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Article

Mediterranean Blue Flowers with a Scent of Cannabis

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Abstract: The aromatic herb *Plectranthus neochilus* with a cannabis smell, widely used in folk medicine, has become the subject of a study of its essential oils of flowers and leaves. GC-MS analysis revealed a unique composition, the oils of flowers, the main component of which is 2-methyl-1-butene, its concentration exceeds 87 percent. On the one hand, this is very unusual data, although, on the other hand, it is known that 2-methyl-1-butene can be a product of thermal decomposition of 1,1-dimethyl-cyclopropane. However from a microbiological point of view, 2-methyl-1-butene can be a product of bacterial reduction of isoprene by a mixture of *Comamonas* sp. and *Acetobacterium wieringae* bacteria. This issue is extremely interesting and requires further careful study, although a preliminary discussion is given in the discussion of this phenomenon. Analysis of the oil from the leaves showed that the main products are α-thujene and α-pinene, the content of which exceeds 75 percent. According to a detailed analysis of the species Plectranthus, it was found that this species is a producer of volatile metabolites containing cyclopropane and cyclobutane rings. It is assumed that the compounds of essential oils containing cyclopropane and cyclobutane rings may be the main biologically active substances in this species or genus of plants. Data on the biological activity of the main identified compounds of this aromatic plant are presented.

Keywords: *Plectranthus neochilus;* volatiles; cannabis; GC/MS; flowers; leaves

1. Introduction

Plectranthus neochilus is an aromatic herb called "boldo" or "boldogambá", used in folk medicine to treat liver failure and dyspepsia [1]. In South Africa, *P. neochilus* tea is traditionally used for medicinal purposes such as digestive disorders, liver problems, hangovers, and, to a lesser extent, respiratory infections. It is one of the "boldo" species used in different parts of the world because it grows all year round, is adaptable and resilient to the environment, tolerates intense sun, and requires little manipulation [2]. This species is widely distributed in sub-Saharan Africa [3].

The fraction from the aerial parts of the plant has shown strong activity against *Staphylococcus epidermidis* and *Mycobacterium smegmatis*, and the essential oil of *P. neochilus* was effective in inhibiting *Bacillus cereus* [4]. In addition, this plant is known for its distinct and unpleasant skunk odor. Interestingly, researchers from different countries point out that this herbal plant has different smells, apparently this is due to the specific composition of monoterpenes, sesquiterpenes, and diterpenes [5]. According to some studies, *P. neochilus* extracts demonstrated cytotoxic effect in head and neck carcinoma cell lines [6], as well as antioxidant, anti-hyperlipidemic, and antimicrobial activity [7–10].

We were attracted by the unusual smell of cannabis, which emanated from the flowers of this plant growing in Israel, and it was decided to study the essential oil from the leaves and flowers.

2. Materials and Methods

2.1. Plant Material

Flowers and leaves of *Plectranthus neochilus* were used for analyses. The plant material was collected in the center of the city Tel Aviv in April 2019, where is planted as decoration.

2.2. Sample Preparation

A quantity of 10 mg of plant material was used for HS.

2.3. Instrument

GC/MS [Agilent 7890B GC, Agilent 5977B MSD, PAL 3 (RSI 85)]

2.4. Column

Agilent Technologies, Inc., Santa Clara, CA, USA, HP-5MS UI, 30 m × 250 µm, film 0.25 µm.

2.5. Standards

Commercially available standards for α-pinene, camphene, β-pinene, myrcene, Δ^3-carene, α-terpinene, *p*-cymene, limonene, 1,8-cineole, α-ocimene, *trans*-β-ocimene, γ-terpinene, terpinolene, linalool, isopulegol, geraniol, β-caryophyllene, α-humulene, *cis*-nerolidol, *trans*-nerolidol, caryophyllene oxide, guaiol, and α-bisabolol were obtained from Restek (Bellefonte, PA, USA).

2.6. Experimental Conditions for Head Space Analysis

The column temperature was initially 35 °C for 5 min, followed by temperature ramping from 35 to 150 °C at 5 °C/min, then to 250 °C at 15 °C/min (inlet: 250 °C; detector: 280 °C; split ratio 5:1;); gas: Helium (flow rate: 1 mL/min). Incubation time: 6 min; Incubation temperature: 80 °C.

