CROP BREEDING AND APPLIED BIOTECHNOLOGY

ARTICLE

Chemical composition and antiprotozoal potential of essential oil from half-sib progenies of *Varronia curassavica* Jacq.

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Abstract: *The aim of this study was to evaluate chemical characteristics and biological activity of progenies from the first cycle of recurrent selection of Varronia curassavica in order to identify promising progenies for the next stages of the breeding program. The seeds were collected from the parental accession VCUR-503 (chemotype E-caryophyllene/viridiflorol) and subsequently sown in seedling containers and transplanted to the field. The essential oil was extracted 300 days after transplanting, and the levels of E-caryophyllene and viridiflorol were analyzed, as well as their activity against the protozoan Ichthyophthirius multifiliis. Application of the recurrent selection method resulted in increased levels of both compounds. The levels of E-caryophyllene (0.00-23.61%) and viridiflorol (0.00-64.74%) exhibited significant variation within the population under study. The essential oil rich in viridiflorol from progenies led to high mortality (72%) of the protozoan. Promising progenies for the second cycle of recurrent selection were identified based on the variables analyzed.*

Keywords: *Germplasm, selection, volatile oil, biocide,* Ichthyophthirius multifiliis

INTRODUCTION

Varronia curassavica Jacq. (syn. *Cordia verbenacea* DC), Cordiaceae family, is a plant native to Brazil that has economic and pharmaceutical value (Miller and Gottschling 2007, Perini et al. 2015). This species exhibits remarkable phenotypic plasticity, allowing its natural occurrence in different Brazilian biomes, such as the Caatinga, Cerrado, and Mata Atlântica (Atlantic Forest). Additionally, its presence has been recorded in regions of other countries (Weeks et al. 2010, Mendes et al. 2015, Carvalho et al. 2017).

The species is a perennial shrub that reproduces through cross-pollination and can reach a height of up to two meters (Hoeltgebaum et al. 2018). Its leaves are characterized by glandular trichomes that produce essential oil used in the development of herbal medicines and flavorings (Ventrella and Marinho 2008, Oliveira 2021). In addition to its medicinal applications, this species has shown promising results in combating pests in fish farming. Nizio et al. (2018) demonstrated its biocidal action against the parasitic protozoan *Ichthyophthirius multifiliis* in freshwater fish.

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The protozoan *I. multifiliis* is responsible for causing significant economic damage in aquaculture worldwide; it is the causative agent of the disease known as "ichthyophthiriasis" or "white spot disease". Its rapid multiplication and resistance to commercial products make disease control challenging, resulting in negative impacts on fish farming for both consumption and ornamental purposes (Matthews 2005, Xu et al. 2012, Cararo et al. 2017, Kwan et al. 2020).

Several studies have emphasized the need to find alternative treatments for ichthyophthiriasis, including the investigation of medicinal species with biocidal properties (Liang et al. 2015, Lin et al. 2016, Baldissera et al. 2018). The essential oil extracted from *V. curassavica* has shown antiprotozoal activity against *I. multifiliis* (Nizio et al. 2018). This activity is mainly attributed to the sesquiterpenes *E*-caryophyllene and viridiflorol. However, the low concentration of these compounds in the essential oil limits the use of this plant as a raw material for the development of future products. Therefore, the application of plant breeding methods becomes necessary to develop cultivars that have higher levels of the active compounds in the essential oil and have agronomic potential (Oliveira et al. 2020a).

Currently, no published studies in plant breeding are available that focus on the biological activity of native aromatic and medicinal species for the development of veterinary or agricultural products. However, recurrent selection among half-siblings is believed to be an effective method for gradually accumulating favorable alleles and improving the levels of chemical compounds through cycles of recombination and selection (Acquaah 2012).

In light of this, the aim of the present study was to evaluate the performance of S1 progenies of *V. curassavica* regarding the levels of *E*-caryophyllene and viridiflorol compounds and the antiprotozoal activity of *V. curassavica* essential oil against *I. multifiliis*. An additional aim was to identify promising progenies for the next cycles of recurrent selection.

