EO extracted via Clevenger hydro-distillation. The other active sample, MG3M (microwave-assisted), was also a fruit oil. In contrast, leaf EOs, both isolated (MG1M, MG2M) and commercial (MG6S) showed no significant activity. Our chemical analysis revealed that *M. gale* oils with higher monoterpene-to-sesquiterpene (M/S) ratios tended to exhibit longer CPTs. For instance, MG4C (M/S 9.89) showed a CPT of 18.45 min, while MG6S (M/S 1.94) had a CPT of just 5.12 min. In effect, CPT correlated strongly with M/S ratios: MG6S (1.94; 5.12 min) < MG2M (2.45; 6.79 min) < MG1M (2.42; 7.22 min) < MG5C (3.14; 7.96 min) < MG3M (9.49; 11.45 min) < MG4C (9.89; 18.45 min).

The CPT values of *M. gale* EOs varied depending on the plant part and time of year in which the material was collected. As shown in **Figure 2B**, the repellence activity of fruit EO decreased from fruit sample MG4C (active) to MG5C (**Table 1**). Specifically, CPT dropped from 18.45 min (MG4C, Derrymore East, 2 November) to 7.92 min (MG5C, Clara, 9 November), which was comparable to the vehicle (OLB) control. This decline in activity corresponded with decreasing levels of key monoterpenes across these samples. This trend may reflect seasonal physiological changes in the plant. By late October, senescence is nearly complete, and fruit tissues begin to dry and lignify, leading to degradation and loss of volatile components. These observations suggest that late October – early November may represent the optimal harvest window for maximising EO repellent activity, although variation may be seen between sites of harvesting. Chemical analysis further supports this, with a marked decline in the M/S ratio from MG4C (9.89) to MG5C (3.14). MG3M although collected in the Autumn season (21 October) is not directly comparable due to the difference in distillation processes. Our results establish that the extraction method bears some significance in the resulting volatile oil's composition and thus its properties as an insect repellent. Oils extracted via Clevenger hydro-distillation showed greater activity than oils extracted using microwave-assisted hydro-distillation. This is shown in the case of MG4C and MG3M (**Fig. 2B**). Extraction methods can strongly influence the phytochemical profile of a plant EO. We found in our previous study [14] that microwave-assisted extraction yields sesquiterpene enriched EOs which in turn makes the oil less volatile. Opposingly, EOs produced by the Clevenger method contain a higher proportion of monoterpenoids which increases the oil's ability to mobilise or volatilise and interact with insects at a distance [33] (**Fig. 1B**). This justifies the higher repellence of MG4C, a fruit EO extracted by the Clevenger technique with a M/S ratio of 9.89, over the microwave extracted MG3M with a M/S ratio of 9.49, the lower ratio demonstrating enrichment in sesquiterpene content. Internal heating caused by microwave irradiation effectively ruptures glands and oleiferous receptacles of the plant matrix, facilitating the release of larger molecular weight sesquiterpene molecules [14].

