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Chemical Compositions of Essential Oils from German, Roman, and Chinese Chamomile Flowers and Their Biological Activities against Three Economically Important Insect Pests

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Abstract: In this study, the essential oils (EOs) of flowers from German chamomile (GCEOs 1-5) *Matricaria chamomilla* L., Roman chamomile (RCEOs 1-2) *Chamaemelum nobile* (L.) All, and Chinese chamomile (CCEO-1) or "Juhua" *Chrysanthemum morifolium* Ramat were characterized by GC-FID and GC-MS analysis. EOs were tested for biting deterrence/repellency against *Aedes aegypti* and hybrid imported fire ants and for toxicity against *Anastrepha suspensa*. GCEOs 1-5 were characterized by the higher contents of α-bisabolol oxide A (43%-66%) and α-bisabolol oxide B (10%-16%) whereas isobutyl angelate (16%-17%), 2-butenyl angelate (12%-13%), isoamyl tiglate (11%-12%), 3-methyl pentylangelate (8%-11%), and *trans*-pinocarveol (6%-7%) were major compounds of RCEOs 1 and 2. The CCEO-1 was rich in borneol (31%), *ar*-curcumene (12%), bornyl acetate (7%) and intermedeol (5%). Biting deterrence of GCEO-2 and -3, and CCEO-1 was similar to *N*,*N*-diethyl-3-methylbenzamide (DEET) whereas the activity of the other EOs was lower than DEET against *Ae. aegypti*. The activity of pure compounds α-bisabolol and 1,6-dioxaspiro[4.4]non-3-en-2-one from German chamomiles was also similar to DEET against *Ae. aegypti*. Repellency of German chamomile EO, GCEO-F against hybrid imported fire ants was higher whereas the activity of Roman chamomile EO, RCEO-PT was lower than DEET. All EOs, GCEO-4, RCEO-2, and CCEO-1 were toxic against female *A. suspensa*. Further research using intensive *in vivo* bioassays will be conducted to explore the potential of these natural products in insect pest management strategies.

Keywords: Chamomile essential oils, α-bisabolol, repellency, imported fire ant; fruit fly; mosquitoes.

1. Introduction

Because of their ability to transmit diseases and world-wide distribution, mosquitoes are important in global public health. As vectors, mosquitoes cause human diseases including malaria, dengue fever, yellow fever, Rift Valley fever, and Chikungunya. High levels of transmissions can result in substantial human morbidity and mortality. Dengue fever is one of the major diseases that has been

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reported to cause severe morbidity and mortality affecting around 50–100 million people yearly [1]. *Aedes aegypti* (L) and *Ae. albopictus* (Skuse) are the known primary vectors of dengue and Zika virus [2, 3]. *Anopheles* spp. of mosquitoes' vector pathogen of malaria which is considered a great threat to global health [4,5] whereas *Culex quinquefasciatus* Say is reported to transmit West Nile virus [6]. Only malaria infects approximately 250 million people around the globe resulting in one million deaths every year. Synthetic insecticides which are common in use to control mosquito has proved to be one of the major components for prevention and reduction of mosquito-borne disease incidence [7]. Synthetic pesticides are commonly used to control mosquitoes. Because of continuous and indiscriminate use, mosquitoes have developed resistance against commercial pyrethroids [8]. Protection from mosquitoes is achieved by preventing the biting using insect repellents [9]. Repellents are used against insect vectors to provide immediate and localized protection from mosquito bites. In general, applying repellent to the skin is the best feasible way to prevent mosquito bites. Given that these diseases can be transmitted by a single infected mosquito, it is important to use an effective repellent with prolonged protection. DEET is a best-insect repellent available in the market. Many consumers who are reluctant to use DEET on their skin, deliberately seek out natural repellent. Plants present a potential source of natural compounds which possess low mammalian toxicity, are biodegradable and are ecologically non-determined. The exploration of new mosquito repellent from plant sources has been focus of research in recent years [10-12].

Imported fire ants (Hymenoptera: Formicidae) have become pests of significant agricultural and medical importance worldwide. *Solenopsis invicta* Buren and *S. richteri* Forel are two imported fire ant species present in the United States. In addition, hybrids are common along the population boundaries between the two species in Southern States [13,14]. The application of insecticides is the common method to control the imported fire ant. Because of potential negative effects, the use of synthetic chemicals in fire ant management has increasingly become a public concern [15] and interest has now developed to explore safer, more benign, and sustainable alternatives. The Caribbean fruit fly, *Anastrepha suspensa* (Loew), is a quarantine pest of *Citrus* spp. and a pest of many fruits like guava in Florida, USA, and has also been found in many countries and regions [16]. Current management of this pest relies on bait spray incorporating conventional insecticides. There has been increasing interest to explore natural products, which are environmentally friendly, because of the potential environmental contamination of insecticides [17]. The discovery of novel insecticides and repellents from non-toxic and biodegradable plant materials that are often safe and environmentally friendly than synthetic pesticides have been the focus of recent research [18,19].

Chamomiles have been used as medicinal plants and herbal remedies for human diseases. The most common chamomiles used in traditional medicine are German chamomile (*Matricaria chamomilla* L. syn: *M. recutita* L.), Roman chamomile (*Chamaemelum nobile* (L.) syn: *Anthemis nobilis* L.) and Juhua or Chinese chamomile (*Chrysanthemum x morifolium* Ramat.) [20]. They all belong to the family Asteraceae, also known as Compositae or the sunflower family. There are numerous cultivars and hybrids available in each of these species of chamomiles. They have close similarities in their floral morphologies, leading to confusion in species identification and challenges in quality control of the traded materials. The products sold as 'chamomile' in commerce can contain materials of any of these or other related species. German chamomile is an important medicinal herb native to Europe. It is now grown in Punjab and other parts of India, North Africa, Asia, North and South America, Australia, and New Zealand [21]. Hungary is one of the major producers of chamomiles [22]. Chamomiles are used mainly as anti-inflammatory agents [23] internally as an infusion for stomach pain and externally as a powder to cure wounds, skin eruptions, and infections of the mouth [24]. Chamomile tea is widely used for stimulatory effects on the secretions of the liver [25]. Our previous study demonstrated that German chamomile essential oils varied in their chemical composition depending on the country of origin and their insecticidal activity against *Ae. aegypti* was also diverse [26]. Therefore, it is important to investigate the chemical composition of different chamomile species, which also likely differ in essential oil composition as a potential source of ingredients for insect pest control. This study was designed to (i) determine morpho-anatomical characteristics of the flower heads of German, Roman, and Chinese chamomiles, (ii) identify the chemical compositions of these chamomile essential oils (EOs) by gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS) and (iii) determine the biting deterrence/repellency against mosquitoes and fire ants and toxicity against fruit fly.

2. Materials and Methods

2.1. Plant Material

Five samples of German chamomile flowers (GCEO 1-5), two Roman chamomile flowers (RCEO 1-2) and one Chinese chamomile (CCEO-1) ("Juhua") flowers were used in this study (Table 1). All the samples were assigned unique ID codes and deposited in the National Center for Natural Products Research (NCNPR) botanical repository at the University of Mississippi (University, MS, 38677 USA). Commercially available German and Roman chamomile EOs (GCEO-F and RCEO-PT) were purchased online from Floracopeia (www.floracopeia.com, Grass Valley, CA, USA) and Plant Therapy (www.planttherapy.com, Twin Falls, ID, USA), respectively.

Table 1. Chamomile samples used in this study

Chamomile Sample Codes	Botanical Name	NCNPR Specimen Number	Source
GC-1	*Matricaria chamomilla* L.	11681	Voucher
GC-2	*Matricaria chamomilla* L.	11680	Voucher
GC-3	*Matricaria chamomilla* L.	9362	Commercial
GC-4	*Matricaria chamomilla* L.	4903	Commercial
GC-5	*Matricaria chamomilla* L.	9359	Commercial
RC-1	*Chamaemelum nobile* (L.) All.	19116	Voucher
RC-2	*Chamaemelum nobile* (L.) All.	9254	Commercial
CC-1	*Chrysanthemum morifolium* Ramat.	9540	Voucher

GC- German chamomile; RC- Roman chamomile; CC- Chinese chamomile

2.2. Essential Oil Isolation

Chamomiles (GCEOs 1-5, RCEOs 1-2, and CCEO-1) in Table 1 were subjected to water distillation using Clevenger type apparatus (2005). EOs were then calculated on a moisture-free basis for German chamomile at 0.3%-0.4% (dark blue oil), Roman chamomile at 0.6%-0.75% (pale blue oil), and Juhua or Chinese chamomile 0.1% (yellow oil).

