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GC-MS/MS and 1H-NMR Analysis of Endemic *Campanula baskilensis Behçet (Campanulaceae) Leaf Fractions*

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Abstract

In this research, 1H-NMR and CG-MS/MS spectrophotometric techniques were used to elucidate the chemical profile of leaf fractions of Campanula baskilensis Behçet (Campanulaceae). A column chromatography system was used for the fractionation of the crude extract. Fifteen separate fractions (Fr1-15) were obtained throughout the fractionation procedure. Thirty-nine molecules were identified using both spectrometry techniques. Thirty-one compounds of these molecules were identified using GC-MS/MS and ^{1}H-NMR techniques, and eight compounds were listed as unknown. The highest quantity as a percentage recorded was by lupeyl acetate as following 77.80% in Fr6-2, 63.21% in Fr3-3, 43.81% in Fr4-3. 1-octadecene was determined as 36.23% in Fr3-2 and 32.14% in Fr4-1. Additionally, levels of 34.88% for hexadecanoic acid and 34.05% for borneol were recorded at Fr8. It is also noteworthy that Fr3-4 is formed at a percentage of 97.28 of unknown molecule. Molecular structures such as hexadecanoic acid (methyl palmitate), benzoic acid, eicosanoic acid, limonene, borneol, and hentriacontanol were supported by 1H-NMR analysis. Understanding the chemical constituents of C. baskilensis plant will provide opportunities to suggest broader research in the future. The identified molecules can be the subject of isolation and further original models for various in-vitro and in-silico bioassays or effective drug development and application.

Key Words: *C. baskilensis*, GC-MS/MS, ^{1}H-NMR, Fractionation

1. Introduction

The endemic *Campanula* species are found in limited regions with rocky terrain and edaphic and microclimatic condition (Yildirim et al., 2019). A wide range of chemical compounds has been identified in *Campanula* species, including flavonoids, phenolics, anthocyanins, polyacetylenes, phenylpropanoids, essential oils, acylated triterpenoids, glycosides, resins, and coumarins. Additionally, a variety of their subunit compounds are present, including fraxin, linalool, α-terpineol, lavandulyl acetate, (E, E)-allo-ocimene, β-pinene, α-cadinene, β-farnesene, β-caryophyllene, and myo-inosito (Anestis et al., 2023; Mohammed et al., 2023; Brandt et al., 2017; Kim et al., 2017). Chemical compounds synthesized and

stored in various parts of the plants have essential functions and possess high biological properties that add to the plant's vitality. Biologically active chemical substances found naturally in plants are called phytochemicals. Several previous studies have emphasized that the chemical components of plants performed extraordinary economic and medical importance (Newman and Cragg, 2016). Phenolics are secondary metabolite products of plants and the most common phytochemical category found in plants, and they also play an important role in the reproduction, growth, and metabolism of plants. They are also responsible for defense mechanisms against pathological viruses and fungal infections, parasites, and predators and contribute to the color of plants. Scattered over Iran-Turan, the Eastern Mediterranean, and Mediterranean phytogeographical areas, *Campanula*, one of the most prominent genera in the Campanulaceae family, it is represented by about 115 species in Türkiye. Several investigations aimed to identify the volatile components of the aerial parts of Campanula species and some of their morphology and phenological aspects. The importance of *Campanula* taxa, which are edible and decorative plants, has been uncovered via expanded research in addition to its medical advantages (Sarıkaya and Kavaklı, 2020). According to the results of epidemiological studies, increasing the consumption of plant-based foods, fruits, and vegetables is extremely important in preventing chronic diseases such as cancer, diabetes, cardiovascular diseases, Alzheimer's disease, and age-related functional decline. Fruits and vegetables contain phytochemicals, such as active phenolic compounds, which act as natural antioxidants (Wen et al., 2015; Liu, 2013; Eberhardt et al., 2000; Sun et al., 2002). The past few decades have seen an increase in passion for investigating secondary metabolites that promote health, such as phenol and carotenoid compounds, from many perspectives. The essential components of *Campanula* species and several secondary metabolism products were quantitatively measured using chemotaxonomic analysis techniques. As several earlier investigations have confirmed, aerial parts of the plant, particularly the leaves, are also thought to be an abundant source of such chemicals (Politeo et al., 2013).

This work aims to characterize polyphenolic and volatile compounds quantitatively for leaf fractions of the newly discovered endemic *C. baskilensis* Behcet. We used chemotaxonomic methods with the application of NMR techniques for the fractions that showed high purity and GC-MS/MS compared to more than 30 volatile and widely researched compounds. Access to the chemical content of this plant may enable us to form a comprehensive idea of the potentially bioactive compounds present in the other species within the same environment, especially since these plants are endemic to Türkiye in an environment that is difficult for other species to live in.

