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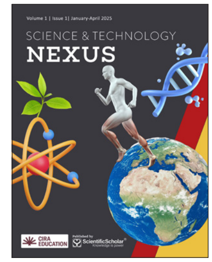

Original Article

Gas Chromatography Mass Spectrometry Profiling of Volatiles from 5 Major Apiaceous Aerial Parts via Headspace solid phase microextraction Compared to Essential Oil, and In Context of Anti-tuberculosis Effect

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ABSTRACT

Objective: Green leafy vegetables from the Apiaceae family are widely consumed due to their distinctive aroma, flavour, and recognized medicinal properties. While the flavour composition of dried Apiaceae fruits has been extensively studied, the phytochemical profiling of the fresh aerial parts remains an area of active research and growing interest. The main goal of the current study was to investigate the phytochemical profile of 5 major Apiaceae green vegetables *viz.,* anise, caraway, celery, dill, and fennel, targeting volatile metabolites.

Material and Methods: Headspace solid-phase microextraction (HS-SPME) and gas chromatography mass spectrometry (GC-MS), were used for volatile analysis comparing fresh aerial parts with extracted essential oils (EOs).

Results: A total of 56 volatiles were detected in the essential oil via GC-MS analysis, compared to 47 volatile compounds detected in the fresh aerial parts via HS-SPME. The fresh aerial parts were further analysed via HS-SPME for comparison to the essential oil. Furthermore, the anti-tuberculosis assay was investigated for the EOs extracted from Apiaceae herbs. EOs most predominant classes were monoterpene hydrocarbons in dill and celery at 82-96%, oxygenated monoterpenes in fennel and anise at 62.5-74.3%, and sesquiterpenes at 100% in caraway.

Conclusion: The fresh aerial parts analysed using HS-SPME revealed monoterpenes as a major class in dill and celery at 30.3-62.2%, oxygenated monoterpenes to amount as a major class in the 5 specimens at 32-98%, represented by anethole, estragole, and fenchone, whereas sesquiterpenes in anise and caraway at 47%**.** Anti-tuberculosis assay of EOs showed effectiveness, especially in the case of celery, dill, and fennel, with MIC values ranging from 0.98 to 3.9 µg/mL.

Keywords: Apiaceae, Essential oil, Gas chromatography-mass spectrometry (GC-MS), HS-SPME, Tuberculosis

1. INTRODUCTION

Culinary herbs and spices are commonly used to improve the taste and aroma of food recipes, attributed to their diverse metabolites with nutritional and health benefits.[1] Due to their richness in essential oils, herbal spices have long been recognized as key food ingredients that impart characteristic Flavors and confer various health benefits, while also enhancing the sensory attributes of diverse food products.[2] Essential oils (EOs) derived from these spices have been extensively utilized in traditional medicine, primarily for their myriad medicinal properties, including antimicrobial[3] and immunomodulatory.[4]

Among plant families employed as spices or consumed as green vegetables, the carrot family (Apiaceae) is one of the most commonly utilized. This family comprises approximately 450 genera and 3100–3200 species of annual herbs, particularly abundant in the Mediterranean region.[5] Eos composition of Apiaceae fruits accounts for their distinct aroma.[6] In addition to their culinary uses, several species within this family are valued for their medicinal properties, notably their antioxidant and antimicrobial activities.[7]

The most widely cultivated members of the Apiaceae family include anise (*Pimpinella anisum* L.), caraway (*Carum carvi* L.), celery (*Apium graveolens* L.), dill (*Anethum graveolens* L.), and fennel (*Foeniculum vulgare* Mill).[8] Beyond their culinary applications as spices, these plants offer a variety of health benefits, including antimicrobial, anti-inflammatory, anti-tumour, digestive, and antioxidant activities.[9] Anise essential oil, rich in (E)-anethole, is valued for its sweet flavour and is commonly used to flavour foods and alcoholic beverages.[10] Caraway oil, containing high levels of limonene and carvone, is widely utilized in the food industry and is employed to alleviate gastric discomfort.[11] Celery is frequently used in traditional medicine for its antifungal and anti-inflammatory properties, with its essential oil showing antibacterial activity largely due to its limonene content.[12] Dill, recognized for its strong aroma, serves both as a flavouring agent and for its pharmacological actions, such as cholesterol-lowering,[13] antiemetic, and antispasmodic effects.[14] Fennel is one of the most commonly used aromatic Apiaceae plants, with essential oils dominated by (E)-anethole, exhibiting antispasmodic properties and typically consumed after meals to aid digestion in Indian cuisine.[15]