Analytical method validation—selectivity, specificity, accuracy, precision, linearity, range, limit of detection, limit of quantification, ruggedness, and robustness were performed. They are beyond the scope of this manuscript and will be published elsewhere.

2.7. Identification

The content compounds were identified by comparison to standards, retention times, retention indices, and the spectral matching of libraries NIST/EPA/NIH Mass Spectral Library 2017, Wiley Registry of Mass Spectral Data 11th Edition, FFNSC3, ©2015, and Adams Essential Oils Library.

3. Results

The essential oils extracted from the flowers and leaves of *Plectranthus neochilus* have been minimally studied, particularly using the modern technique of chromatography-mass spectrometry. If the leaves of this species have been studied, however long ago and on old gas-liquid equipment, and essential oil of flowers has not been studied at all and is of great interest [11–13].

3.1. Plant Flowers Analysis

A total of 16 volatile components were detected in the essential oil of *P. neochilus* flowers, with the majority of the identified compounds present in concentrations of less than one percent. GC-MS analysis of essential oil of *P. neochilus* flowers showed an unexpected result, the content of two main compounds was 95.34 percent, of which 87.35 percent was 2-methyl-1-butene, 7.99 percent was *cis*-1,2-dimethyl-cyclopropane (see Figure 1 and Table 1). Such an unusual variant required explanations,

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and they were found. It turns out that more than 50 years ago, Flowers and Frey [14,15] studied the thermal behavior of 1,1-dimethyl-cyclopropane, and found that this compound undergoes gas-phase thermal isomerization with the formation of 3-methyl-1-butene, 2-methyl-2-butene, and 2-methyl-1-butene. More than 30 years later, Baldwin and Shukla [16] showed that 1,1-dimethyl-cyclopropane at 420°C undergoes gas-phase thermal isomerization to the same products, but this occurs during gas-liquid chromatography (see Figure 2). So, using these data, we can assume that 87.35 percent was 1,1-dimethyl-cyclopropane (**1**). It turns out then that this is a rare case where the essential oil of a plant consists of more than 95 percent of terpenes containing a cyclopropane ring. As can be seen from the chromatogram (Figure 1), the dominant peak is a single peak (2-methyl-1-butene), which makes up 87.35 percent, which is a rare case in plant analysis. 2-methyl-1-butene has an odor described as sweet, wine-like, penetrating, musty, disagreeable, and suffocating. Such an unusual combination of mono-, sesquiterpenes, and diterpenes in the essential oil of the flowers and leaves of *P. neochilus* creates such a specific smell.