2. Material and Methods

2.1. Plant material and chemicals

A regionally localized and widespread species *C. baskilensis* Behçet is reported to exist over the Baskil (Elazığ) area in Türkiye 's Eastern Anatolia region. It was first collected by Prof. Dr. Lütfi Behçet, Bingol University's Faculty of Arts, Department of Biology (Behçet and İlçim, 2018).

2.2. Fractionation

Fractionation processes using solvents with different polarities, from lowest to highest polarity, were applied to obtain separate fractions that contain a single or few compounds that carry the same physical properties as the extract. Also, in order to reach pure compounds, it is necessary to go through this step, which is considered somewhat preliminary to obtain individual compounds after performing the second

fractionation process, second fractions containing a group of compounds that may be close in polarity or size. Firstly, the triplet extraction process was applied to the *C. baskilensis* leaves (251 grams) using a solution mixture of methanol-chloroform 1:1. Once the 32 g of crude extract was obtained, it was applied to the impregnated silica column (100 grams of silica gel (silica 60) was prepared for chromatographic processes by eluting it with hexane) with hexane. Additionally, based on the increasing polarity of the elution, methanol 6.6, ethyl acetate 4.4, and chloroform 2.7 solvents were utilized in that order. Nine significant fractions were obtained from this rudimentary fractionation (Basar et al., 2023). Then, based on the results of thin-layer chromatography, the second stage of fractionation was applied for more accurate separation using the Sephadex (LH-20) column to reach isolated compounds, using mixed solvent systems (methanol, chloroform, ethyl acetate, and hexane) as shown in Figure 1.

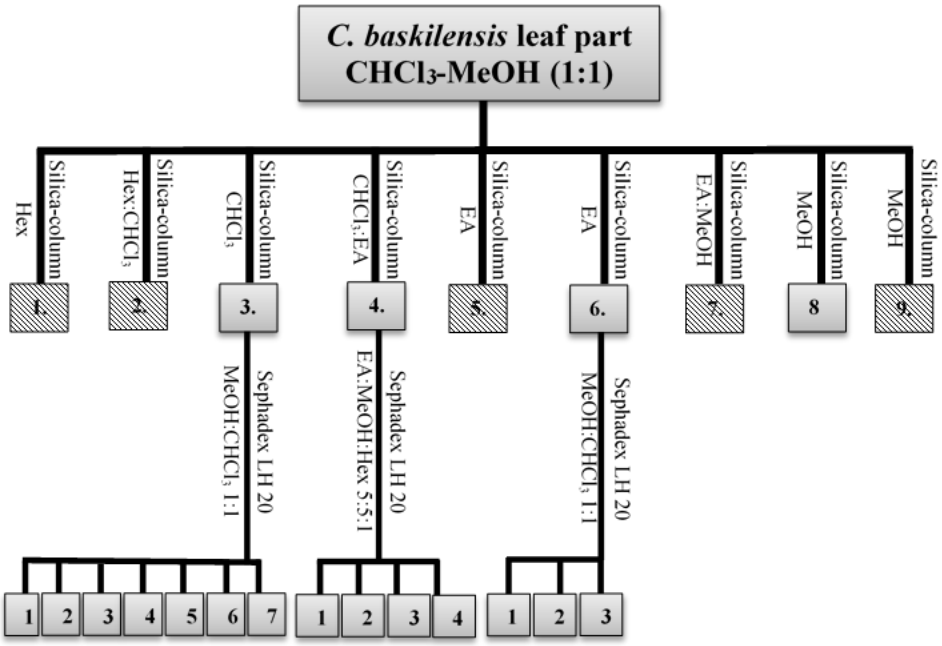

Figure 1. Chemical characterization fractionation scheme; methanol-chloroform (MeOH:CHCl3), hexane (Hex) and ethyl acetate (EA)

Comparing the thin layer chromatography findings for each sub-fraction allowed the combination of the similar ones. Fractions with similar content were combined by applying thin-layer chromatography (TLC), and nine sub-fractions were obtained. A 60-40 ethyl acetate-hexane solvent system was used for TLC. Anisaldehyde was used as the TLC reagent. Subfractions of each fraction were combined with similar ones by TLC. These re-obtained fractions were subjected to chromatography with Sephadex in a solvent system using only methanol (Ipek et al., 2017). These applications allowed us to get fifteen highly pure fractions that could be subjected to characterization methods Figure 1.