The analysis of food and spices has lately started to ensure their quality and verify sensory attributes to enhance consumer satisfaction. Recently, modern metabolomics approaches were applied for the assessment of food metabolome in a comprehensive and untargeted manner.[16] Gas chromatography-mass spectrometry (GC–MS) is well adopted for the characterization of volatiles and nutrients (post-silylation) in herbal samples.[17] Head space solid-phase microextraction (HS-SPME) is a favoured, robust, and solvent-free tool without thermal decomposition tool adopted for volatiles recovery.[18] To envisage such large data sets and identify key metabolites, multivariate data analyses are employed, including principal component analysis (PCA) that simplifies the complexity of metabolite data, ensuring better sample classification.[19]

Increasing interest in plant essential oils (EOs) acting as antimicrobials. Such interest is owing to their richness in oxygenated terpenes, i.e., phenolics of potential action against several microorganisms.[20] Furthermore, owing to their hydrophobic nature, EOs move across the cell membrane lipids of bacteria, damage the cell wall structures, and make them more permeable.[21] Tuberculosis (TB) is a highly infectious disease affecting almost one billion people in the last 200 years.[22] *Tuberculosis bacilli* are mycobacteria which, in most cases, invade the lungs, and in the rest, invade other extra-pulmonary organs, *i.e.*, lymph nodes, bones, and genitourinary system.[23] Subsequently, there is an urgent need to discover new treatments to reduce the burden of this disease and overcome its multi-drug-resistant tuberculosis problem.[24]

The main goal of the current study is to investigate the aroma profile of 5 Apiaceae vegetables aerial parts, including anise, caraway, celery, dill, and fennel, comparing them with their essential oil composition via SPME-GC-MS analysis for the first time with their anti-tuberculosis activity. This comparative approach seeks to identify the most promising Apiaceae herbs based on their chemical composition and biological activity, particularly their anti-tuberculosis potential.

2. MATERIAL & METHODS

2.1. Plant material

Five Apiaceae aerial parts, viz., anise (*Pimpinella anisum*), caraway (*Carum carvi*), celery (*Apium graveolens*), dill (*Anethum graveolens*), and fennel (*Foeniculum vulgare*) were collected during 2022 from plants grown at the Faculty of Pharmacy Herbarium, Cairo University, Giza, Egypt. A voucher specimen was deposited at the Herbarium library. The lyophilized fresh aerial parts were powdered and stored at -20°C till analysis.

2.2. Chemicals and materials

SPME Stableflex fibers coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm), PDMS (polydimethylsiloxane) were purchased from Supelco (Oakville, ON, Canada). All chemicals, solvents, and standards were purchased from Sigma Aldrich, USA.

2.3. Essential oil extraction by hydrodistillation clevenger apparatus

The aerial parts (100 mg) for each specimen, viz., anise, caraway, celery, dill, and fennel were distillated by a modified clevenger apparatus with distilled water for 3h. The distilled EOs were stored at -20°C until further analysis.

2.4. Volatiles analysis of aerial parts and essential oil of Apiaceae vegetables

Volatile analysis was carried out using HS-SPME. Dried powdered aerial parts (20 mg) were mixed with 10 μg (Z)-3-hexenyl acetate in a 1.5 ml screw cap vials with SPME fiber inserted manually and volatile compounds were extracted at 50°C for 30 min. SPME fiber was subsequently removed and injected manually in the injection port of the gas chromatography-mass spectrometer (GC-MS). GC-MS analysis was performed on EOs and SPME of fresh aerial parts. Schimadzu GC-17A gas chromatogram equipped with DB-5 column (30 m × 0.25 mm i.d. × 0.25 µm film thickness; Supelco) was coupled to a Shimadzu QP5050A mass spectrometer. The temperature of the interface and the injector both held at 220°C. The temperature program used for volatiles analysis followed a gradient program. Oven temperature was held first at 40°C for 3 min, then raised to 180°C at a rate of 12°C min^{-1}, kept at 180°C for 5 min, and finally reached a rate of 40°C min^{-1} to 240°C and kept for another 5 min. Helium was used as a carrier gas at a flow rate of 0.9 ml/min. SPME fiber was prepared to the next injections by placing it in the injection port for 2 min at 220°C to for complete elution of volatiles. Blank runs were made during samples analyses. HP quadruple mass spectrometer was operated in EI mode at 70 eV; a scan range was set at m/z 40-500.

2.5. GC-MS data processing and multivariate analysis

Identification of volatile and silylated metabolites in the five apiaceae plants was carried out by comparing their (RI) retention indices relative t n-alkanes (C6-C20), matching mass spectra to NIST, WILEY library database, and with standards whenever available. AMDIS software (www.amdis.net) was used for peaks deconvolution prior to mass spectral matching.