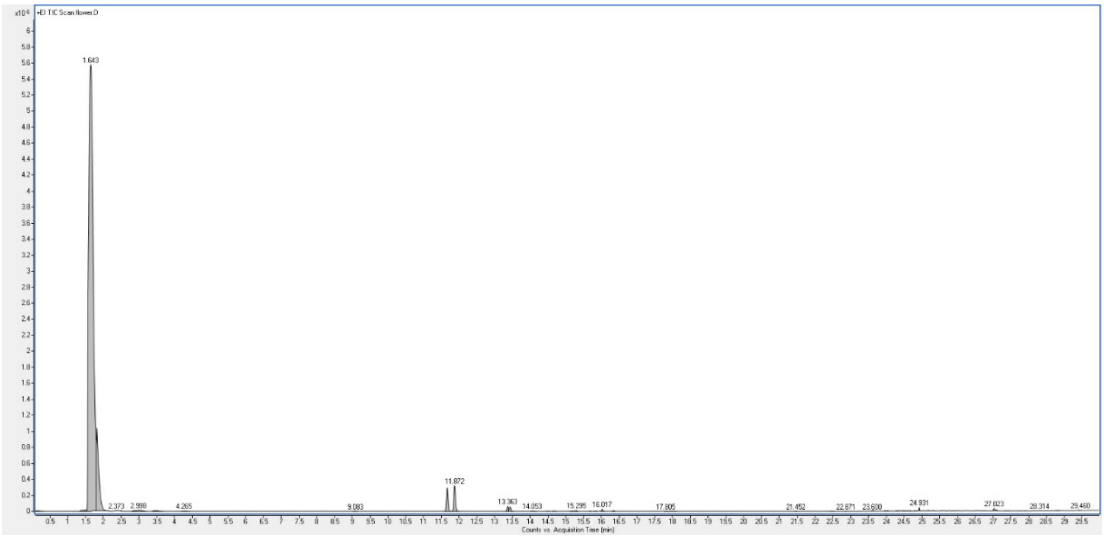

Figure 1. GC-MS chromatogram of the analysis of essential oil of *P. neochilus* flowers.

Table 1. *P. neochilus* plant flowers - relative areas of identified compounds to the main peak (2-methyl-1-butene area = 100 %) on chromatogram and quantitative content of some compounds from calibration curves of their standards.

Peak	RT	%	Compound	RI
1	1.643	87.35	2-methyl-1-butene	488
2	1.820	7.99	cis-1,2-dimethyl-cyclopropane	516
3	2.373	0.10	2-methyl-3-buten-2-ol	614
4	2.870	0.07	3-methyl-butanal	652
5	2.998	0.21	2-methyl-butanal	662
6	11.664	1.58	alpha-thujene	931
7	11.872	1.77	alpha-pinene	937
8	13.363	0.29	sabinene	976
9	13.428	0.29	beta-pinene	980
10	14.654	0.01	delta-3-carene	1011
11	15.167	0.02	p-cymene	1022
12	15.295	0.04	limonene	1030
13	15.680	0.01	cis-beta-ocimene	1035

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14	16.017	0.12	trans-beta-ocimene	1037
15	16.337	0.03	gamma-terpinene	1060
16	24.931	0.12	caryophyllene	1419

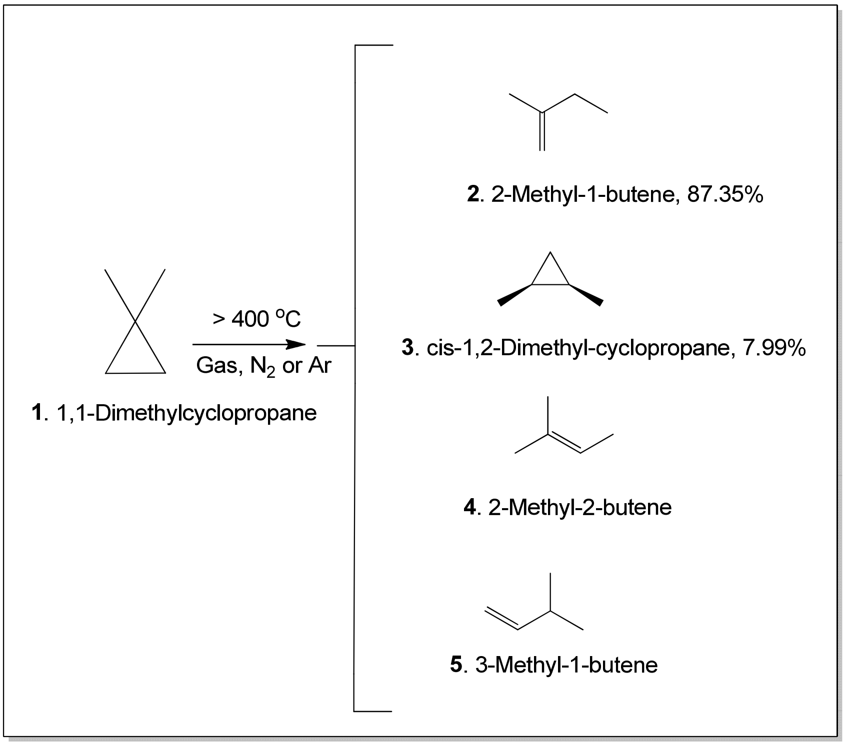

Figure 2. During gas-liquid chromatography, thermal isomerization of 1,1-dimethyl-cyclopropane (**1**) into several products occurs in the gas phase, the main one being 2-methyl-1-butene (**2**), and *cis*-1,2-dimethyl-cyclopropane (**3**). Volatile 2-Methyl-2-butene (**4**) and 3-Methyl-1-butene (**5**) were not detected, although their oxidized products, 2-methyl-butanal, and 3-methyl-butanal, were found.