2.3. GC-MS/MS analysis

All applied samples underwent esterification to determine the chemical constituents of the *C. baskilensis* fractions' leaf part. Samples at the ratio of 1:10 were mixed with hexane for the esterification procedure. 5 mL of 1M KOH (dissolved in MeOH) was then added, and the mixture was forcefully agitated for 30 seconds using a vortex device. The outcome

of the reaction indicated that 1 mL of the upper phases, which contained fatty acid methyl esters that had formed in the mixtures (the hexane phase), should be taken and filtered into vials using a syringe to pass through a 0.45-micron filter. The mixture's constituents were then subjected to a phytochemical analysis using GC-MS. Analyses were performed using a mass detector model Agilent 5975C with the triple-axis detector and Agilent Technologies Brand 7890A model GC-MS equipment. The following analytical parameters and GC temperature programming were used in GC analyses of fractions: the initial temperature was set to 100°C for 10 minutes, then a constant 20°C/minute to 180°C for 15 minutes, followed by a constant 20°C/minute to 300°C for 30 minutes. The ion temperature of the MS detector is 280°C. Agilent J&W HP-5ms Ultra Inert on GC column (5%-phenyl-methylpolysiloxane) specifically tested for inertness in the analysis of active compounds (30m X 320 µm X 0.25 µm) has been carried out (Yenigun et al., 2024; Ozen et al., 2017).

2.4. ^{1}H-NMR analysis

The Agilent-Premium Compact 14.1 Tesla 600 MHz Frequency NMR equipment was utilized to identify the structures of the fractions' molecules. The amount of protons in a molecule and how each proton interacts with its neighbor protons may be seen in the ^{1}H-NMR spectrum (Ipek et al., 2017; Ozen et al., 2017; Yenigun et al., 2023; Yenigun et al., 2024).

3. Results and Discussion

Identifying the chemical composition of the fractions' contents and/or isolating molecules, as well as determining the chemical structures using appropriate analytical processes, paves the way for future studies regarding the biological effectiveness of our species. Here in our study, all *C. baskilensis* leaf` fractions and sub-fractions were analyzed using GC-MS/MS and ^{1}H-NMR analysis. The main reason for performing a second fractionation was to obtain separate molecules whose composition is easy to determine using the analysis as mentioned earlier methods. Based on the results of TLC, we selected the Fr8 from the initial fractionation and the remaining fractions from the second fractionation by merging similar fractions after the second stage.

3.1. GC-MS/MS analysis

As seen in Table 1 and Figure 2, GC-MS/MS data for the volatile components for all of the fractions indicates the existence of approximately forty compounds, most of which could be identified. The compounds that were most prevalent among the fractions were as follows: borneol, 1-hexadecene, and hexadecanoic acid where these compounds determined in eleven fractions, followed by tetradecanoic acid, 1-octadecene and 1-octadecanol where this compounds determined in ten fractions. Another remarkable finding was the high level of lupeyl acetate detected in multiple fractions: Fr6-2 at 77.80%, Fr3-3 at 63.21%, and Fr4-3 at 43.81%, followed by 1-octadecene at 36.23% for Fr3-2 fraction and 32.14% for Fr4-1 fraction. In addition, Fr8 recorded high levels of hexadecanoic acid at 34.88% and borneol at 34.05%. It is also striking that Fr3-4 has a large proportion of unknown molecules as 97.28% in addition to borneol as 2.72% . As for the rest of the compounds, they were less prevalent, lower than 30% in all remain fractions. By comparing the results obtained with the results of previous studies for different Campanula species in the literature, we note that these species contain a wide chemical diversity: In a previous study, 53 prominent compounds were identified as the volatile components of C. portenschlagiana detected by GC-MS/MS; such as Linalool, Nonanal, α-terpineol, Pentadecane, and Caryophyllene oxide (Politeo et al., 2013).