In this study, although the duration of repellence differed between the Y-tube olfactometer and arm-in-cage assays, the same *M. gale* EOs were significant across both with their relative efficacy preserved, with the exception of MG6S. EOs that demonstrated significant activity in the arm-in-cage assay (> 8 min compared to the control) also exhibited significant repellence in the Y-tube olfactometer assay. Despite the significant results for MG4C (arm-in-cage: 18.45 min, Y-tube: 150 min) and MG3M (arm-in-cage: 11.45 min, Y-tube: 90 min) in both assays, these EOs, and most natural repellents relying on terpenes for their repellence activity, will inherently be more successful in the Y-tube olfactometer model than the arm-in-cage. Mosquito host-seeking is a multi-step behavioural process guided first by long-range olfactory cues, particularly carbon dioxide, lactic acid and other human skin volatiles, and then by short-range signals such as heat and humidity [34]. These cues are detected by specialised olfactory receptor neurons (ORNs) located on the antennae and maxillary palps [35]. They activate orientation and odour-plume tracking long before a mosquito is close enough to assess thermal gradients or moisture [9,36], which are controlled by a whole different suite of sensory organs such as thermosensitive neurons located in sensilla on the antennae and at the tip of the proboscis or labellum [37]. Heat acts as the final landing and probing cue, and without heat, odour alone is insufficient for final host contact. It is the integration of all the signals which drive efficient host seeking. Since Y-tube olfactometer assays isolate this early olfactory phase, they are highly sensitive to volatile natural repellents that interfere with long-range host detection. In contrast the arm-in-cage model evaluates repellence under far more challenging short-range conditions [38]. Here, mosquitoes are already in host-seeking mode and integrate multiple cues simultaneously, making it more difficult for volatiles alone to prevent landing and probing [9]. Moreover, the direct application of the test substance to the skin, introduces additional constraints. Factors such as body heat can accelerate the evaporation of plant volatiles, potentially influencing duration of protection. As a result, although EO repellents rich in terpenes may show activity in both assay types, they typically will produce a stronger and more sustained avoidance response in a spatial repellence model (Y-tube) compared to a close-range repellence one (arm-in-cage), which creates a more complex multi-modal sensory environment. Similarly, samples with no significant activity in the arm-in-cage test showed no effect in the Y-tube assay (MG1M, MG2M), with the exception of MG6S, which demonstrated an effect up to 30 min in the Y-tube olfactometer assay and no significant effect in the arm-in-cage. The shorter avoidance response in the Y-tube for MG6S, compared with MG4C and MG3M, would indicate that the repellence activity of the chemical constituents of this oil was too weak to provide an effect in the arm-in-cage assay. Furthermore, clove oil (SA1S), also displayed differences between the two methods i.e. arm in cage (CPT = 67.93 min) and Y-tube assays (MPA = 15 min), suggesting that its performance may be more sensitive to assay conditions, potentially due to its chemical composition, volatility, or mode of action. Mitra et al. found no significant repellence with 500 µL of clove oil in a Y-tube assay [39] and additionally Nentwig et al. showed clove oil had some repellence at lower doses (1 – 6 mg), but higher doses (> 15 mg) were less effective [40]. Our dose (100 µL or 105 mg) exceeds 15 mg, suggesting that higher concentrations may limit efficacy in this assay. Additionally with eugenol as the primary component in clove oil and a phenylpropanoid, it is less likely to volatilise readily for prolonged spatial repellence action in the Y-tube olfactometer as opposed to the arm-in-cage where it remains persistent on surfaces and may act as a successful contact deterrent as well as a spatial repellent [41].

To clarify the contribution of individual terpenoid constituents to the observed repellence of *M. gale* EOs, S-plots correlating CPT values with GC–MS data were generated. **Figure 4D** show that eugenol (data point 1) positively correlated with repellence, as indicated by the strong intensity of the green colour and its position on the plot. This clearly demonstrates that eugenol is the principal contributor to the contact repellence against *Ae. aegypti* [37,38]. Owing to its high repellence activity (CPT = 67.93 min), clove bud oil (SA1S) dominated the model and masked the activity profile of the *M. gale* oils, further highlighting eugenol as the primary compound for insect repellence. To identify active compounds within *M. gale*, clove oil samples were removed, and the data reanalysed, generating a new S-plot (data not shown) using only *M. communis* and *M. gale* EO samples. However, this model exhibited low predictive performance ($Q^2 = 0.402$) and did not reveal any reliable correlations. One likely explanation is the small differences in CPT values between active *M. gale* samples (MG4C, 18.45 min; MG3M, 11.45 min) and inactive samples

(MG5C, 7.96 min; MG1M, 7.22 min; MG2M, 6.79 min; MG6S, 5.12 min), in contrast to the much stronger activity of clove oil (SA1S, 67.93 min). Consequently, the model was unable to detect variables associated with this relatively small variation. To investigate further, the major constituents—α-pinene, eucalyptol, and limonene—present at high levels in MG4C (161.77 mg/mL; 88.84 mg/mL; 75.48 mg/mL) and MG3M (176.88 mg/mL; 115.31 mg/mL; 122.04 mg/mL) were tested individually and in 10% combinations to assess their repellence. However, none of these compounds or combinations demonstrated significant activity (**supplemental Fig. S2**).