2.3. Gas Chromatography Coupled with Flame Ionization Detection (GC-FID) and Gas Chromatography Mass Spectrometry analysis

The GC-MS analysis was carried out using an Agilent 5975 GC-MSD system coupled with an Agilent 6890N GC-FID system. The HP-Innowax FSC column was used. The GC oven temperature was kept at 60°C for 10 min and then programmed to 220°C at a rate of 4°C/min and maintained constant at 220°C for 10 min. The volatile substances were identified via relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to a series of *n*-alkanes [27], library search in Wiley GC-MS Library, Mass-Finder 4 Library [28], MS literature data [29-30], and in-house "Baser Library of EO Constituents" database [31]. The principal component analysis (PCA) and hierarchical cluster analysis (HCA) were carried out with the JMP 16.0 Pro software (SAS Institute, Cary, NC, USA). Data used for the statistical analysis of the chamomile EO compositions was a 66 x 8 matrix (66 individual compounds x 8 samples = 528 data). The PCA was performed by selecting the two highest principal components (components 1 and 2) obtained by the linear regressions operated on mean-centered, unscaled data, as an unsupervised method. The HCA was performed using Ward's method.

2.4. Isolation of Trans-tonghaosu

The *trans*-tonghaosu was isolated from German chamomile (GC-1 and 2, Table 1). Liquid-liquid fractionation was followed by sequential purifications on silica gel using an Isolera Four system (Biotage,

Uppsala, Sweden) equipped with Biotage silica gel flash cartridges and a mobile phase composed of 10% EtOAc in hexane. *trans*-Tonghaosu presented as a pale light-yellow oil, and the structure was confirmed by ^{1}H and ^{13}C NMR experiments, by LC-MS and by comparison with literature data [32].

2.5. Synthesis of 1,6-dioxaspiro[4.4]non-3-en-2-one

The 1,6-dioxaspiro[4.4]non-3-en-2-one was obtained as previously described by Avonto et al. [33]. The alkaline methanol solution was cooled to − 20 °C (NaOH 25.0 mmol in 20 mL of MeOH), then 3-(furan-2-yl) propan-1-ol (2.02 g, 16.01 mmol in 20 mL MeOH) was added dropwise and saturated with oxygen by bubbling for 20 min. Rose bengal (155 mg, 0.16 mmol in 1 mL MeOH) was then added and the reaction was irradiated using a halogen lamp (500 W). After 40 min the reaction was acidified to pH 3 by addition of concentrated HCl. The resulting mixture was concentrated under vacuum extracted by addition of brine and $CHCl_3$ (3 × 25 mL). The organic phase was treated with $MgSO_4$, filtered and the solvent was evaporated. The compound of interest was purified by column chromatography on neutral alumina with 30% EtOAc in hexanes and followed by vacuum distillation using Kugelrohr equipment (120 °C at 0.7 mm Hg) to yield 46% of 1,6- dioxaspiro[4.4]non-3-en-2-one as a colorless liquid.

2.6. Mosquito Bioassays

Adult mosquitoes used in these studies were from the laboratory colonies maintained at the Mosquito and Fly Research Unit at the Center for Medical, Agricultural and Veterinary Entomology, USDA-ARS, Gainesville, Florida. For biting deterrence bioassays, eggs were hatched and the insects were reared to the adult stage in the laboratory and maintained at 27 ± 2 °C and 60 ± 10% RH with a photoperiod regimen of 12:12 h (L: D). 6-15-d-old adult females were used. Experiments were conducted using Klun and Debboun (K&D) module bioassay system developed by Klun et al. [34] for quantitative evaluation of biting deterrence of candidate compounds. Briefly, this assay system consists of a six-well reservoir with each of the 3 × 4 cm wells containing 6 mL of feeding solution. As described by Ali et al. [19], a feeding solution containing green-fluorescent tracer dye (www.blacklightworld.com) was used to assess the feeding by the females. Essential oils from chamomile species and selected pure compounds were tested in this study. Treatments of the essential oils were applied at 10 µg/cm^2 and the pure compounds and DEET (97%, *N*, *N*-diethyl-*meta*-toluamide) (Sigma Aldrich, St. Louis, MO) at 25 nmol/cm^2 was used as the positive control. All the treatments were prepared in molecular biology grade 100% ethanol (Fisher Scientific Chemical Co. Fairlawn, NJ) at the time of bioassay.

The temperature of feeding solution was maintained at 37 °C by passing warm water through the reservoir using a circulatory bath. The reservoirs were covered with a layer of collagen membrane (Devro, Sandy Run, SC) and treated organdy cloth was positioned over collagen covered solution with a Teflon separator between organdy and six-celled module. A six-celled K&D module containing five female mosquitoes per cell was positioned over cloth treatments and trap doors were opened to expose the mosquito females to the treatments. The number of mosquitoes biting through organdy treatments in each cell was recorded after a 3 min exposure and mosquitoes were prodded back into the cells. Mosquitoes were squashed and the presence of green-fluorescent tracer dye (or not) in the gut was used as an indicator of feeding. Two sets of 5 replications each with 5 females per treatment were conducted on 2 different days using a new batch of females in each replication. Treatments were replicated 10 times in total.

2.7. Fire Ants

Fire ant used in this study were from the mounds located under natural field conditions (University Field Station, 93 University of Mississippi, 15 County Road 2078, Abbeville, MS 38601). The species and hybrid imported fire ant identification was performed by using venom alkaloid and hydrocarbon indices [14]. Workers from an imported hybrid fire ant colony were used in these studies.

2.8. Digging Bioassay

Repellency of chamomile EOs against fire ants was determined by using the digging bioassay described by Ali et al. [35]. To avoid the escape of ants, the inner side of the arena petri dish was coated with Insect a Slip (BioQuip Products 2321 Gladwick Street Rancho Dominguez, CA 90220, USA). The sand sieved through a #35 USA standard testing sieve (Thomas Scientific, Swedesboro, NJ) with a uniform 500-micron size was used in this study. Four grams of disinfected sand was treated with different concentrations in a volume of 400 µL in a 45 mL fluted aluminum weighing dish (Fisher Scientific, 300 Industry Drive Pittsburgh, PA 15275) and thoroughly mixed with a small spatula. After evaporating the solvent, de-ionized water was added at a rate of 0.65 µL/g of sand to moisten the sand. The vials were filled with treated sand while the sand in the control treatment was treated only with ethanol. The vials were then screwed to the caps attached to the bottom of the arena. Each vial contained a mean 3.6 g of sand measured on a dry weight basis. Fifty hybrid fire ant workers were introduced in the center of the arena petri dish. The experiment was conducted at 25 ± 2 °C temperature and 50 ± 10% relative humidity. After 24 h, the sand was collected back into aluminum dishes, dried at 150°C for 1 h, and weighed. A series of concentrations were tested until the sample failed the test of repellency. Each experiment was replicated 3 times.

2.9. Toxicity of Chamomile EOs to Anastrepha Suspensa

The toxicity of German chamomile oil (GCEO-4), Roman chamomile oil (RCEO-2), and Chinese chamomile oil (CCEO-1) were determined on adult females of Caribbean fruit fly, *A. suspensa*, via topical bioassays using thoracic application under laboratory conditions at 26 ± 1 °C, 70 ± 5% RH, and 12:12 L:D photoperiod. For each EO, the stock solution was prepared by adding 100 µg of EO to 1 µL of dimethyl sulfoxide (DMSO). The stock solution was then diluted with acetone to establish 5, 10, 15, 20, 30, and 50 µg/µL solutions, and each dilution was tested in topical bioassays to evaluate the toxicities against *A. suspensa*.