MATERIAL AND METHODS

The experiment was conducted at the "Campus Rural da UFS" experimental farm (lat 11° 00' S, long 37° 12' W) in the county/municipality of São Cristóvão, Sergipe, Brazil. To obtain the progenies, seeds were collected from the VCUR-503 accession, which is part of the *V. curassavica* collection maintained in the active germplasm bank (*banco ativo de germoplasma* – BAG) of medicinal and aromatic plants at the Universidade Federal de Sergipe (Federal University of Sergipe). This collection is registered in the National Genetic Heritage Management System (*Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* - SISGEN) under the number A8CCB3B. It should be noted that some of the accessions in this collection have *E*-caryophyllene and viridiflorol as major components, as described by Oliveira et al. (2020b).

Before seed collection, the inflorescences of the VCUR-503 accession were monitored daily to identify the presence of fertilized flowers and fruit at the stage of physiological maturity, characterized by a red color. When more than 50% of the flowers of the inflorescences were fertilized, the inflorescences were protected by non-woven fabric (TNT) bags to ensure the integrity of the fruit and prevent damage to the seeds during collection.

The collected fruit was placed in paper bags and then washed under running water to remove all the pulp. The seeds were individually sown in germination trays filled with medium-texture soil collected from the *V. curassavica* collection of the Active Germplasm Bank of medicinal and aromatic plants of the Federal University of Sergipe. The trays were transferred to a greenhouse with an irrigation system. After formation of the third pair of leaves, the plants were transplanted into polyethylene cups filled with the same substrate mixed with cow manure at a ratio of 3:1. Each plant was identified with the code of the maternal parent, VCUR-503, followed by a cardinal number corresponding to the progeny (e.g., VCUR-503-01). Simultaneously, a cutting from the maternal parent was grown during the same developmental period of the progenies in the greenhouse. In all, 88 half-sibling progenies were obtained.

Seeds were sown in January 2018. After 60 days of growth in the greenhouse, the progenies and the maternal parent were transferred to the field in March 2018. In the field, the plants were spaced at a distance of 1.0 × 1.0 meter and identified with wooden stakes. Prior to planting, the soil was tilled by plowing and harrowing. For base fertilization, each plant hole received 1.0 liter of well-cured cow manure. Additionally, fertilizer was top dressing by applying 3.0 liters of well-cured cow manure per plant. Throughout the experiment, the plants did not receive irrigation, so as to create higher selection pressure and identify drought-tolerant materials.

The aerial parts of the plants were harvested 300 days after transplanting by cutting them at 40 cm above ground level.

The harvested material was defoliated and placed in a forced-air circulation drying chamber, where it was maintained at a temperature of 40 ± 1 °C for five days, following the method described by Ehlert et al. (2006). For extraction of the essential oil, 35 g of leaves and inflorescences were weighed and transferred to 3000 mL glass flasks containing 1500 mL of distilled water. The essential oil was extracted by hydrodistillation using a Clevenger apparatus, with a duration of 140 minutes, and performed in triplicate, as described by Nizio et al. (2018).

After extraction, the essential oils were stored in amber bottles and kept in a freezer until chemical analysis. Chemical analysis was performed using a GC/MS system (GCMSQP2010 Ultra, Shimadzu Corporation, Kyoto, Japan), following the methodology described by Oliveira et al. (2020b). The components were identified by comparing the retention index (Van den Dool and Kratz 1963).

A completely randomized experimental design was used, with three replications. Essential oils from the progenies VCUR-503-119, VCUR-503-91, VCUR-503-34, VCUR-503-66, and VCUR-503-101 were tested, as well as from the parental accession VCUR-503. Initial concentrations tested included 0.0, 10.0, 25.0, 50.0, and 75.0 mg L^{-1}, along with a water + tween 80 control, following the protocol described by Nizio et al. (2018). Mortality observed at the 10 mg L^{-1} concentration was used for progeny differentiation. Initially, a solution of essential oil and Tween 80 was obtained in a 2:1 ratio to enable solubilization of the essential oils in water. Aliquots of this mixture were pipetted and mixed with certain volumes of distilled water in Falcon tubes, according to the intended concentrations of essential oils. The experimental unit consisted of a Petri dish of 5.0-mL capacity containing 10 parasites (in the trophon phase) obtained from infested fish. The parasites were exposed to solutions containing the essential oil, and mortality was assessed after 1 hour of exposure.