3.2. Products of Bacterial Action

The high abundance of 2-methyl-1-butene (**2**) can probably be explained from a microbiological point of view. It is known that isoprene is the most abundant biogenic volatile organic compound in the Earth's atmosphere and plays an important role in atmospheric chemistry. Despite this, little is known about the microbiological processes that serve as terrestrial sinks for isoprene. However, aerobic bacteria that degrade isoprene have been identified [16–20]. Thus, many aerobic microorganisms that degrade isoprene have been isolated from soil and leaves of isoprene-producing trees (e.g., poplar, willow), including strains of *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Gordonia*, *Klebsiella*, *Leifsonia*, *Loktanella*, *Micrococcus*, *Methylobacterium*, *Mycobacterium*, *Nocardia*, *Nocardioides*, *Pseudomonas*, *Ramlibacter*, *Sphingopyxis*, *Sphingobacterium*, *Sphingobium*, *Shinella*, *Stappia*, *Pantoea*, *Rhodococcus*, and *Variovorax*, indicating that a diversity of aerobic bacteria contributes to isoprene turnover [21].

Recently, an *Acetobacterium* dominated acetogenic mixed culture was reported that could reduce isoprene to a mixture of three methylbutene isomers (i.e., 2-methyl-1-butene (**2**), 2-methyl-2-butene (**4**), and 3-methyl-1-butene (**5**), in the presence of hydrogen and bicarbonate [22,23]. Physiological studies showed that the *Acetobacterium* population in this mixed culture utilized isoprene as an electron acceptor to conserve energy under anoxic conditions. Axenic *Acetobacterium* cultures such as *A. woodii* DSM 1030, *A. malicum* DSM 4132, and *A. wieringae* DSM 1911 did not utilize isoprene, suggesting that the ability to metabolize isoprene is not a common trait among *Acetobacterium* spp. [23,24].

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A new strain of *Acetobacterium wieringae*, designated strain Y, demonstrated that strain Y was able to reduce isoprene to *three* methylbutene isomers (2-methyl-1-butene, >97%), 3-methyl-1-butene, < 2%), and 2-methyl-2-butene, < 1%) during growth with H_2 and CO_2/HCO_3^- [25]. In the presence of isoprene, 40% less acetate was formed, suggesting that isoprene reduction is associated with energy conservation in *Acetobacterium* spp. (the bacteria in this enrichment culture have recently been identified as *Comamonas* sp. and *A. wieringae*, named MAG ISORED-1 and ISORED-2, respectively [24]). In comparison, strain *Acetobacterium wieringaes* DSM1911 was unable to reduce isoprene [26], whereas an enriched culture containing *Acetobacterium* obtained from a wastewater treatment plant in Sydney, Australia, could readily biotransform isoprene [22]. Although not all *Acetobacterium* spp. can biotransform isoprene under anoxic conditions, *Acetobacterium wieringaes* strains from different geographic locations possess this ability, suggesting that *Acetobacterium* strains containing the gene reductase(s) play a role in the global isoprene cycle [18]. It cannot be excluded that other microorganisms are also involved in isoprene reduction in enriched cultures. For example, it has been suggested that members of Comamonadaceae reduce isoprene [27,28]. Therefore, further studies are needed to investigate the diversity of microorganisms capable of biotransforming isoprene under anaerobic oxygen conditions.

Thus, the high content of 2-methyl-1-butene (>87%) can be explained by the presence of *Acetobacterium wieringae* or other active strains of *Acetobacterium* spp. in *P. neochilus* flowers, which reduced isoprene to three isomers of methylbutene: 2-methyl-1-butene, 3-methyl-1-butene, and 2-methyl-2-butene. Further studies are required to clarify this phenomenon.