To start the topical bioassay on *A. suspensa*, the pupae were first collected and placed in a tray inside a screen cage (30 cm × 30 cm × 30 cm) under the laboratory conditions as mentioned above to allow for adult emergence. Emerged female adults inside the cage were supplied with food and water. At 10 days after emergence, females were collected using an aspirator into a plastic vial (3 cm in diameter × 8 cm in height) and used for topical bioassay. The procedure of topical assay of female adults was the same as described by Kurtca et. al. [31]. Vials containing female adults were first chilled at 4°C in a refrigerator for 5 min to calm the flies, and then transferred out to a petri dish under the laboratory condition to conduct the topical application. On the dorsal thorax of female adults, 1 µL dilution of each of the three EO was applied by using a repeating dispenser equipped with gastight and microliter syringe (50 µL) (PB600, Hamilton Company, Reno, NV, USA). The flies were immediately transferred into a plastic cup (6 cm in diameter × 7.4 cm in height) after topical application and covered with a mesh screen for post-treatment observation. After 24 h, numbers of live and dead flies were recorded and mortality in each treatment was calculated. Untreated females and those treated with acetone alone were used as controls. For each dilution and control, 10 female flies were treated, and each treatment was replicated 3 times. For GCEO-4, concentrations of 5, 10, 15, and 20 µg/µL were used and a total of 180 flies were tested. For RCEO-2, concentrations of 5, 10, 15, 20, and 30 µg/µL were used and a total of 210 flies were tested. For RCEO-2, concentrations of 5, 10, 15, 20, 30, and 50 µg/µL were used and a total of 270 flies were tested.

2.10. Statistical Analyses

Proportion not biting (PNB) was calculated using the procedure described by Ali et al. [19]. As the K&D module bioassay system can handle only four treatments along with negative and positive controls, in order to make direct comparisons among more than four test compounds and to compensate for variation in overall response among replicates, biting deterrent activity was quantified as biting deterrence index (BDI) [19]. The BDI's were calculated using the following formula:

$$\left[BDI_{i,j,k}\right] = \left[\frac{PNB_{i,j,k} - PNB_{c,j,k}}{PNB_{d,j,k} - PNB_{c,j,k}}\right]$$

Where $PNB_{i,j,k}$ denotes the proportion of females not biting when exposed to test compound *i* for replication *j* and day *k* (*i*=1-5, *j*=1-5, *k*=1-2), $PNB_{c,j,k}$ denotes the proportion of females not biting the solvent control "*c*" for replication *j* and day *k* (*j*=1-5, *k*=1-2) and $PNB_{d,j,k}$ denotes the proportion of females not biting in response to DEET "*d"*(positive control) for replication *j* and day *k* (*j*=1-5, *k*=1-2). This formula makes an adjustment for inter-day variation in response and incorporates information from the solvent control as well as the positive control.

A BDI value of 0 indicates an effect similar to ethanol, while a value significantly greater than 0 indicates biting deterrent effect relative to ethanol. BDI values not significantly different from 1, are statistically similar to DEET. BDI values were analyzed using SAS Proc ANOVA [single factor: test compound (fixed)], (SAS Institute, Cary, NC 2012). To determine whether confidence intervals include the values of 0 or 1 for treatments, Scheffe's multiple comparison procedure with the option of CLM was used in SAS.

Data of the fire ants were analyzed using the analysis of variance and means were separated using Ryan-Einot-Gabriel-Welsch multiple range test at $P \leq 0.05$ [36]. Mortality data of *A. suspensa* for each treatment in toxicity bioassays were corrected using Abbott's formula [37] prior to the analysis. The lethal doses (LC_{50} and LC_{99}) for each chamomile oil (GCEO-4, RCEO-2, and CCEO-1) were calculated based on mortality data. A probit analysis was then used to calculate the lethal dose corresponding to a 50% (LC_{50}) and 99% (LC_{99}) reduction in the *A. suspensa*'s survival based on the regression curve. The statistical analysis was performed using SAS [36].

3. Results and Discussion

Of the three chamomile types (Figure 1), *M. chamomilla* and *C. nobile* have similar floral characteristics. The capitula or the flower-heads are radiate, up to 2.5 cm in diameter; ray florets white and disc florets yellow. However, the two species can be distinguished by their morpho-anatomical characters. In brief, the flower-heads of *M. chamomilla* (Figure 1A, B) are cone-like, usually borne in corymbs, without paleae, with the ligules of ray florets generally down-curving, and the receptacle is hollow inside whereas in *C. nobile* (Figure 1C, D), the heads are broadly conical, usually solitary, with paleae, and nearly flat ligules, and the receptacle is solid inside. The flower-heads in *C. morifolium* (Figure 1E, F) are more than an inch in diameter and larger than the other two types. The ray florets are conspicuous, usually in many rows, variable in size, shape, and color; disc florets yellow and often hidden.

The chemical composition of chamomiles is shown in Table 2. The German chamomile EOs 1 to 5 were characterized to contain α-bisabolol oxide A (43-66%), α-bisabolol oxide B (10-19%), (*E*)-β-farnesene (5-13%), α-bisabolone oxide A (6-11%), chamazulene (2-6%) and α-bisabolol (2-3%) whereas RCEOs 1-2 were characterized with a high content of esters such as isobutyl angelate (16-17%), 2-methyl-2-propenyl angelate (12-13%), 2-methylbutyl angelate (11-12%), 3-methyl pentylangelate (8-11%). The main constituents of the CCEO-1 were borneol (31%), *ar*-curcumene (12%), bornyl acetate (7%), and intermedeol (5%).

Many studies have reported the chemical compositions of chamomiles. Hoeferi et al. [26] recently investigated six German chamomile EOs from various origins. Commercial samples from Hungary, South Africa, Serbia and India were high in (*E*)-β-farnesene (19% to 39%), α-bisabolol oxide A (6% to 24%) along with α-bisabolol oxide B (4% to 12%), α-bisabolone oxide A (3% to 9%), α-bisabolol (1% to 12%), chamazulene (1% to 8%) and *cis*-tonghaosu (1% to 10%) whereas wild-grown chamomiles from Hungary and India were rich in α-bisabolol (38%), chamazulene (22%) and *trans*-tonghaosu (17%) while EO from India had α-bisabolol oxide A (25%) and α-bisabolol oxide B (17%) as main components. Additionally, bisabolone oxide A (7%), chamazulene (7%), (*E*)-β-farnesene (7%), α-bisabolol (6%) and *cis*-tonghaosu (6%) were also present [26].