Analysis of variance was used on the percentage data for the compounds E-caryophyllene and viridiflorol, and the means were grouped using the Scott Knott test ($p \leq 0.05$). Analysis of variance was used on the *I. multifiliis* mortality data, and the means were compared using Tukey's test ($p \leq 0.05$). The Sisvar statistical software was used. A correlation analysis was carried out between mortality and the percentage of certain compounds present in the essential oils of the progenies tested. The correlation between mortality and the sum of viridiflorol with these compounds was also analyzed to verify probable synergism.

RESULTS AND DISCUSSION

A total of 36 chemical compounds were detected and identified in the essential oils of the progenies and parental *V. curassavica*, including α-pinene, sabinene, β-pinene, β-phellandrene, terpinen-4-ol, *E*-caryophyllene, γ-elemene, α-zingiberene, germacrene D, β-selinene, bicyclogermacrene, δ-amorphene, espatulenol, globulol, caryophyllene oxide, and viridiflorol. Despite the variability of the chemical compositions, the compounds that showed the highest average levels were *E*-caryophyllene (10.71%) and viridiflorol (12.69%). The levels of *E*-caryophyllene (0.00-23.61%) and viridiflorol (0.00-64.74%) exhibited significant variation within the population under study, and *E*-caryophyllene was absent only in the progeny VCUR-503-42. In contrast, approximately 43.18% of the 88 half-sibling progenies analyzed did not contain viridiflorol in their essential oil (Figure 1 and Table 1). The highest level of *E*-caryophyllene was observed in the progeny VCUR-503-06 (23.61%). For viridiflorol, only the progenies VCUR-503-66 (64.74%) and VCUR-503-119 (62.84%) showed levels exceeding 60%.

The high variability in levels of compounds within the population may be related to polyploidization in *V. curassavica*. Polyploidization is the process of chromosomal duplication that can alter phenotypic characteristics in aromatic and medicinal plants (Niazian and Nalousi 2020, Scarrow et al. 2021). One such species is *V. curassavica*, which may exhibit polyploidization mechanisms during crossbreeding, as reported in experiments by Hoeltgebaum et al. (2017). Additionally, it is possible that hexaploid chemotypes may show greater segregations in the early cycles of recurrent selection due to the larger number of gene loci (Frey and Holland 1999, Liu et al. 2007, Batista et al. 2017, Shmeit et al. 2020).

Molecular studies conducted by Brito et al. (2016) validate the existence of wide genetic variation in the *V. curassavica* collection, with polymorphic bands (97.98%). These data corroborate the present study, as they indicate a strong tendency for the emergence of progenies with high diversity. This phenotypic variability can be observed in the offspring of crossbreeding, as chromosomal recombination during gene exchange can facilitate the introduction of new genes responsible for the expression of novel traits in interaction with the environment, which is common in native species (Stower et al. 2012, Wang et al. 2020, Haile et al. 2020, Talebi et al. 2021, Muravnik et al. 2021).