2.6. Anti-tuberculosis activity

Hence the anti-tuberculosis activity was performed against the four apiaceous essential oils including anise, celery, dill, and fennel except caraway due to low oil yield. Anti-mycobacterial activity of the samples was evaluated against Mycobacterium tuberculosis (ATCC 27294) using microplate Alamar blue assay (MABA) with slight modification performed in black, clear-bottomed, 96 well microplates. Initial essential oils stock solutions were prepared in dimethyl sulfoxide, and subsequent two-fold dilutions were performed in microplates. 0.1 Ml of 105 CFU/mL M. tuberculosis inoculum was added to wells, additional control wells consisted of bacteria only as control. Plates were incubated at 37°C. On day 4th post incubation, 20 µL of alamar Blue solution (Alamar Biosciences/Accumed, Westlake, OH, USA) and 12.5 µL of 20% Tween 80 were added to the entire plate, Isoniazide was used as the reference drug. Plates were then incubated at 37°C, and results were recorded at 24 h post-reagent addition at 590 nm. Percent inhibition was defined as: 1 – (mean of test well/mean of B wells) × 100. Visual MICs were defined as the lowest concentration of drug that prevented a color change. MIC90 was defined as the concentration that prevents 90% of mycobacterial growth. The MIC was defined as the lowest concentration of drug that prevented change in color.

3. RESULTS AND DISCUSSION

The main goal of this study was to assess the aroma composition of the aerial parts of 5 Apiaceae species grown in Egypt, *viz.*, anise, caraway, celery, dill, and fennel, compared to their extracted essential oils. However, the previous studies on Apiaceae species were focused on fruit aroma,[10] with less reports on its aerial parts for aroma composition.

3.1. GC-MS volatile profiling in apiaceae aerial parts and EOS

HS-SPME-GC-MS analysis of Apiaceae aerial parts aroma profiles led to the identification of 47 volatile compounds [Table S1, Figure 1] belonging to 4 major chemical classes, including monoterpenes, hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and aromatic hydrocarbons [Figure 2a]. EOs aroma analysis led to the identification of 56 volatile compounds [Table 1, Figure S1] belonging to 6 major chemical classes, including monoterpenes and sesquiterpene hydrocarbons alongside their oxygenated conjugates [Figure 2b].

3.1.1. Oxygenated monoterpenes

Oxygenated monoterpene hydrocarbons were detected as the most abundant class in all Apiaceae aerial parts analysed using HS-SPME-GC/MS at 98.4, 69.6, 52.9, 49.6, and 36.4% in fennel, dill, anise, caraway, and celery, respectively. Anethole (peaks 22 and 28) was detected at high levels in anise and fennel compared to much lower levels in other specimens at 21% - 31%. Anethole constituted the major Apiaceae aroma compound with a distinct sweet taste commonly found in oral hygiene products and alcoholic beverages.[10] Fenchone with bitterness sensation was detected only in fennel at 44.8% comparable to reported levels,[25] whereas menthone was found exclusively in caraway, though at a very low level ≤1%. Citral, an oxygenated monoterpene with potent antimicrobial activity[26] was detected only in caraway at 12.3%

Compared to fresh herbs, extracted EOs of only three Apiaceae specimens [Table 1] contained oxygenated monoterpenes, which constituted the most abundant class in anise and fennel at 74.3 and 62.5%, respectively, compared to much lower levels in dill EO at 17.9%, which contributes to their antioxidant and antimicrobial activities.[27] Anethole was detected as the major volatile in anise compared to much lower levels in fennel EO at 70.6% and 15.7%, respectively. Positional isomer of (*E*)-Anethole, estragole, was detected in contrast at higher levels in fennel versus anise at 46.7% and 3.7% respectively, compared to trace levels in fennel using SPME at 1.1% and following previous results from fruit.[10,28] Estragole is a flavouring agent that is less safe than anethole due to its carcinogenic and potential genotoxic effects.[29] Preparation of fennel fruit using infusion was found to lower