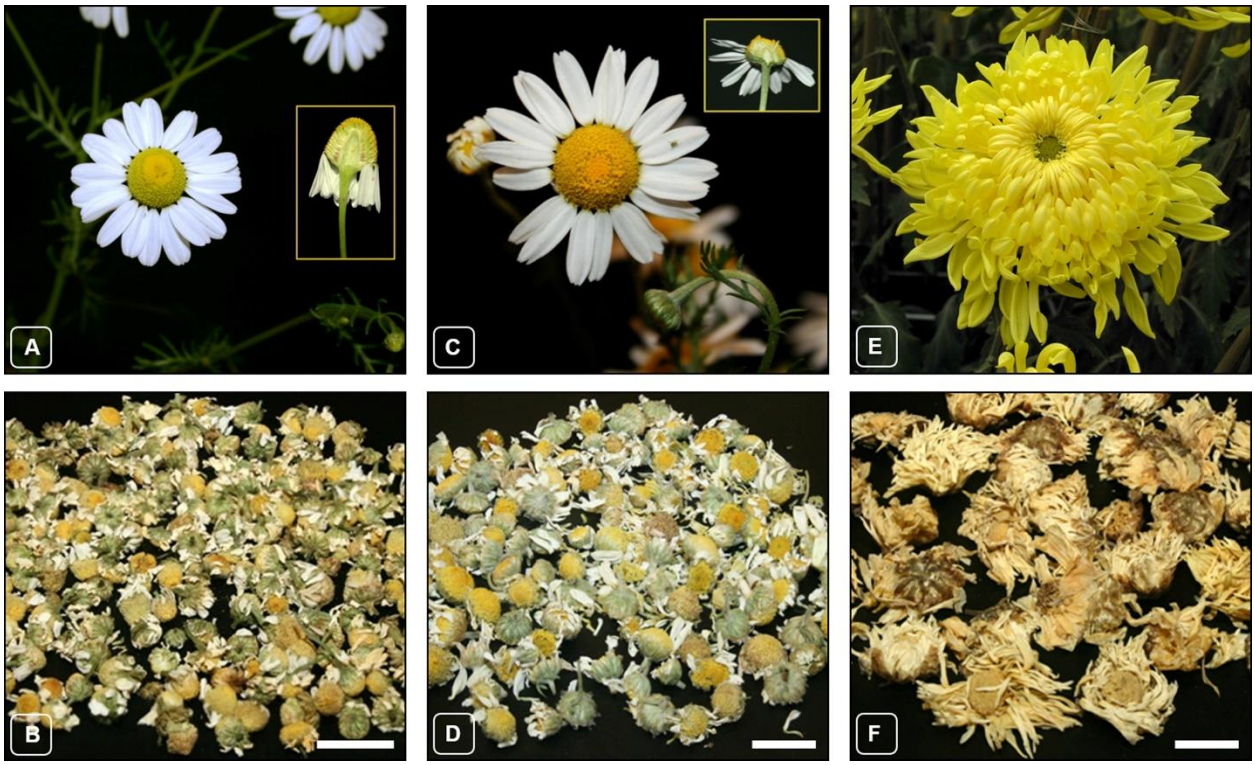

Figure 1. Fresh and dried flower-heads of three chamomile types. A, B- German chamomile (*Matricaria chamomilla*); C, D- Roman chamomile (*Chamaemelum nobile*); E, F- Chinese chamomile "Juhua" (*Chrysanthemum x morifolium*). Bars: B, D, F = 1 cm. [Photo credits: Fig. 1E- KHQ Flower Guide/ Flickr; all others- V. Raman].

According to results obtained from commercial Pharmacopeia (PhEur) grade German chamomile EO, the EO of PhEur chamomile seemed to be attributable to the α-bisabolol oxide A (48%) and (*E*)-β-farnesene (22%) chemotype, followed by α-bisabolol oxide B (6%), α -bisabolon oxide A (6%), chamazulene (4%) and α-bisabolol (2%) [38]. A detailed study on 27 authenticated plants, 35 commercial products, and 11 essential oils of German, Roman, and Chinese chamomile samples showed that these three chamomiles have distinguishable patterns [39]. For example, farnesene, bisabolol oxide B, α-bisabolol, bisabolol oxide, and *trans*-tonghaosu were characterized as the main constituents of German chamomile EOs. High contents of volatile esters, isobutyl isobutyrate, 2-methyl butyl isobutyrate, butyl 3-methyl-2-butenoate, 3-methylbutyl 3-methylbut-2-enoate, 3-methylbut-2-enyl 3-methylbut-2-enoate, hexadecan-4-yl 3-methylbut-2-enoate, were found in Roman chamomile samples. Among the monoterpenes and sesquiterpenoids, borneol, *ar*-curcumene, caryophyllene oxide, alloaromadendrene, eudesm-7(11)-en-4-ol and isoaromadendrene epoxide, showed relatively high amounts in eight Chinese chamomile samples. Data from the current study and all the other previous studies evidenced that German, Roman and Chinese chamomile EOs demonstrated that oxygenated sesquiterpenes, oxygenated monoterpenes and angelic ester profiles can be successfully used for profiling chamomile species. Additionally, this study suggests that multivariate statistical analysis can be used to further differentiate for different types of chamomile samples.

Table 2. The chemical composition of essential oils of German chamomiles GC 1-5; RC- Roman chamomiles RC 1-2 and Chinese chamomile CC -1

#	KI[a]	RRI[b]	Compound	GCEO-1 %	GCEO-2 %	GCEO-3 %	GCEO-4 %	GCEO-5 %	RCEO-1 %	RCEO-2 %	CCEO-1 %	IM
1	1025[c]	1032	α-Pinene	-	-	-	-	-	5.0	5.1	-	RRI, MS
2	1043-1086[c]	1076	Camphene	-	-	-	-	-	0.4	0.6	-	RRI, MS
3	1092[d]	1078	Isobutyl isobutyrate*	-	-	-	-	-	3.1	3.2	-	MS
4	1085–1130[c]	1118	β-Pinene	-	-	-	-	-	0.2	0.4	-	RRI, MS
5	1162, 1173[e]	1145	Isobutyl methacrylate*	-	-	-	-	-	0.8	1.0	-	MS
6	1168-1185[e]	1153	Isobutyl 2-methylbutyrate*	-	-	-	-	-	1.5	1.2	-	MS
7	1154-1195[c]	1188	α-Terpinene	-	-	-	-	-	-	-	t	RRI, MS
8	1185-1203[e]	1203	2-Methylbutyl isobutyrate	-	-	-	-	-	3.4	2.9	-	MS
9	1186-1231[c]	1213	1,8-Cineole	-	-	-	-	-	-	-	3.4	RRI, MS
10	1222-1266[c]	1255	γ-Terpinene	-	-	-	-	-	-	-	0.5	RRI, MS
11	1246-1291[c]	1280	*p*-Cymene	-	-	-	-	-	0.2	0.4	0.5	RRI, MS
12	1260-1305[e]	1286	2-Methylbutyl 2-methylbutyrate	-	-	-	-	-	0.8	1.1	-	MS
13	1287,1293[e]	1292	Isobutyl angelate*	-	-	-	-	-	16.8	15.7	-	MS
14	1252-1319[e]	1294	1,2,4-Trimethyl benzene	-	-	-	-	-	-	-	0.9	MS
15	-	1319	(*E*)-2-Methyl-2-butenyl isobutyrate*	-	-	-	-	-	1.4	1.2	-	MS
16	-	1335	2-Methyl-2-propenyl angelate *	-	-	-	-	-	12.5	12.1	-	MS
17	1301-1382[e]	1355	1,2,3-Trimethyl benzene	-	-	-	-	-	-	-	0.6	MS

18	-	1348	Isoamyl angelate*	-	-	-	-	-	6.5	6.0	-	MS
19	1395[e]	1353	2-Methylbutyl angelate*	-	-	-	-	-	12.3	10.8	-	MS
20	1320-1358[c]	1358	Artemisia ketone	tr	0.1	0.7	t	-	-	-	-	MS
21	1377-1405[c]	1403	Yomogi alcohol	tr	0.8	0.7	t	-	-	-	-	MS
22	-	1452	α,*p*-Dimethylstyrene	-	-	-	-	-	-	-	0.4	MS
23		1455	3-Methylamyl angelate*	-	-	-	-	-	7.8	11.3	-	MS
24	1438-1480[c]	1466	α-Cubebene	-	-	-	-	-	-	-	2.0	MS
25	-	1467	Angelyl angelate*	-	-	-	-	-	2.3	1.7	-	MS
26	1462-1522[c]	1497	βα-Copaene	-	-	-	-	-	-	-	0.5	MS
27	1515[c]	1532	Camphor	-	-	-	-	-	-	-	4.0	RRI, MS
28	1507-1564[c]	1553	Linalool	-	-	-	-	-	-	0.7	-	RRI, MS
29	1533-1590[c]	1582	*cis*-Chrysanthenyl acetate	-	-	-	-	-	-	-	1.0	MS
30	1545-1590c	1586	Pinocarvone	-	-	-	-	-	2.9	2.6	-	RRI, MS
31	1579[c]	1591	Bornyl acetate	-	-	-	-	-	-	-	7.3	RRI, MS
32	1601[c]	1611	Terpinen-4-ol	-	-	-	-	-	-	-	1.9	RRI, MS
33	1580⁻1616[c]	1616	Hotrienol	-	-	-	-	-	-	-	2.9	MS
34	1597-1648[c]	1648	Myrtenal	-	-	-	-	-	-	0.8	-	MS
35	-	1669	Sesquisabinene	-	-	-	-	-	-	-	2.0	MS
36	1643-1671[c]	1670	*trans*-Pinocarveol	-	-	-	-	-	6.9	6.3	0.5	RRI, MS
37	1643-1684[c]	1695	(*E*)-β-Farnesene	10.8	13.3	11.1	5.4	5.9	-	-	0.8	MS
38	1694[c]	1706	α-Terpineol	-	-	-	-	-	-	-	0.3	RRI, MS