3.3. Plant Leaves Analysis

Essential oils of the leaves contained 18 volatile components, with the main compounds being substances containing cyclopropane and cyclobutane rings in their structure. GC-MS analysis of *P. neochilus* leaf essential oil showed that the major components were α-pinene (42.82%), α-thujene (31.9%), and β-pinene (7.29%). The chromatogram (see Figure 3) of the essential oil of the leaves showed that it was dominated by three main compounds, with other volatile compounds making up minor amounts. The analysis data of leaf essential oil are shown in Table 2.

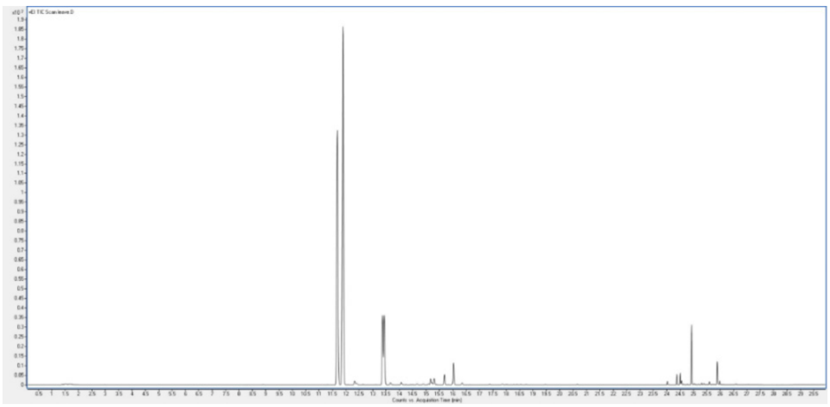

Figure 3. Chromatogram of essential oil from *P. neochilus* leaves.

Table 2. *P. neochilus* plant leaves - relative areas of identified compounds to the main peak (α-pinene area = 100 %) on chromatogram.

Peak	RT	%	Compound	RI
1	11.68	31.90	α-thujene	931
2	11.888	42.82	α-pinene	937
3	12.329	0.44	dehydrosabinene	947
4	13.371	6.91	sabinene	976

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5	13.436	7.29	β-pinene	980
6	14.069	0.27	β-myrcene	991
7	15.167	0.59	*p*-cymene	1022
8	15.303	0.64	limonene	1030
9	15.688	0.90	*cis*-β-ocimene	1040
10	16.025	2.12	*trans*-β-ocimene	1050
11	16.346	0.21	γ-terpinene	1060
12	24.017	0.21	α-cubebene	1351
13	24.378	0.55	copaene	1376
14	24.506	0.61	β-bourbonene	1384
15	24.554	0.26	β-copaene	1418
16	24.931	3.04	caryophyllene	1419
17	25.885	1.29	aromadendrene	1440
18	25.981	0.01	β-cadinene	1520

4. Discussion

The essential oils of *Plectranthus neochilus* flowers and leaves are dangerous, and their consumption can cause nausea, vomiting, and diarrhea in humans. Cats and dogs face similar risks, showing symptoms such as gastrointestinal upset. Contact with or ingestion of the essential oil can cause headaches, dizziness, or skin irritation due to its toxicity [29,30]. It is known that non-contact toxicity is determined by the volatile components of essential oils. As a rule, volatile components are low-molecular substances containing derivatives of sulfur, nitrogen, or phosphorus, although light hydrocarbons with unusual architecture containing cyclic fragments can be toxic [31–34].

We tried to explain this unusual result in the analysis of volatile compounds from flowers in terms of thermal isomerization in GC-MS analysis or a bacterial version. And this version seems to us the most realistic. The eternal question that arises in a person when he smells. Why are odors concentrated in flowers? This is part of the strategy that helps flowering plants reproduce and spread their species. Certain odors help these flowers solve a big problem. Flowers that need the help of insects, bats, and birds go one step further, producing a floral scent that acts as a stinky greeting to the right pollinator. Like perfumes, floral scents are made up of a large and varied number of volatile chemicals that easily evaporate and become airborne. The type of chemical, its amount, and its interaction with other chemicals give a flower its unique scent. The scents or aromas of flowers can be sweet and fruity, or they can be musky, even foul or putrid depending on the pollinator they are trying to attract [35–37].