Table 1. The volatile composition (%) of the fractions obtained by GC-MS/MS

Compounds	RT (min.)	3-1	3-2	3-3	3-4	3-5	3-6	3-7	4-1	4-2	4-3	4-4	6-1	6-2	6-3	8
Limonene	12.28	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	4.26	nd	29.81	nd
Benzoic acid	14.67	nd	nd	nd	nd	nd	3.64	nd	nd	nd	nd	nd	nd	nd	nd	nd
Borneol	17.21	nd	nd	nd	2.72	2.18	nd	5.60	16.06	3.42	1.35	1.07	8.82	1.02	15.00	34.05
2-tert-Butyl-4-isopropyl-5-methylphenol	27.71	nd	nd	nd	nd	nd	2.63	8.33	3.02	3.15	1.48	nd	nd	nd	nd	nd
Isoaromadendrene epoxide	29.59	nd	nd	nd	nd	nd	nd	nd	nd	1.26	nd	nd	nd	nd	nd	nd
Tetradecanoic acid	30.89	1.87	0.98	nd	nd	nd	1.91	3.66	5.17	13.69	7.03	4.37	3.38	nd	8.49	nd
Unknown	31.57	nd	nd	nd	nd	2.04	nd	nd	4.07	nd	1.92	nd	19.33	nd	nd	nd
1-Hexadecene	31.74	1.18	22.40	2.23	nd	9.71	1.85	16.68	6.39	5.89	7.28	3.70	8.47	nd	nd	nd
Pentadecanoic acid	32.08	0.20	nd	nd	nd	nd	nd	nd	4.44	nd	nd	4.48	nd	nd	nd	nd
2-Pentadecanone, 6,10,14-trimethyl-	33.19	1.22	0.36	nd	nd	4.95	nd	nd	nd	1.76	2.67	nd	15.99	nd	nd	nd
Hexadecanoic acid	33.16	11.97	3.59	2.97	nd	nd	14.10	13.53	13.50	18.46	10.32	16.85	nd	nd	8.69	34.88
1-Octadecene	33.90	nd	36.23	7.02	nd	6.88	13.57	23.16	32.14	6.61	6.91	12.33	nd	nd	9.67	nd
9-Octadecenoic acid	34.91	nd	nd	nd	nd	nd	nd	2.51	nd	nd	8.02	nd	nd	nd	nd	6.89
Octadecanoic acid	35.16	8.16	1.30	nd	nd	nd	nd	2.28	nd	6.30	nd	3.69	nd	nd	nd	8.21
1-Octadecanol	37.05	1.08	nd	nd	nd	1.75	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Eicosanoic acid	37.70	24.30	nd	nd	nd	2.14	7.49	nd	nd	1.75	1.54	4.50	9.88	nd	nd	8.13
Unknown	37.92	nd	nd	nd	nd	nd	nd	1.84	nd	nd	nd	nd	nd	nd	nd	nd
1-Octadecanol	38.69	1.03	14.83	1.99	nd	1.68	6.43	4.88	9.44	1.48	2.16	nd	nd	nd	nd	nd
Heneicosanoic acid	39.27	0.89	nd	nd	nd	11.64	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Stigmast-5-en-3-ol	39.80	nd	nd	nd	nd	nd	21.88	nd	nd	nd	nd	nd	nd	nd	nd	nd
Unknown	40.20	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.12	8.77	nd	nd	nd
1-Tricosanol	40.65	29.04	nd	nd	nd	nd	2.55	5.77	nd	nd	nd	nd	nd	nd	nd	nd
Docosanoic acid	41.44	nd	nd	nd	nd	nd	2.28	nd	nd	nd	nd	2.30	nd	10.16	nd	nd
Viminalol	41.76	nd	nd	10.77	nd	12.68	nd	1.87	nd	nd	3.81	nd	nd	nd	nd	nd
Unknown	42.40	nd	nd	nd	nd	3.01	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
1-Pentacosanol	43.18	1.28	7.44	nd	nd	nd	3.00	1.78	5.78	nd	nd	nd	nd	nd	nd	nd
Unknown	43.75	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.16	7.50	nd	nd	nd
Unknown	44.81	nd	nd	1.65	97.28	1.95	nd	1.78	nd	nd	nd	nd	nd	nd	nd	nd
1-Tetracosanol	46.62	5.44	2.15	nd	nd	2.23	nd	1.79	nd	nd	nd	4.60	nd	nd	nd	nd
Tetracosanoic acid	48.06	7.44	nd	nd	nd	nd	nd	nd	nd	nd	1.70	nd	nd	nd	nd	nd
Viminalol isomer	48.90	nd	nd	10.17	nd	nd	nd	nd	nd	nd	nd	nd	5.92	11.02	nd	nd
9,12-Octadecadienoic acid,2,3-bis[(trimethylsilyl)oxy]propyl, ester	49.67	nd	nd	nd	nd	nd	6.95	nd	nd	nd	nd	nd	nd	nd	nd	7.84
Lupeyl acetate	50.10	nd	nd	63.21	nd	17.37	nd	nd	nd	nd	43.81	nd	nd	77.80	nd	nd
Nonacosanol	51.17	nd	3.71	nd	nd	nd	nd	nd	nd	nd	nd	30.11	nd	nd	nd	nd
Octacosanoic acid	52.83	nd	nd	nd	nd	nd	nd	nd	nd	17.98	nd	9.74	nd	nd	nd	nd
Unknown	54.86	nd	nd	nd	nd	19.80	nd	nd	nd	18.25	nd	nd	nd	nd	nd	nd
Unknown	56.53	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	7.67	nd	nd	nd
1-Hentriacontanol	57.21	3.78	6.99	nd	nd	nd	8.79	1.96	nd	nd	nd	nd	nd	nd	28.33	nd
Hexacosanoic acid	59.64	1.11	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