39	1653-1728[c]	1719	Borneol	-	0.6	-	-	-	-	-	31.1	RRI, MS
40	1686-1743[c]	1742	β-Selinene	-	-	-	-	-	-	-	1.6	MS
41	1699-1751[c]	1751	Carvone	-	-	0.2	-	-	-	-	-	RRI, MS
42	1751-1765[c]	1764	*cis*-Chrysanthenol	-	-	-	-	-	-	-	1.8	MS
43	1722-1774[c]	1773	δ-Cadinene	-	-	-	-	-	-	-	3.3	MS
44	1743-1788[c]	1786	*ar*-Curcumene	-	-	-	-	-	-	-	12.4	MS
45	1743-1808[c]	1804	Myrtenol	-	-	-	-	-	-	1.1	-	MS
46	-	1805	2-Hydroxy-2-methyl-3-butenyl angelate*	-	-	-	-	-	2.0	1.0	-	MS
47	1802-1846[c]	1845	(*E*)-Anethole	-	-	0.5	-	-	-	-	-	RRI, MS
48	-	1849	Calamenene	-	-	-	-	-	-	-	0.4	MS
49	1893-1941[c]	1941	α-Calacorene	-	-	-	-	-	-	-	0.8	MS
50	1936-2023[c]	2008	Caryophyllene oxide	-	-	-	-	-	-	-	3.0	RRI, MS
51	1978-2037[c]	2020	Methyl tetradecanoate (=*M. myristate*)	2.3	-	-	-	-	-	-	-	RRI, MS
52	1995-2055c	2050	(*E*)-Nerolidol	-	0.5	-	-	-	-	-	-	MS
53	2074-2150[c]	2144	Spathulenol	3.3	3.5	3.0	2.4	2.4	-	-	-	MS
54	-	2156	α-bisabolol oxide B	18.7	15.9	12.7	10.3	9.6	-	-	-	MS
55	2170-2187[e]	2174	Fokienol	-	-	-	-	-	-	-	0.2	MS
56	2165[d]	2187	T-Cadinol	-	0.5	0.9	0.7	0.8	-	-	-	MS
57	-	2200	α-Bisabolone oxide A	7.1	10.5	7.5	6.2	6.3	-	-	-	MS
58	-	2214	*ar*-Turmerol	-	-	-	-	-	-	-	0.3	MS

59	2170-2254[e]	2226	Methyl palmitate	5.8	-	-	-	-	-	-	-	RRI, MS
60	2178-2234[c]	2232	α-bisabolol	-	2.6	1.9	1.6	1.7	-	-	-	RRI, MS
61	2227-2301[c]	2298	Decanoic acid	-	0.7	0.4	1.7	1.5	-	-	-	RRI, MS
62	2218-2264[c]	2264	Intermedeol	-	-	-	-	-	-	-	4.9	MS
63	2334-2452[c]	2430	Chamazulene	3.1	5.5	4.2	3.1	2.0	-	-	-	MS
64	-	2438	α-bisabolol oxide A	15.0	42.6	53.2	65.5	66.4	-	-	-	MS
65	2400-2476[c]	2456	Methyl oleate	27.7	-	-	-	-	-	-	-	RRI, MS
66	2476-2523[c]	2509	Methyl linoleate	4.8	-	-	-	-	-	-	-	RRI, MS
			Monoterpene Hydrocarbons (MH)	-	-	-	-	-	5.8	6.5	1.0	
			Oxygenated Monoterpenes (OM)	-	1.5	1.6	-	-	9.8	11.5	46.9	
			Sesquiterpene Hydrocarbons (SH)	10.8	13.3	11.1	5.4	5.9	-	-	23.8	
			Oxygenated Sesquiterpenes (OS)	44.1	76.1	79.2	86.7	87.2	-	-	8.4	
			Fatty acid+methyl esters (FA+ME)	40.6	0.7	0.4	1.7	1.5	-	-	-	
			Esters	-	-	-	-	-	71.2	69.2	7.3	
			Others	3.1	5.5	4.7	3.1	2.0	-	-	1.9	
			Total	98.6	97.1	97.0	96.9	96.6	86.8	87.2	89.3	

[a]KI from literature [27-30]. (Babushok et al. [40][c], www.pherobase.com[d], PubChem[e]); [b]RRI: Relative retention indices calculated against *n*-alkanes; % calculated from FID data; t: Trace (< 0.1 %) *: tentative identification; IM: Identification method based on the relative retention indices ([b]RRI) of authentic compounds on the HP Innowax column; MS, identified on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data.

The profile of the GCEOs, by groups of chemical compounds, contained a high concentration of oxygenated sesquiterpenes (OS, 44-87%) whereas ester compounds (69-71%) were predominant in RCEOs. The oxygenated monoterpenes (OM, 47%) and sesquiterpene hydrocarbons (SH, 24%) were the main class of CCEO (Figure 2).

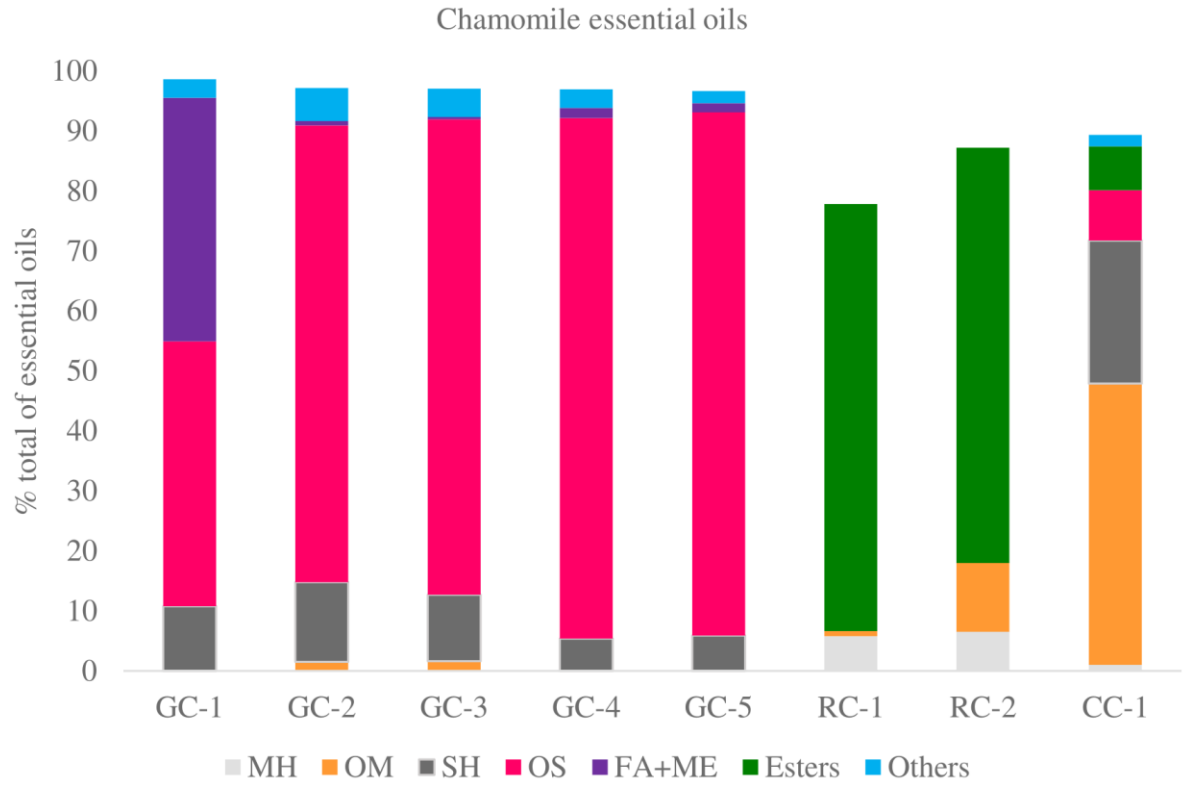

Figure 2. Main class of compounds in the GCEOs, RCEOs and CCEO. MH: monoterpene hydrocarbons, OM: oxygenated monoterpenes, SH: sesquiterpene hydrocarbons, OS: oxygenated sesquiterpenes.

Multivariate statistical analysis was used to determine underlying patterns among the chamomile EO compositions, by both the principal component (PC) and hierarchical cluster (HC) analyses. Table 3 demonstrates that the six components had eigenvalues greater than one which explains most of the variables in component 1 and component 2. Factor loads of individual variables are presented in Supplementary Material (Table S1), which shows how strongly each variable influenced component 1 and component 2.