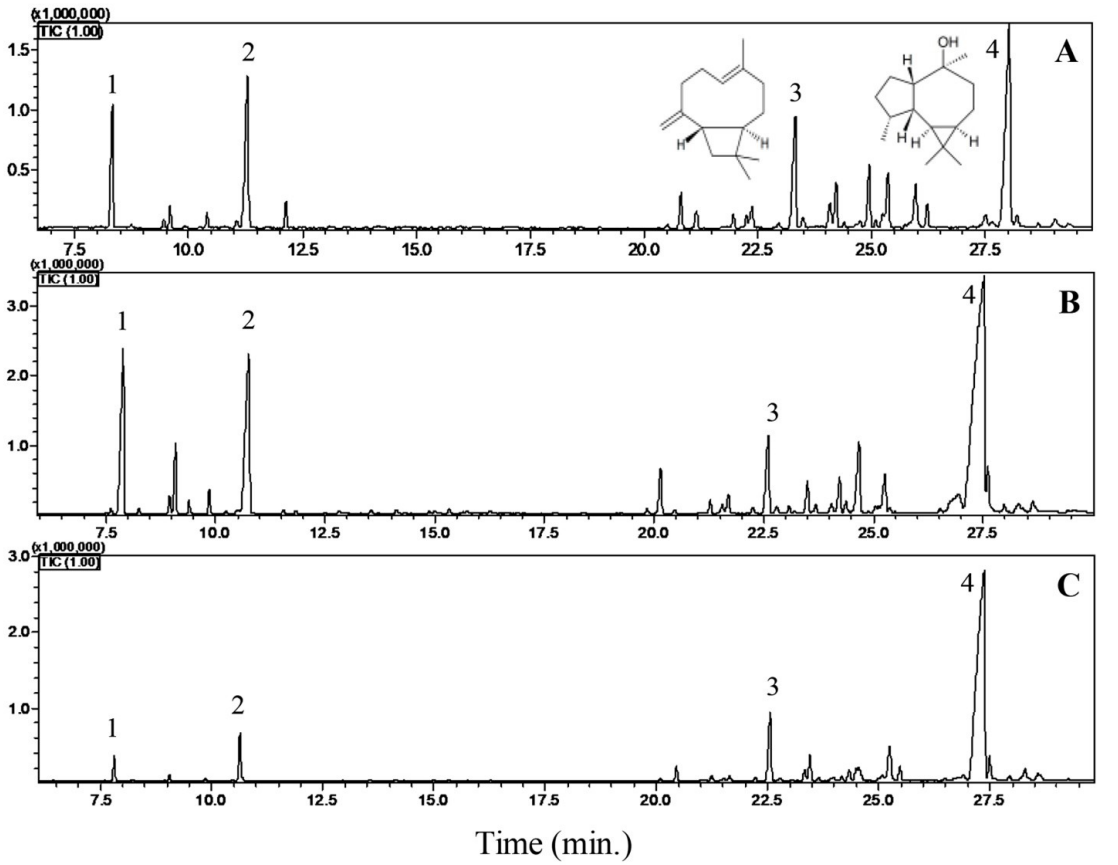

Figure 1. Chromatographic profile of essential oils of *Varronia curassavica*. A: parental accession VCUR-503; B: VCUR-503-101, and C: VCUR-503-119. Major peaks – 1: α-pinene, 2: β-phellandrene, 3: *E*-caryophyllene, and 4: viridiflorol. Structure of the compounds *E*-caryophyllene and viridiflorol.

The variability of chemical compounds in the plant is a direct effect of enzymes involved in secondary metabolism (Durazzini et al. 2019). Additionally, the origin of these constituents is directly related to the major compounds that serve as precursors for the biosynthesis of minor compounds or directly participate in production of minor compounds in the plant, such as *E*-caryophyllene and viridiflorol (Barros et al. 2009, Ramak et al. 2014).

The high levels of major compounds directly affected biological activity against *I. multifiliis* (Table 2). In this study, the high toxicity of the essential oils with high levels of viridiflorol in association with minor compounds such as δ-elemene, *E*-caryophyllene, α-humulene, and bicyclogermacrene was observed, which in synergy may have potentiated the mortality of this protozoan. This result was confirmed by Nizio et al. (2018), who observed increased membrane rupture and leakage of cellular content after exposure of individuals to the *E*-caryophyllene + viridiflorol complex. However, when evaluating the isolated compounds alone, a higher lethal concentration was required, with no mortality observed for *E*-caryophyllene at a concentration of 14.90 mg L^{-1} and only 42.2 ± 22.5 % mortality for viridiflorol at a concentration of 11.4 mg L^{-1}. The effect of synergy from these compounds is evident due to the wide range of mortality of *I. multifiliis*, from 35-72% at the concentration of 10 mg L^{-1} in the essential oils of the progenies.

Furthermore, it can be observed that mortality coincided with the levels of viridiflorol and the sum of viridiflorol + *E*-caryophyllene. The highest mortality was caused by VCUR-503-119, exceeding 70%. A possible explanation for this phenomenon lies in the chemical composition of this progeny; it expressed viridiflorol above 60% in phenotypic evaluation. However, due to the low level of *E*-caryophyllene (7.70%), it is believed that its antiprotozoal potential

Table 1. Content of *E*-caryophyllene and viridiflorol in 88 half-sibling progenies and the parental accession (P) of *Varronia curassavica*