Blooming apple or cherry trees are known to emit a sweet aroma that attracts bumblebees, honeybees, and other bees, while the beautiful flowers of the pear tree, a close relative of apples and cherries, smell musky or rotten, attracting flies as pollinators. The corpse flower, native to the Indonesian rainforest, emits a foul odor reminiscent of rotting flesh to attract flies and beetles to pollinate its flowers [38,39].

Apparently, in our case, the combination of terpenes isolated from *P. neochilus* flowers resembles the smell of cannabis (see chemical composition in Table 1). Volatiles associated with off-flavours and odours found in *P. neochilus* flowers have also been detected in other plant and animal samples. For example, off-flavours in tuna oil were successfully identified using supercritical carbon dioxide extraction. GC-MS was used to identify the main volatiles contributing to off-flavours and odours, which included 2-methyl-2-butene (27.7%), 1,1-dimethyl-cyclopropane (2.2%), cyclopropane, 1,1,dimethyl-2-allylcyclopropane (6.2%), and octadiene [40]. GC-MS analysis of volatile components of canned sockeye and pink salmon showed that they contained 2-methyl-1-butene (2%) [41].

Volatile compounds in Romanian wines are an under-researched area. In their study, 2-methyl-1-butene (around 3%) was found in the wines "*Tămâioasă românească*" and "*Busuioacă de Bohotin*" from the Pietroasa area in Dealu Mare, vintage 2008 [42].

Thiol derivatives of 2-methyl-1-butene, 3-methyl-1-butene, and 2-methyl-2-butene in roasted sesame seeds. Interestingly, active thiols with sulfurous, meaty, and/or catty odors similar to blackcurrant were identified in roasted sesame seeds. Among them, 2-methyl-1-propene-1-thiol, (Z)-3-methyl-1-butene-1-thiol, (E)-3-methyl-1-butene-1-thiol, (Z)-2-methyl-1-butene-1-thiol, (E)-2-methyl-1-butene-1-thiol, and 4-mercapto-3-hexanone were not previously known as food components. Their structures were confirmed by comparing their mass spectra and retention indices as well as their sensory properties with those of the synthesized reference compounds. Relatively unstable 1-alkene-1-thiols represent a new class of food flavorings and have been proposed as key contributors to the characteristic but fleeting aroma of freshly ground roasted sesame seeds [43,44].

Three methylbutenes, 2-methyl-2-butene, as well as two other isomers of this compound (2-methyl-1-butene and 3-methyl-1-butene) inhibit the germination of spores of the pathogenic Mucorale *Rhizopus arrhizus* NCCPF 710004, although to a lesser extent than 2-methyl-2-butene [45].

Analyzing the composition of terpenes of flowers and leaves, we conclude that *P. neochilus* is a powerful producer of compounds cyclopropane and cyclobutane rings. Figure 4 shows the most common terpenes containing cyclopropane and cyclobutane rings produced by *P. neochilus* growing in different countries and on different continents and in different climatic conditions [46,47]. Environmental conditions, mineral nutrition, and other factors can determine the composition of essential oils in flowers and leaves [48,49].

However, the most important question is who synthesizes cyclopropane and cyclobutane metabolites in the genus *Plectranthus*? It is known that the endophytic bacterium with probiotic properties, *Rhodococcus globerulus* is an endosymbiont of *Plectranthus amboinicus* [50,51], and colonies of endophytic bacteria such as *Bacillus* sp., *Bacillus megaterium, Bacillus pumilus, Bacillus licheniformis, Micrococcus luteus, Paenibacillus* sp., *Pseudomonas* sp., and *Acinetobacter calcoaceticus* have been isolated from *Plectranthus tenuiflorus* [52]. Fungal endophytes *Chaetomium subglobosum, Alternaria alternata,* and *Fusarium oxysporum* were found in *Plectranthus amboinicus* [53].

No reliable data on *endo*-bacteria or fungal endophytes isolated from *P. neochilus* have been found, and the question of the true producers of the bioactive terpenes found in this species remains open. It is known that *endo*-bacteria or fungal endophytes make a significant contribution to the biosynthesis of many biologically active metabolites. This applies to both lichens, consisting of two fungal symbionts, or microflora and cyanobacteria, and to plants containing colonies of *endo*-bacteria or fungal endophytes [54–56].