Table 3. Originally determined principal components with matrix eigenvalues and percentage of variance

Number of Component	Eigenvalue	% of Total Variance Explanation	Cumulative Variance [%]
1	29.1583	44.179	44.179
2	24.2765	36.783	80.962
3	5.4906	8.319	89.281
4	2.9529	4.474	93.755
5	2.1641	3.279	97.034
6	1.9270	2.920	99.954
7	0.0306	0.046	100.000

The score and the loading plots of the PCA performed on the chemical composition and PCA provided a clear separation of GCEOs from the RCEOs and CCEO (Figure 3a). German and Roman chamomile EOs were plotted in the leftmost area of the left quadrants: GC 1-5 in the lower quadrant (PC1 < 0; PC2 <0), and RC 1-2 in the upper quadrant (PC1 <0; PC2 > 0). The sample CC-1 was loaded alone in the right upper quadrant with a positive score on PC1 > 0. Figure 3b shows that chamomile EOs were separated into three main groups. GCEOs 1-5 loaded in the bottom of left quadrant, which was characterized by (*E*)-β-farnesene (#**37**), α-bisabolol oxide B (#**54**), α-bisabolene oxide A (#**57**), α-bisabolol oxide A (#**64**) and chamazulene (#**63**) whereas RCEO 1and 2 loaded in the upper of left quadrant and had unique group of esters isobutyl isobutyrate (#**3**), isobutyl methacrylate (#**5**), isobutyl 2-methylbutyrate (#**6**), 2-methylbutyl isobutyrate (#**8**), 2-methylbutyl 2-methylbutyrate (#**12**), isobutyl angelate (#**13**), (*E*)-2-methyl-2-butenyl isobutyrate (#**15**), 2-methyl-2-propenyl angelate (#**16**), isoamyl angelate (#**18**), 2-methylbutyl angelate (#**19**), 3-methylamyl angelate (#**23**), angelyl angelate (#**25**) and 2-hydroxy-2-methyl-3-butenyl angelate (#**46**). CC-1 was distinguished positive loading with PC1 in the upper right quadrant, which was characterized by the highest amount of borneol (#**39**), *ar*-curcumene (#**44**) and bornyl acetate (#**31**). The dendrogram obtained by the hierarchical cluster analysis (HCA) carried out on the chemical composition of eight chamomile samples were divided into three macro- clusters (Figure 4); (i) red ones comprised all the German chamomiles (GCEO 1 to 5), (ii) the green cluster comprised the two Roman chamomile samples (RCEO 1 and 2), and (iii) Chinese chamomile (CCEO-1, blue) was loaded far from the GC 1-5 and RC-1-2 samples, thus evidencing that the sample CC-1 had different chemical composition than samples from GCEOs 1-5 and RCEOs 1-2, which was a bit far from the hub due to rich in oxygenated monoterpenes (OM) and sesquiterpenes hydrocarbons (SH). The result of both PCA and HCA indicated that chamomile EOs were significantly influenced by the chemical composition.

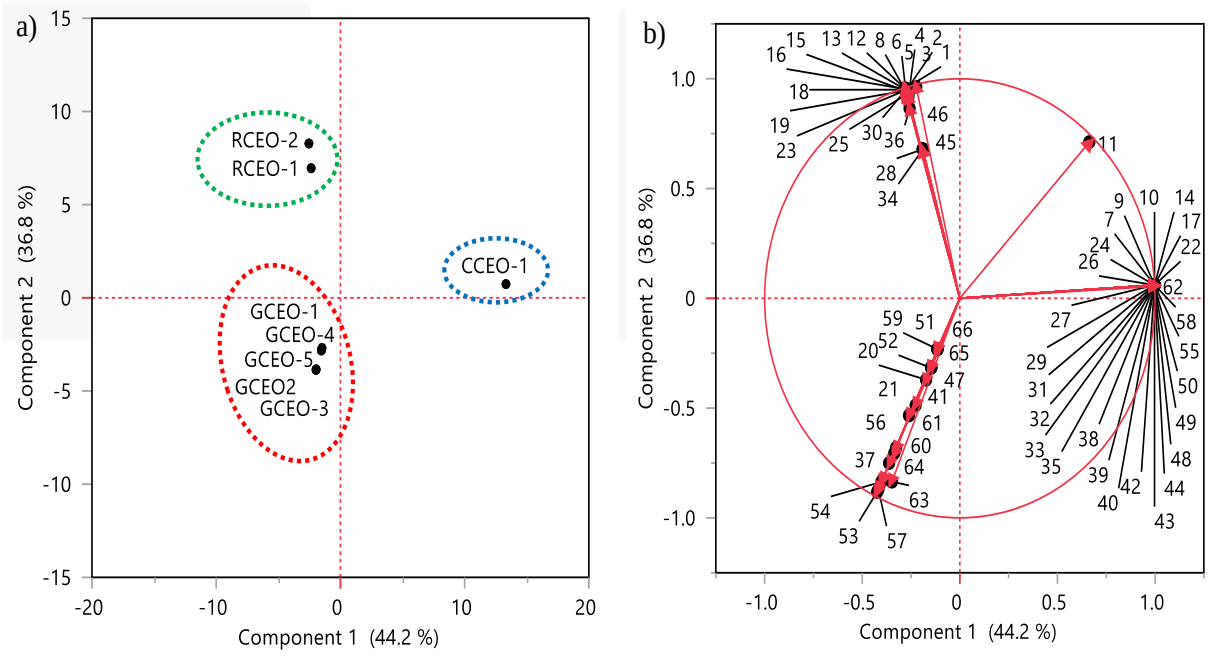

Figure 3. The score (**a**) and the loading plot (**b**) of the principal component analysis of chamomile volatile samples.

*GCEO 1-5 German chamomiles; RCEO 1-2- Roman chamomiles; CCEO-1 Chinese chamomile.

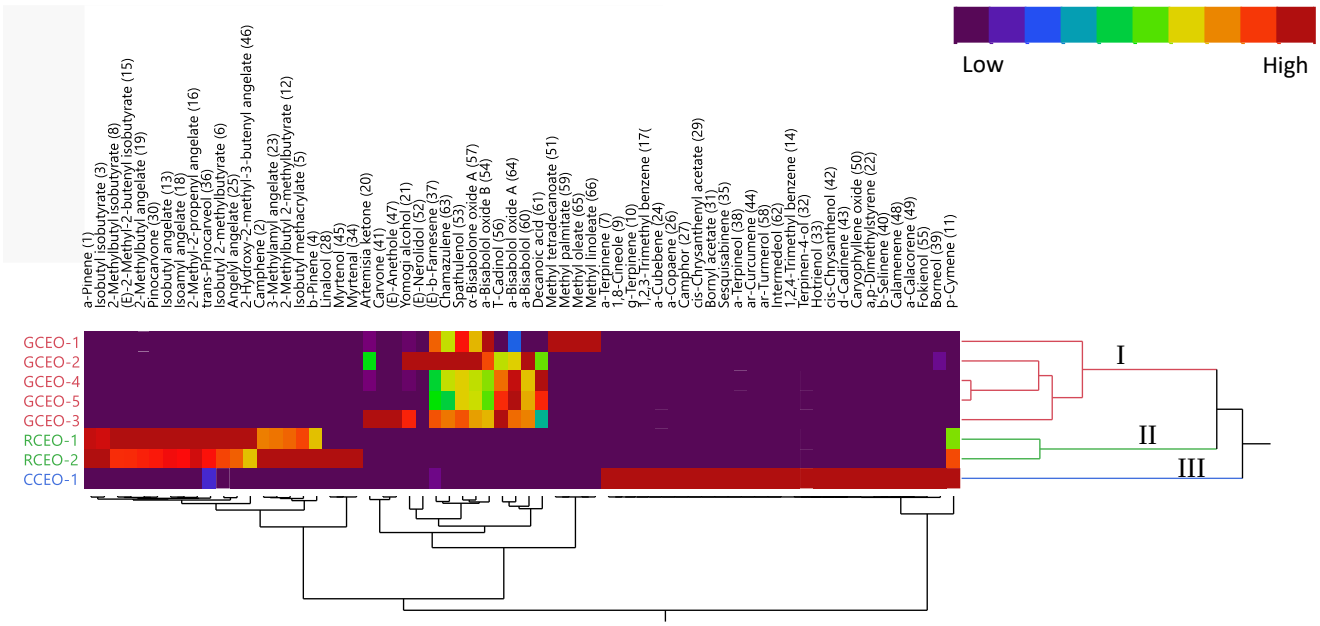

Figure 4. Two-way dendrogram of the hierarchical cluster analysis (HCA) was performed on the chemical compositions of the five German chamomiles (GC 1-5) samples, and two Roman chamomiles (RC 1-2) samples, and one Chinese chamomile (CC-1) sample.
*The color box indicated the standardized abundance of each compound. The red represents high density of compounds and blue represents low density.