Genotype	***E*-caryophyllene** (%)*		**Viridiflorol** (%)		**Genotype**	***E*-caryophyllene** (%)		**Viridiflorol** (%)	
VCUR-503 (P)	10.81	a16	27.07	a15	VCUR-503-61	8.03	a10	3.02	a8
VCUR-503-01	8.03	a10	53.14	a32	VCUR-503-62	5.03	a5	0.00	a1
VCUR-503-02	5.13	a5	44.64	a24	VCUR-503-65	8.25	a11	0.00	a1
VCUR-503-03	10.44	a15	2.82	a7	VCUR-503-66	3.40	a3	64.74	a35
VCUR-503-04	4.74	a5	0.00	a1	VCUR-503-67	3.53	a3	0.34	a2
VCUR-503-05	20.46	a26	0.00	a1	VCUR-503-69	16.31	a23	0.00	a1
VCUR-503-06	23.61	a30	0.00	a1	VCUR-503-70	15.19	a22	0.00	a1
VCUR-503-07	14.66	a21	0.00	a1	VCUR-503-71	14.64	a21	0.74	a4
VCUR-503-10	6.71	a8	45.29	a25	VCUR-503-72	22.62	a28	0.00	a1
VCUR-503-11	3.68	a3	0.00	a1	VCUR-503-74	6.72	a8	10.40	a12
VCUR-503-12	15.07	a22	0.00	a1	VCUR-503-75	4.84	a5	44.43	a23
VCUR-503-13	8.33	a11	0.45	a3	VCUR-503-77	14.08	a20	0.00	a1
VCUR-503-14	8.66	a12	48.91	a30	VCUR-503-78	9.34	a13	0.00	a1
VCUR-503-15	19.04	a24	0.00	a1	VCUR-503-79	7.12	a9	42.63	a21
VCUR-503-23	19.84	a25	6.27	a10	VCUR-503-81	14.97	a21	36.58	a16
VCUR-503-24	7.78	a10	0.00	a1	VCUR-503-82	15.16	a22	0.00	a1
VCUR-503-25	16.12	a23	0.61	a3	VCUR-503-84	8.67	a12	0.00	a1
VCUR-503-26	14.64	a21	0.75	a4	VCUR-503-86	2.31	a2	0.00	a1
VCUR-503-28	10.02	a15	2.04	a6	VCUR-503-88	7.23	a9	47.39	a29
VCUR-503-30	14.90	a21	38.64	a18	VCUR-503-89	3.59	a3	46.39	a27
VCUR-503-31	19.84	a25	0.73	a4	VCUR-503-90	6.72	a8	0.00	a1
VCUR-503-32	11.61	a17	0.00	a1	VCUR-503-91	11.39	a16	41.80	a19
VCUR-503-34	6.61	a8	58.56	a33	VCUR-503-92	10.66	a15	0.00	a1
VCUR-503-35	19.17	a24	0.00	a1	VCUR-503-93	15.15	a22	0.00	a1
VCUR-503-36	8.73	a12	43.44	a22	VCUR-503-94	7.61	a10	2.94	a7
VCUR-503-38	10.06	a15	0.00	a1	VCUR-503-95	9.55	a14	0.00	a1
VCUR-503-40	8.21	a11	0.00	a1	VCUR-503-97	10.15	a15	0.00	a1
VCUR-503-42	0.00	a1	0.00	a1	VCUR-503-98	11.39	a16	0.57	a3
VCUR-503-43	13.46	a19	0.00	a1	VCUR-503-99	12.05	a17	0.54	a3
VCUR-503-44	6.92	a8	0.62	a3	VCUR-503-101	4.86	a4	43.74	a22
VCUR-503-45	15.44	a22	42.63	a21	VCUR-503-102	22.18	a28	0.00	a1
VCUR-503-46	12.55	a18	14.66	a14	VCUR-503-103	14.69	a21	0.64	a3
VCUR-503-47	4.64	a5	50.29	a31	VCUR-503-104	15.43	a22	0.00	a1
VCUR-503-48	11.16	a16	3.09	a8	VCUR-503-106	3.12	a3	0.00	a1
VCUR-503-49	13.68	a19	0.87	a4	VCUR-503-107	6.79	a8	47.06	a28
VCUR-503-50	16.54	a23	5.08	a9	VCUR-503-108	5.58	a6	1.75	a5
VCUR-503-51	9.68	a14	45.56	a26	VCUR-503-109	5.00	a5	0.00	a1
VCUR-503-52	6.00	a7	0.63	a3	VCUR-503-110	11.70	a17	0.56	a3
VCUR-503-53	10.15	a15	10.92	a13	VCUR-503-111	3.85	a3	0.00	a1
VCUR-503-54	9.03	a13	0.00	a1	VCUR-503-112	3.75	a3	0.00	a1
VCUR-503-55	19.54	a25	0.35	a2	VCUR-503-115	10.31	a15	0.56	a3
VCUR-503-56	14.11	a20	0.55	a3	VCUR-503-116	3.55	a3	37.28	a17
VCUR-503-57	23.11	a29	9.04	a11	VCUR-503-118	21.01	a27	0.00	a1
VCUR-503-58	7.57	a10	0.00	a1	VCUR-503-119	7.72	a10	62.84	a34
VCUR-503-60	7.61	a10	45.13	a25					
Mean	10.72		12.81						
CV (%)	2.65		0.79						