M. gale oils were found to perform far better in spatial repellence settings compared to contact repellence. Even those oils with low CPT values (e.g. MG6S) performed better in the Y-tube assay exhibiting repellence of up to 30 min with MPA varying from 20% to 55% between the first and fourth time points (**Fig 3A**). Since the sample number reduces with increasing timepoints in this assay, the data collected for six samples at the 1st timepoint were taken for correlation analysis. S-plot revealed myrcene (datapoint 2) and α-phellandrene (datapoint 3) to exhibit negative correlation with MPA and to be contributing to the spatial repellence activity of *M. gale* EOs (**Fig 5C**). Several studies have examined the insect repellent effects of myrcene against *Ae. aegypti* mosquitoes with significant results observed for both feeding deterrence [42], and larval uptake and toxicity [43]. A study by Duarte et al. found that as well as feeding deterrence, myrcene showed interactions with the *Ae. aegypti* odorant binding protein 22 (AeOBP22) [42]. Myrcene content for MG4C is equivalent to 13.71 mg/mL in 100 µL of neat EO dosage exposed to around 25 mosquitoes, and 18.82 mg/mL for MG3M. The S-plot (**Fig. 5C**), as aforementioned, highlights α-phellandrene (datapoint 3) as another contributor to the repellent activity of *M. gale* EOs, present at 21.10 mg/mL and 60.82 mg/mL in MG3M and MG4C (**supplemental Table S3**) respectively, equating to 21100 and 60820 ppm in the 10% EO emulsions used in arm-in-cage assays. α-Phellandrene is known for its larvicidal activity against *Ae. aegypti* mosquitoes (lethal concentration, 90% (LC_{90}) = 19.3 ppm) and *An. quadrimaculatus* mosquitoes (LC_{90} = 36.4 ppm), as well as other insect pests [44,45]. Molecular docking studies have shown it binds spontaneously to *Ae. aegypti* odorant-binding protein AaegOBP1 ($\Delta G_{binding}$ = –7.069 kcal/mol) [46] and it has a moderate biting deterrent index (BDI = 0.52) [47]. Jaenson et al. also reported that *M. gale* inflorescence volatiles reduced *Ae. aegypti* mosquito probing by 82.1% after 15 min exposure, comparable to a DEET-based repellent MyggA (90% reduction), with α-phellandrene comprising 25.4% of the active extract [48]