All the chamomile EOs showed biting deterrent activity greater than the ethanol against *Ae. aegypti* (Figure 5). Based on 95% CI, biting deterrence of GCEOs 2-3 and CCEO-1 at 10 µg/cm^2 showed activity similar to DEET at 25 nmol/cm^2 whereas all the other tested samples showed activity lower than DEET. Since GCEOs 2-3 showed deterrence, we selected characteristic compounds like α-bisabolol oxide A, (*E*)-β-farnesene, and α-bisabolol for biting deterrent activity using K&D bioassay system against *Ae. aegypti*. We also included *trans*-tonghaosu and 1,6-dioxaspiro[4.4]non-3-en-2-one in this study because polyacetylenes have a history of insecticidal activity and polyacetylenes accumulate in some German chamomile chemotypes [41,42]. Based on 95% CIs, activity of α-bisabolol and 1,6-dioxaspiro[4.4]non-3-en-2-one was similar to DEET at 25 nmol/cm^2 (Figure 6). α-bisabolol oxide A, (*E*)-β-farnesene and (*E*)- β -farnesene with the BDI values of 0.66, 0.82 and 0.70 were also very active but the activity was significantly lower than DEET, respectively.

There are many reports in the literature on the biting deterrence/repellency of natural products against different species of mosquitoes. Some of these are essential oils of *C. longa* rhizome and leaf and *ar*-turmerone [17], *Matricaria discoidea* [12], *Prangos heyniae* essential oil [43], *Cannabis chemovars* volatile oils [44] and *Magnolia grandiflora* essential oil [45]. There are only few studies on chamomile essential oils against mosquitoes. *Matricaria recutita* volatile oil was reported to show reduction of oviposition and repellency against *Culex pipiens* [46]. Results from this study suggested that α-bisabolol and 1,6-dioxaspiro[4.4] non-3-en-2-one which showed biting deterrence similar to DEET might provide natural source of lead compounds as potential sources for mosquito repellency. Only CCEO-1 contained *ar*-curcumene (12%) and intermedeol (5%) as major contents which are documented as repellents against mosquitoes [18] and ticks [47]. These two compounds appear to be responsible for the activity of this essential oil. α-bisabolol showed biting deterrence similar to DEET and spathulenol has been reported to have biting deterrent activity against mosquitoes [12]. These two compounds are present in the samples of GCEO-2 and 3 and in combination with other compounds appear to be responsible for the activity of these essential oils. Further research, through intensive *in vivo* bioassays, is needed to explore the market potential of these natural products in pest management applications.

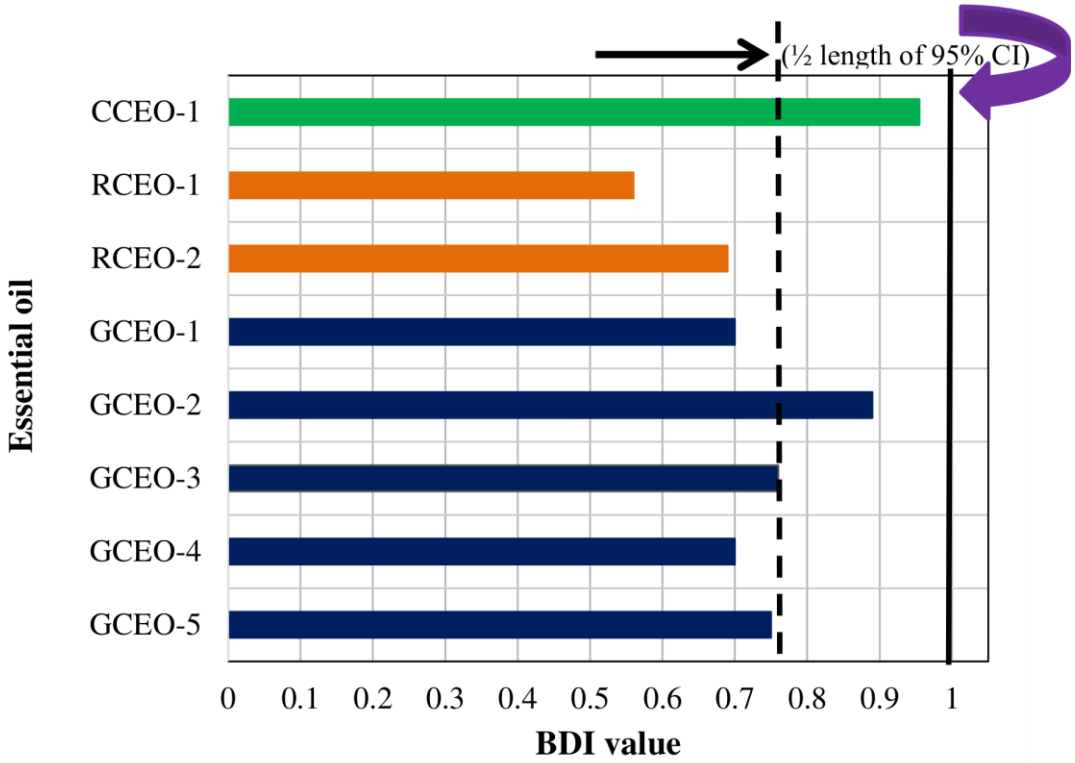

Figure 5. Biting deterrent indices (BDI) of five essential oils (10 µg/cm^2) of 8 chamomiles essential oils against female *Ae. aegypti*.

[*] Ethanol was the solvent control and DEET at 4.8 µg /cm^2 was used as positive control. Mean BDI values falling between 1/2 length of 95% CI and 1 are statistically similar to DEET.GCEO 1-5 are *Matricaria chamomilla* (German chamomiles), RCEO 1-2 are *Chamaemelum nobile* (Roman chamomiles), CCEO-1 is *Chrysanthemum morifolium* (Chinese chamomile).

Mean weight (g) of treated sand removed by the hybrid imported fire ant workers released in multiple-choice digging bioassay with different concentrations of GCEO-F and RCEO-PT is given in Table 4. Based on the sand removal, GCEO-F treatments at dosages of 156 - 39 µg/g showed significantly higher repellency against hybrid imported fire ant workers than the solvent control whereas the repellency at 19.5 µg/g was similar to the solvent control. In RCEO-PT treatments, hybrid imported fire ant workers showed repellency similar to ethanol control at dosages of 156 - 39 µg/g. In DEET treatments, repellency was significantly higher than solvent control at 156, and 78 µg/g whereas the repellency of DEET at 39 µg/g was similar to solvent control.

Based on the sand removal data, GCEO-F showed better repellency whereas RCEO-PT was significantly less active than GEEO-F and DEET against hybrid imported fire ants. Natural products have been reported to show repellency against fire ants. Plant products including mint oil and cinnamon oil have been reported to be effective against red imported fire ants [48, 49]. Chen et al. [50] reported repellency of intermedeol and callicarpenal against fire ant workers in the laboratory. Kafle and Shih [51] reported the repellency of various compounds of clove (*Syzygium aromaticum*) against red imported fire ants. Borneol and α-terpineol had shown toxicity against red imported fire ant workers of *S. invicta* [52]. *Magnolia grandiflora* essential oil and its pure compounds 1-decanol and 1-octanol have been reported to show significant repellency against hybrid imported fire ant. This is the first report on the repellency of chamomile essential oils against hybrid imported fire ants.