Means followed by the same number followed by the letter "a" in the column do not differ statistically from each other using the Scott-Knott test ($p \leq 0.05$). * Relative percentage (peak area) – Flame Ionization Detector (FID).

originates from synergy with other compounds present in the essential oil. These compounds have radicals attached to their chemical structure that react with components of the cell membrane and destabilize it, leading to its rupture (Dewick et al. 2015).

The analysis of Table 3 revealed that the compound viridiflorol was positively correlated with the mortality of *I. multifiliis*. Furthermore, examination of the synergy between the major compounds showed that all combinations involving viridiflorol were positively correlated with mortality of the protozoan. It is noteworthy that the correlation between viridiflorol together with *E*-caryophyllene and mortality was 0.88, which was higher than the correlations observed for the compounds individually. These results indicate that the presence of viridiflorol, especially in conjunction with *E*-caryophyllene, is associated with a more pronounced mortality effect on *I. multifiliis*.

Table 2. Toxicity of the essential oil from progenies and parent of the breeding program of *Varronia curassavica* against the protozoan *Ichthyophthirius multifiliis* at a concentration of 10 mg L^{-1} after 1 hour of exposure

Genotypes	**Mortality** (%)
VCUR-503-119	72.00 a
VCUR-503-91	52.00 ab
VCUR-503-34	51.67 ab
VCUR-503-66	48.33 ab
VCUR-503-101	35.00 b
VCUR-503	20.00 b
Mean (%)	46.50
CV (%)	26.07

Means followed by the same letters do not differ from each other using Tukey's test ($p \leq 0.05$).

Viridiflorol has a hydroxyl group linked to the carbon chain, which, according to Buonanno et al. (2019), provides a stronger biocidal effect on protozoan cells, targeting polar structures. However, *E*-caryophyllene has a carbon chain without hydroxyl groups. Therefore, it is believed that the compounds act through different mechanisms of action, with E-caryophyllene targeting nonpolar structures in the plasma membrane of *I. multifiliis* (Baranović and Segota 2018, Wang et al. 2018). A study published by Dias et al. (2022) confirmed the biocidal effect of the essential oil of *Psidium cattleianum*, rich in β-caryophyllene and viridiflorol, on foodborne bacteria, supporting the findings of the present study.

Application of the recurrent selection method resulted in improvement of important phenotypic traits in the selection of elite progenies for the second cycle of recombination and selection. The promising progenies with high levels of *E*-caryophyllene and viridiflorol were VCUR-503-30, VCUR-503-66, VCUR-503-75, VCUR-503-79, VCUR-503-101, and VCUR-503-119, as they exhibited chemical characteristics and biological activity of interest to the breeding program. However, breeding programs targeting biological activity for aromatic species is a relatively new field and requires further genetic studies to deepen our understanding.

Table 3. Correlation between the compounds present in the parent (VCUR-503) and progenies (VCUR-503-44, VCUR-503-66, VCUR-503-91, VCUR-503-101, VCUR-503-119) of *V. curassavica* and mortality of *Ichthyophthirius multifiliis*

Correlation between mortality and content		**Correlation between mortality and the sum of contents**	
Tricyclene	0.13	Viridiflorol + tricyclene	0.79
α-pinene	-0.71	Viridiflorol + α-pinene	0.67
β-phellandrene	-0.55	Viridiflorol + β-phellandrene	0.87*
δ-elemene	-0.75	Viridiflorol + δ-elemene	0.79
E-caryophyllene	-0.15	Viridiflorol + *E*-caryophyllene	0.88*
α-humulene	-0.12	Viridiflorol + α-humulene	0.82*
Germacrene D	-0.86	Viridiflorol + germacrene D	0.76
Bicyclogermacrene	-0.65	Viridiflorol + bicyclogermacrene	0.80*
Viridiflorol	0.79		

* Probable synergism.

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