Cyclopropane metabolites are quite widespread, and their main producers are bacteria and/or fungal endophytes [57–59]. This topic was widely discussed in literature in the late 90s and is still relevant today [60,61]. Metabolites containing the cyclopropane ring exhibit a wide range of biological activities, including antibacterial, antimicrobial, and anticancer activities [62,63]. Natural metabolites containing the cyclobutane fragment represent unique and interesting architectural molecular assemblies, and their formation largely depends on the action of UV radiation [64,65]. Analysis of the biological activity of compounds containing the cyclobutane group demonstrates a wide range of activities, including anticancer properties [66–68].

Interesting is the activity of the dominant compounds in both flowers and leaves. Volatile 2-methyl-2-butene is the main component in the essential oils of the flowers, which is detected by GC-MS analysis. In this study, we cannot say for sure whether this compound is a metabolic product of the plant or whether it is a degradation product under the conditions of GC-MS analysis. Thus, Kaur and Singh [45] showed that 2-methyl-2-butene is a potent inhibitor and significantly impaired the emergence and elongation of the fungal germ tube during germination of the pathogenic Mucorale *Rhizopus arrhizus* (family Mucoraceae). This is a very interesting function of endogenous 2-methyl-2-butene.

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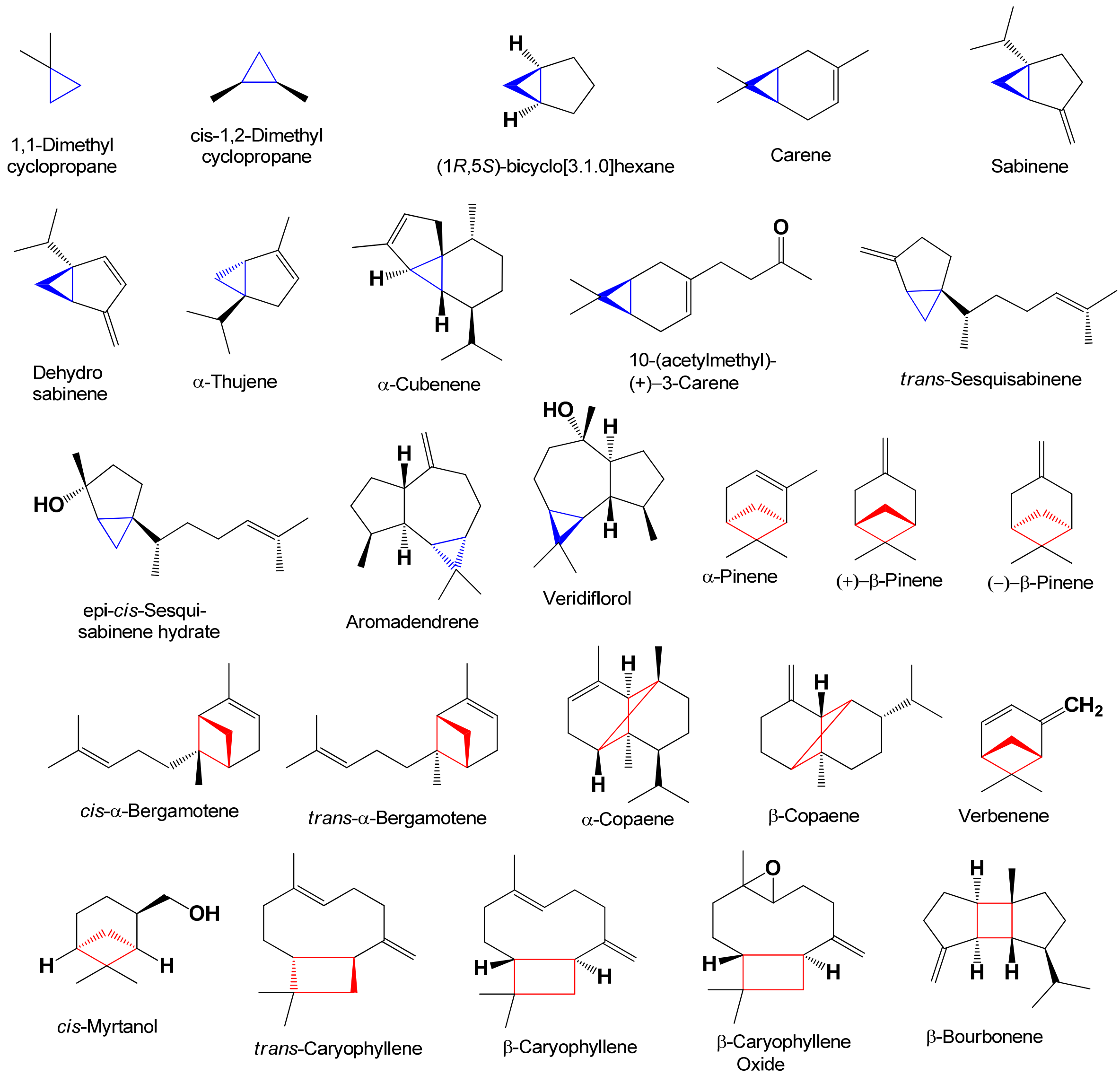