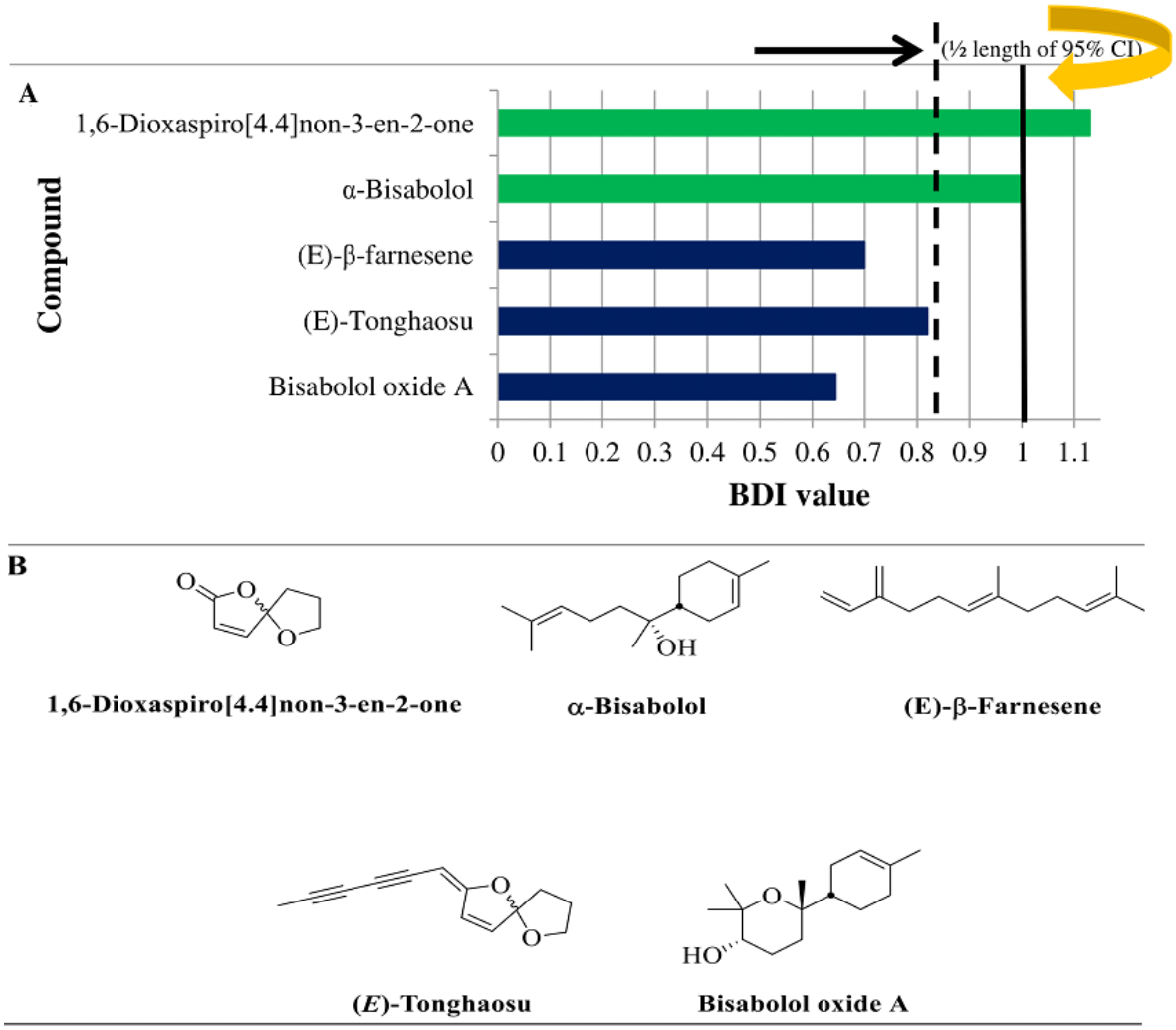

Figure 6. A: Biting deterrent indices (BDI) of five pure compounds (25 nmol/cm^2) isolated from chamomiles essential oils against female *Ae. aegypti*. Ethanol was the solvent control and DEET at 25 nmol/cm^2 was used as positive control. Mean BDI values falling between 1/2 length of 95% CI and 1 are statistically similar to DEET. **B**: Structure of compounds presented in Figure 6A.

All the three chamomile EOs showed toxicity to adult female *A. suspensa* (Table 5). The LC_{50} values of the three EOs against *A. suspensa* were 9.30, 8.57, and 7.58 µg/fly for GCEO-4, RCEO-2, and CCEO-1, respectively. The LC_{99} values of the three oils against *A. suspensa* were 18.68, 22.38, and 58.49 µg/µL for GCEO-4, RCEO-2, and CCEO-1, respectively. The female adults in untreated control had 0% mortality whereas mortality in acetone treatment was 6.69%. Based on LC_{50} values, there were no significant differences among the three essential oils tested against *A. suspensa*. However, Based on LC_{99}, GCEO-4 (18.68 µg/fly) and RCEO-2 (22.38 µg/fly) were 2-3 times more toxic than CCEO-1 (LC_{99} 58.49 µg/fly).

There has been reports on the effectiveness of natural products against fruit flies. *Juniperus foetidissima* essential oil has been reported to be toxic to *A. suspensa* female adults [31]. The differences in toxicity among the three oils against female *A. suspensa* can be due to differences in the chemical compositions of these oils. For example, 1,8-cineole and borneol were present only in CCEO-1 (Table 2), and 1,8-cineole had mild toxicity against insect pests, but had a strong toxicity against rice weevil in stored grain [53]. Therefore, it appears that 1,8-cineole, α-terpineol, and borneol

may have contributed to toxicities against female *A. suspensa*. GCEO-4 and RCEO-2 had lower LC_{99} than CCEO-1 which may be because of differences in chemical composition. Although it was not clear which components contributed primarily to the mortality of female *A. suspensa*, synergistic effects of these compounds may have played an important role in improving the toxicity. Our results of GCEO-4, RCEO-2, and CCEO-1 demonstrated a strong contact toxicity against female *A. suspensa*. Further research, through intensive *in vivo* bioassays, is needed to explore the potential of these natural products in development of biopesticides against fruit fly.

Table 4. Mean weight (g) of treated sand removed by the hybrid red imported fire ant workers released in multiple-choice digging bioassay treated with GCEO-F and RCEO-PT

Concentration (µg/g)	Sand removed* (Mean ± SEM)	*F*-value	*P* value
GCEO-F			
Control	1.47 ± 0.39 A	1.19	0.3735
19.5	0.74 ± 0.12 A		
9.8	1.06 ± 0.10 A		
4.8	1.2 ± 0.36 A		
Control	1.88 ± 0.21 A	52.91	0.0001
156	0.01 ± 0.01 B		
78	0.05 ± 0.009 B		
39	0.24 ± 0.13 B		
RCEO-PT			
Control	1.34 ± 0.25 A	0.31	0.8208
156	0.98 ± 0.35 A		
78	1.11 ± 0.25 A		
39	1.13 ± 0.21 A		
DEET			
Control	1.45 ± 0.19 A	19.8	0.0005
156	0.04 ± 0.04 B		
78	0.24 ± 0.2 B		
39	1.11 ± 0.15 A		

*Sand removed is in grams. Means within a column in an experiment not followed by the same letter are significantly different (Ryan-Einot-Gabriel-Welsch multiple range test $P \leq 0.05$).

Table 5. Toxicity of three chamomile essential oils against adult female Caribbean fruit fly, *A. suspensa*, under laboratory conditions.

Chamomile EO	*n*	Slope (±SE)	LC_{50} (95% FL), g/fly	LC_{99} (95% FL), g/fly	χ^2	df
GCEO-4	150	7.68 ± 0.67	9.30 (8.71–9.86)	18.68 (16.86-21.46)	4.1531	2
RCEO-2	180	5.58 ± 0.43	8.57 (7.93-9.19)	22.38 (19.79-26.27)	4.3698	3
CCEO-1	210	2.62 ± 0.33	7.58 (5.16-9.66)	58.49 (37.21-144.25)	8.4710	4

Essential oils GCEOs 2 and 3, CCEO-1 and pure compounds, α-bisabolol and 1,6-dioxaspiro[4.4] non-3-en-2-one isolated from GCEO-2 and -3 showed biting deterrence similar to DEET against *Ae. Aegypti.* GCEO-F showed better repellency than DEET against hybrid imported fire ants. All EOs showed contact toxicity to female *A. suspensa*. Based on these data some of these chamomile essential oils and pure compounds showed a strong potential to be developed as repellents against mosquitoes and fire ants. Further research through intensive laboratory bioassays and field trials will be conducted to explore the potential of these natural products against these pests.

Acknowledgments

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