*nd: not detected

In another study, the essential oil components obtained from *C. glomerata* L. subsp. were analyzed using GC-MS, and 48 compounds representing 89.0% of the total volatile components were characterized, the main components were hexadecanoic acid (24.51%), docosane (15.9%), isocitronellene (12.6%), heneicosane (4.6%), hexahydrofarnesyl acetone (3.2%), 9-tricosene (1.6%), octadecanol (1.4%), caryophyllene oxide (1.3%), α-funebrene (1.2%), β-thujaplicinol (1.1%), pentadecanoic acid (1.1%), tricosane (1.1%), (2E,4E)-decadienal (1.0%), (E)-β-damascenone (1.0%) and (E)-caryophyllene (1.0%) (Sinek et al., 2012). In another study, the volatile components composition of *C. olympica* Boiss were analyzed using GC-MS; 19 components representing 94.0% of the total volatile components were characterized and the main components were 2E,6Z-farnesol (14.8%), 3,3-dimethyl-2[5-methoxy-3-methyl-2-pentylidenen]-1-cyclohexanone (12.1%), dihydro aromadendrane (11.6%), tetracosane (9.0%), pentacosane (7.9%), epoxy alloaromadendrene (5.9%) and cyclohexadecanolide (5.8%) (Tosun et al., 2011). The most noticeable peaks in the spectra of the different fractions were investigated in order to determine whose components were the most common, as shown in Figure 2 and Figure 3.