In addition to three active compounds (eugenol, myrcene and α-phellandrene) identified in correlation analyses (Fig. 4D, 5C), 21 major constituent compounds of *M. gale, M. communis* and clove EOs (α-pinene, p-cymene, limonene, eucalyptol, myrtenyl acetate, eugenol acetate, β-pinene, γ-terpinene, camphene, linalool, delta-3-carene, borneol, benzaldehyde, 4-terpinenol, α-terpineol, citronellol, β-caryophyllene, trans-nerolidol, germacrone, and both β-cis- and β-trans-ocimene were quantified (**supplemental Table S3**). In the 10% v/v EO emulsions used in the arm-in-cage assays, MG4C presents 5 terpenes with the highest amounts comparative to other sampled EOs. These are, β-pinene (3.12 ppm), α-pinene (6.08 ppm), camphene (0.95 ppm), delta-3-carene (0.75 ppm) and γ-terpinene (0.84 ppm) Additionally, p-cymene (4.4 ppm) presented the highest content for MG3M in this assay. A higher content of these constituents may suggest they bear some influence in the activity observed for these two samples in both assays, literary evidence further pertains to this.β-pinene studies have demonstrated repellence for a 3% formulation in Vaseline against *Culex pipiens* of 22 min [49] and larvicidal activity against *Ae. aegypti* (LC_{50}= 21.02 ppm for eggs, 108.39 ppm for larvae) after a 72 h exposure [50]. β-Pinene is readily detected by the antennae of female *Ae. aegypti* mosquitoes [51] and has demonstrated moderate biting deterrence ((+)-β-pinene - BDI = 0.57, (-)-β-pinene - BDI = 0.51)[47]. Camphene exhibits a strong antennal response in female *Ae. aegypti* as determined by gas chromatography-electroantennographic detection [52] and shows larvicidal activities against *Culex pipiens* (LC_{50}= 450 ppm, 48 h exposure)[53]. Delta-3-carene showed ~65% repellency against *Ae. aegypti* and *Ae. albopictus* in a 1 h cage repellency assay [54], a strong biting deterrence index (BDI = 0.75) versus DEET (BDI = 1.0) and larvicidal activity against *An. quadrimaculatus* (LC_{50} = 42.9 ppm; LC_{90} = 80.0 ppm) after 24 h exposure [44]. It also synergised the insecticidal effects of clothianidin against *Ae. aegypti*, enhancing knockdown and mortality by 17.7- and 4.1-fold after 1 h and 24 h exposure [55]. Gu et al. demonstrated that γ-terpinene exhibited ~55% repellence against *Ae. aegypti* and ~65% repellence against *Ae. albopictus* over 1 h exposure time in a short-distance cage assay [54]. p-Cymene has been shown to be mildly effective as a feeding deterrent in a membrane-feeding model [42]. There is no literature pertaining to the insect repellent activity of α-pinene and furthermore, our own results consolidate this, with no significant effect of α-pinene in the arm-in-cage assay observed (**supplemental Fig. S2**)

Conclusion

In conclusion, the use of *M. gale* in the repellence of insects has been validated. A range of factors impact the EO's chemical composition and therefore its activity. Of the *M. gale* oils in this study, EOs extracted by Clevenger hydro-distillation from fruit, exhibited the best contact and spatial repellent effects. OPLS-DA model-based correlations between the chemical profiles of EOs and spatial repellence in the Y-tube revealed that α-phellandrene and myrcene may contribute to the repellence activity of *M. gale* EOs, while eugenol and eugenol acetate were found to be associated with repellence of clove oil when OPLS-DA model-based correlations between the chemical profiles and contact repellence from the arm-in-cage were examined. Quantification of the individual constituents and reports in the literature further direct attention towards β-pinene, camphene, delta-3-carene and γ-terpinene, as possibly contributing to the overall observed effects. This is the first report using targeted metabolomic tools to decipher individual components in *M. gale* EOs that contribute to insect repellence at varying ranges. The components in EOs contributing to spatial repellence may, however, differ from those contributing to contact repellence. Future studies will focus on strategies to optimise the efficacy of formulations, through combining *M. gale* oil with other EOs proven to be effective repellents (e.g. clove oil) and through improving formulations for slowed release of the repellent ingredients.

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Authors contribution: SW: conceptualisation; plant material collection; DNA extraction, sequencing and analysis; plant identification; essential oil extraction by Clevenger hydro-distillation; performed arm-in-cage and Y-tube olfactometer assays; manuscript writing, editing and reviewing. SN: conceptualisation; GCMS analysis; data curation; multivariate data analysis; manuscript writing, editing and reviewing. HAL: conceptualisation; performed arm-in-cage and Y-tube olfactometer assays; manuscript editing and reviewing. ADL: assisted in the primary arm-in-cage study; manuscript editing and reviewing. MP: plant material collection; essential oil extraction by microwave–assisted hydro-distillation; manuscript editing and reviewing. TH: provided lab facility for DNA isolation and sequencing; manuscript editing and reviewing. HS: conceptualisation; funding acquisition; manuscript editing and reviewing. IAH: conceptualisation; provided infrastructure for performing arm-in-cage and Y-tube assays; manuscript reviewing and editing. JW: conceptualisation; manuscript editing and reviewing.

Data availability statement: The sequence dataset generated and analysed during the current study is available in the GenBank repository, under accession numbers PV150706, PV150707, PV150708, PV150709, PV150710 [27-31]. Other datasets generated and analysed during this study are available from corresponding authors on reasonable request.

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