Figure 4. The most common terpenes containing cyclopropane and cyclobutane rings are produced by *P. neochilus* growing in various countries and on different continents. Environmental conditions, nutrition and other factors can determine the composition of the essential oil of flowers and leaves.

The other two dominant compounds found in the leaves were α-pinene (42%) and α-thujene (32%). α-Pinene has been used to treat respiratory tract infections for centuries. Furthermore, it plays a crucial role in the fragrance and flavor industry. In vitro assays have shown an enantioselective profile of (+)- and (−)-α-pinene for antibacterial and insecticidal activity, respectively [69]. Previous studies have shown that α-pinene, a common volatile plant metabolite, may exert anti-inflammatory effects on human chondrocytes, thus demonstrating potential anti-osteoarthritic activity. Recent data suggest that α-pinene has isomer- and enantiomer-selective anti-inflammatory and anti-catabolic effects in human chondrocytes, with (+)-α-pinene being the most promising candidate for further studies to determine its potential value as an anti-osteoarthritic drug [70].

However, the oil from *Cistus libanotis* (*Cistus clusii*), is used in many industries, mainly in perfumery and, more recently, as a raw material for food additives. The volatile fraction of fresh plant material of *C. libanotis* was investigated by headspace solid-phase microextraction coupled with GC-MS. The analysis revealed that sabinene (25.3%) and α-thujene (23.8%) as the main components showed significant cytotoxic effect against DLD-1 and CAPAN-1 cell lines, while it was less active against healthy L929 cell line. Thus, it can be speculated that two metabolites with cyclopropane rings, sabinene, and α-thujene, may exhibit cytotoxic effects against DLD-1 (colorectal adenocarcinoma) and CAPAN-1 (human pancreatic ductal adenocarcinoma) cell lines [71].

5. Conclusion

Thus, we studied the composition of essential oils of flowers and leaves with Cannabis scent, an aromatic herb *Plectranthus neochilus*, which is widely used in folk medicine. GC-MS analysis showed that the main component of the flower oil is 2-methyl-1-butene, its concentration exceeded 87 percent. On the one hand, we found in the literature that 2-methyl-1-butene may be a product of the thermal decomposition of 1,1-dimethylcyclopropane, while on the other hand 2-methyl-1-butene may be a product of the activity of bacteria or other microorganisms. This issue is extremely interesting and requires further study. Analysis of the leaf oil showed that the main products are α-thujene and α-pinene, the content of which exceeds 75 percent. Data on the biological activity of the dominant compounds are given.

Author Contributions: Conceptualization, L.O.H., and V.M.D.; methodology, L.O.H., L.N.M., N.S., A.O.T., and V.M.D.; software, L.N.M., N.S., A.O.T.; investigation, L.O.H., and L.N.M.; resources, L.O.H., N.S., and V.M.D.; writing—original draft preparation, L.O.H., N.S., and V.M.D.; writing—review and editing, L.O.H., L.N.M., N.S., A.O.T., and V.M.D. All authors have read and agreed to the published version of the manuscript.

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