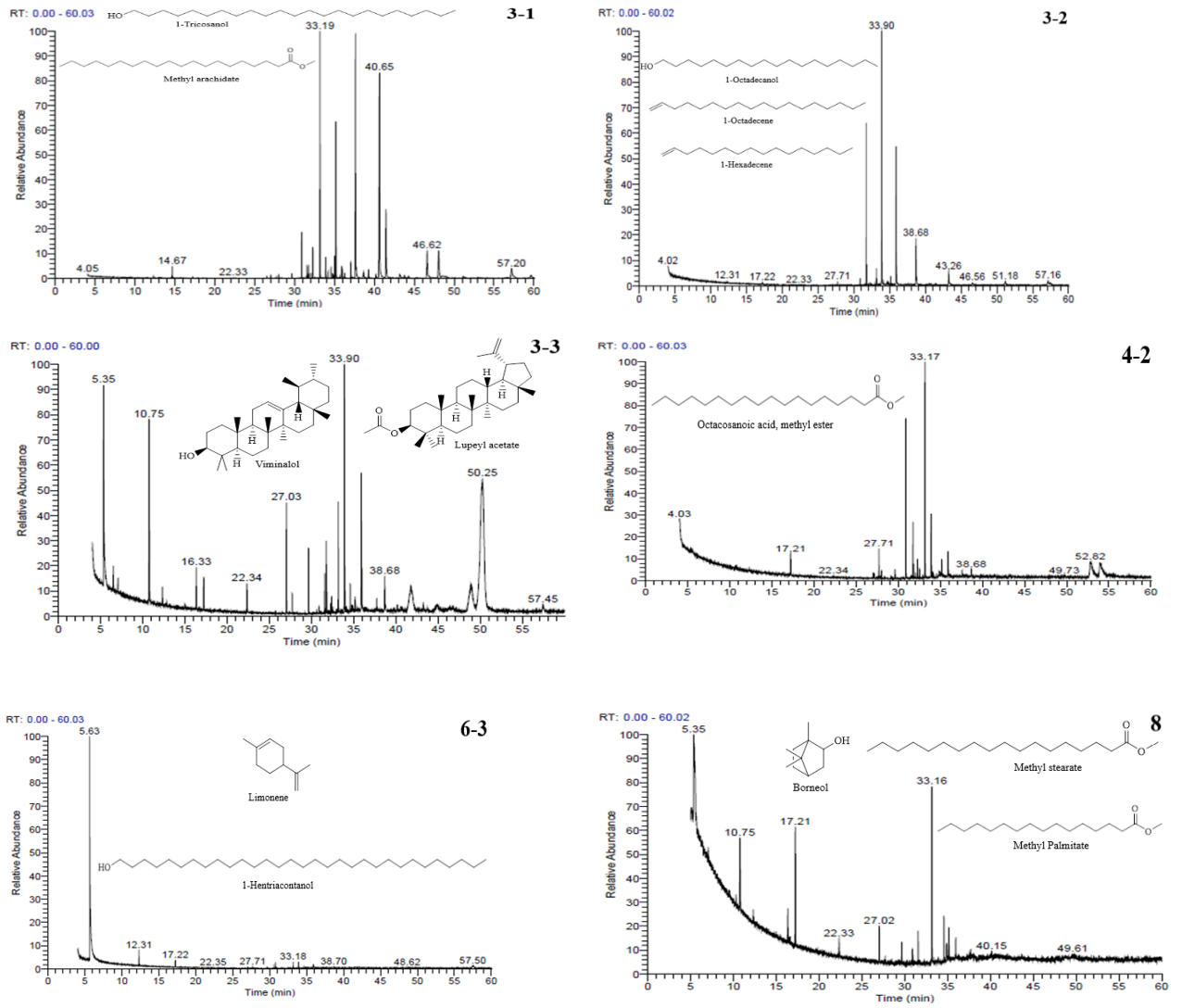

Figure 1. GC-MS/MS Spectrums for the most abundant molecule

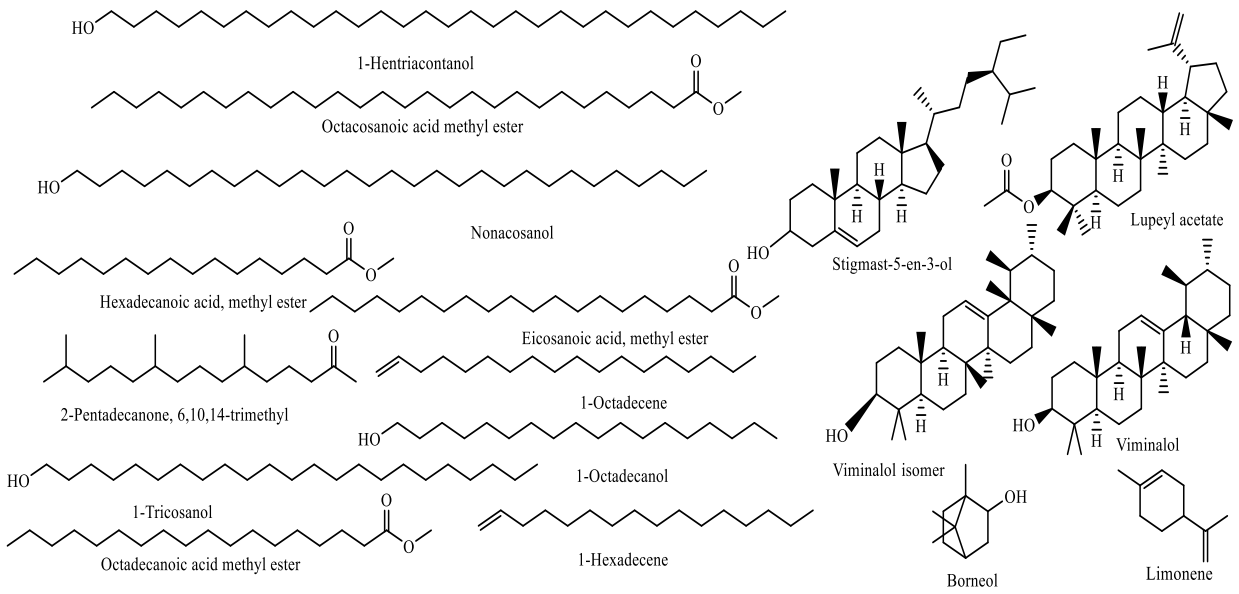

Figure 2. Molecular structures of the most abundant compounds in GC-MS/MS analysis

3.2. ^{1}H-NMR analysis

NMR analysis was performed on *C. baskilensis* leaf fractions depending on the most prominent peaks in conjunction with GC-MS/MS to determine the structures of the different volatile components, Table 1 and Figure 4. CH2 groups in the volatile compounds; as hexadecanoic acid, eicosanoic acid, and 1-tricosanol were resonate as multiplets between 1.2-1.5 ppm, CH3 groups were in the area below 1 ppm, and for the peaks above 2 ppm were s for those CH2 groups adjacent to the carbonyl group, CH2 groups in Fr3-1 were in resonance. For benzoic acid, we inferred through the characteristic hydrogen signals attached to carbon are split into triblets by the effect of neighboring CH2 protons, the other CH3 and CH2 groups appear to resonate between 0.5 to 1.8 ppm, respectively. Fr3-2 has the same groups and it was detected also via the characteristic hydrogen signals attached to carbon 3 split into triblets with the effect of neighboring CH2 protons, the other CH3 and CH2 groups appear to resonate between 0.5 to 1.8 ppm. Fr3-3 spectrum. Fr4-2 spectrum gave 0.9 ppm, the last carbon of the -CH3 fatty acid resonated as a triplet, and between 2.3-2.5 ppmCH2 group appeared to be adjacent to the carbonyl group and resonated like a triplet, Also, at 1.3 ppm hydrogens belonging to saturated hydrocarbons were resonated in the form of muliplets, and finally a mixture of hexadecanoic acid and octadecanoic acid has been observed in the same spectrum. Further, limonene, borneol, and hentriacontanol appear as follows in Fr6-3: olefinic protons resonated around 5.5 ppm, methyl groups resonated around 1 ppm, and hydrogens bonded to carbon adjacent to the hydroxyl group resonated around 4 ppm, while the CH2 groups in the molecules resonated in multiples around 1.5 ppm, the OH groups gave a signal around 3.5 ppm. Finally, Fr8 fraction spectrum showed sugar groups with high amounts due to the hydrogen groups that resonated between 3-4 ppm and the hydroxyl hydrogens that appeared between 4 to 5 ppm. However, it is worth mentioning that sugar groups are not seen in GC-MS/MS because they are not volatile. The reason for detecting some common volatiles found in multiple fractions, although with different solvents, is that the separation techniques used in this research can be considered classical techniques. It also has a kind of complexity, as there is a possibility of more than one compound mixing or coming down of more than one

compound that is different in degree of polarity from the solvent used due to the influence of many factors, including its molecular size and/or weight.

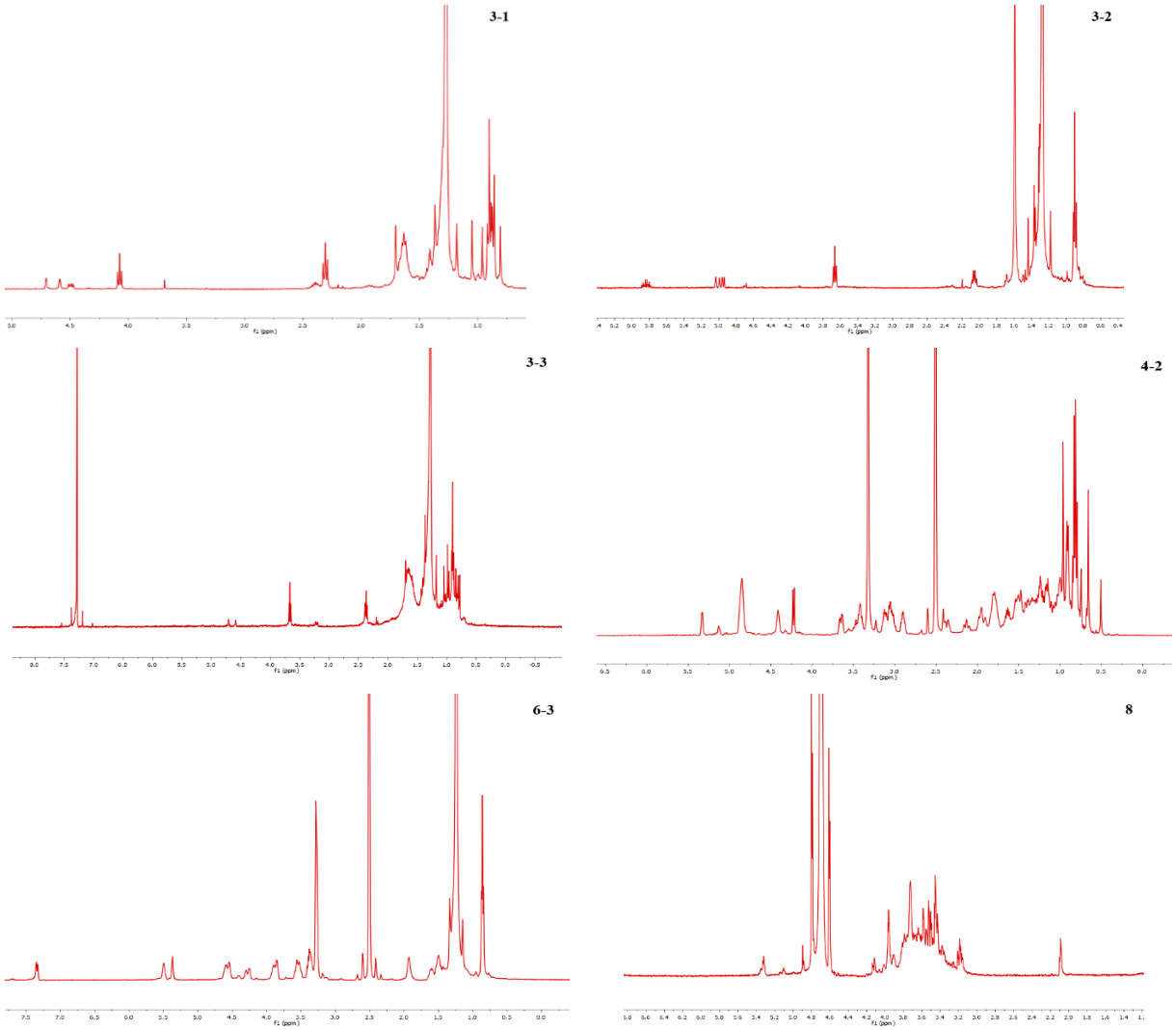

Figure 3. ^{1}H-NMR Spectrums for the most abundant molecules

The following observations were reached by comparing the 1H-NMR data obtained in this study with the data for the same chemicals published in the literature: for borneol in another study they track down δH values as; 1.81 (ddd, J = 16.2, 12.6, 10.5, 5.6 Hz, 2H, –CH–CH2-) 1.64 (t, J = 4.6 Hz, 1H, -CH-) 1.53–1.27 (m, 2H, –CH2-CH2-C-) 1.26–0.92 (m, 2H, –CH2-CH2-C-) 0.91–0.83 (m, 9H, 3 × -CH3) (Dong et al., 2021); 0.85 (3H, s, -CH3), 0.86 (3H, s, -CH3), 0.87 (3H, s, -CH3), 0.95 (1H, dd, H-6b), 1.25 (2H, m, H-5b, H-6a), 1.62 (1H, t, -OH), 1.70 (2H, m, H-3b, H5a), 1.90 (1H, m, H-3a), 2.28 (1H, m, H-4), 4.04 (1H, m, H-2) (Wang et al., 2014). Hexadecanoic acid (methyl palmitate) has been tracked using ^{1}H-NMR (CDCl3, 400 MHz) as the δ found to be: 3.59 (3H, s, OCH3), 2.23 (2H, t, J=8.0 Hz, CH2), 1.55 (2H, t, J=8.0 Hz, CH2), 1.19 (s, -(CH2)n-), 0.82 (3H, t, J= 8.0 Hz, CH3) (Mir et al., 2021). For the Stigmast-5-en-3-ol (β-sitosterol) in a previous study the findings of 1H-NMR (CDCl3, 400MHz) shown signals at δ 3.2 (1H, m, H-3), 5.26 (1H, m, H-6), 5.19 (1H, m, H-23), 4.68 (1H,m, H-22), 3.638 (1H, m, H-3), 2.38 (1H, m, H-20), 1.8-2.0 (5H, m) ppm, in addition to the other peaks were observed at δ 0.76-0.89 (m, 9H), 0.91-1.05 (m, 5H), 1.35-1.42 (m, 4H), 0.69-0.73 (m, 3H), 1.8-2.00 (m, 5H), 1.07-1.13 (m, 3H), 1.35-1.6

(m, 9H) ppm (Kamboj and Saluja, 2011). Moreover, for octacosanoic acid (methyl octacosanoate), 1H-NMR outcomes were recorded as δ = 3.65 (s, OCH3), 2.10 (t, J = 7.7 Hz, H-2), 1.41–1.11 [brs, -(CH2)24-], 0.72 (t, J = 7.3 Hz, CH3)(Wansi et al., 2009).

4. Conclusion

This study represents an exhaustive analysis of the volatile contents via GC-MS/MS and 1H-NMR for diverse fractions of the *C. baskilensis*' leaves. The fractions selected for these analyses were conducted based on their purity using the thin-layer chromatography results. More than thirty volatile compounds were identified due to the component analysis, and a few unknown chemicals with similar properties have been detected, too. Relying on spectroscopic techniques, such as NMR and GC-MS, is greatly beneficial for creating a volatile component and phytochemical content library of what natural sources possess. The quantitative and qualitative analysis of *C. baskilensis*'s volatile components provides information regarding the effectiveness of their extracts or chemical compositions and determines whether or not it is worthy of forward isolation and/or bioassay application. Our research has revealed that *C. baskilensis* possesses a diverse range of volatile components. Further investigation into its biological activity may be achieved using enhanced isolation techniques and experimental and theoretical evaluation.

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Author Contribution

All authors declare equal contribution to the design and experimental work, interpretation of the resultsand editing the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest during the accomplishment of this research. None of the authors has any financial and/or personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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