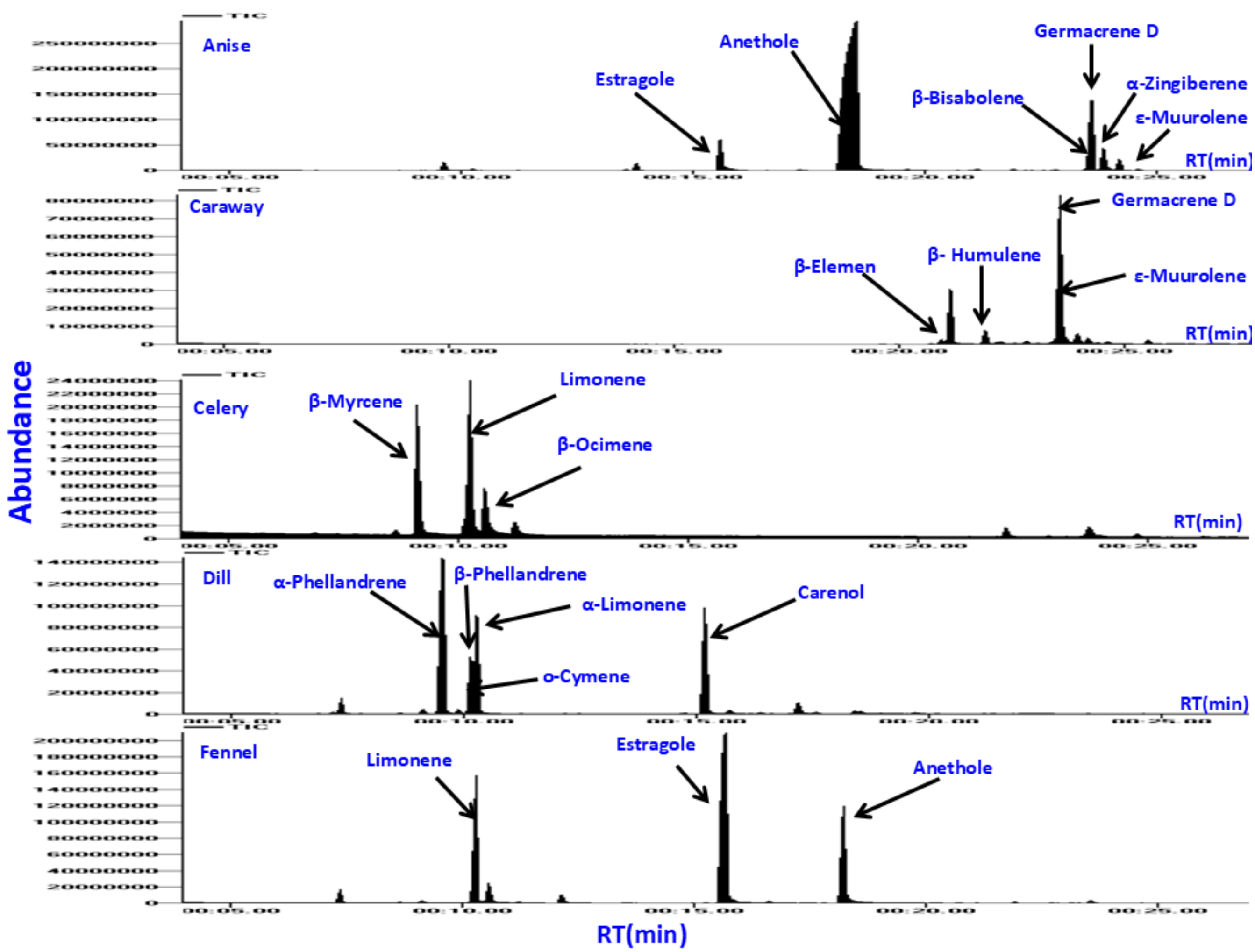

Figure 1: Representative chromatograms of analysed Apiaceae herbs using Head space solid Phase micro extraction coupled with gas chromatography mass spectrometry (HS-SPME-GC-MS).

its toxic side effects,[10] and has yet to be tested in aerial parts. 2-Caren-4-ol, an oxygenated monoterpene with antimicrobial properties,[30] was detected only in dill EO at 12.8%.

3.1.2. Monoterpenes hydrocarbons

Monoterpene hydrocarbons represented the most abundant class in celery at 62% followed by 30% in dill, and at much lower levels in caraway and fennel at 2.5% and 1%, respectively. Limonene, ocimene, and β-myrcene were detected as the major forms in celery at 23.8, 21.7, and 15.2 %, respectively. Limonene is reported for its strong antimicrobial activity.[31] β-phellandrene was detected as the major monoterpene hydrocarbon in dill at 21.4%, followed by cymene at 8.9 %. β-phellandrene was reported to exhibit various biological activities such as antibacterial, antifungal, and antioxidant.[32]

Likewise, monoterpene hydrocarbons were detected as the major class in celery and dill EOs at 95.5 and 81.6%, respectively, followed by 37.4% in fennel EO, compared to trace levels in anise EO. Limonene was detected as the most abundant compound in celery, fennel, and dill EOs at 39.8, 31.9, and 10.2, respectively. Limonene is used as a flavour additive, in addition to its potential antibacterial activity against several Gram-negative and Gram-positive bacteria.[31] β-myrcene was detected at higher abundance in celery EO at 33%, compared to trace levels in other EOs. EOs rich in phellandrene exhibit potential properties as antimicrobial[33] found highest levels in dill EO represented by α- and b-phellandrene at 39.6% and 14.5%, respectively.

3.1.3. Sesquiterpene hydrocarbons

Sesquiterpene hydrocarbons constituted the second most abundant volatile class in caraway and anise aerial parts accounting for 47% compared to trace levels in celery and fennel at 0.7-0.8%. Germacrene D, a sesquiterpene hydrocarbon reported in several plants with potent antimicrobial potential[34] was detected in anise and caraway at

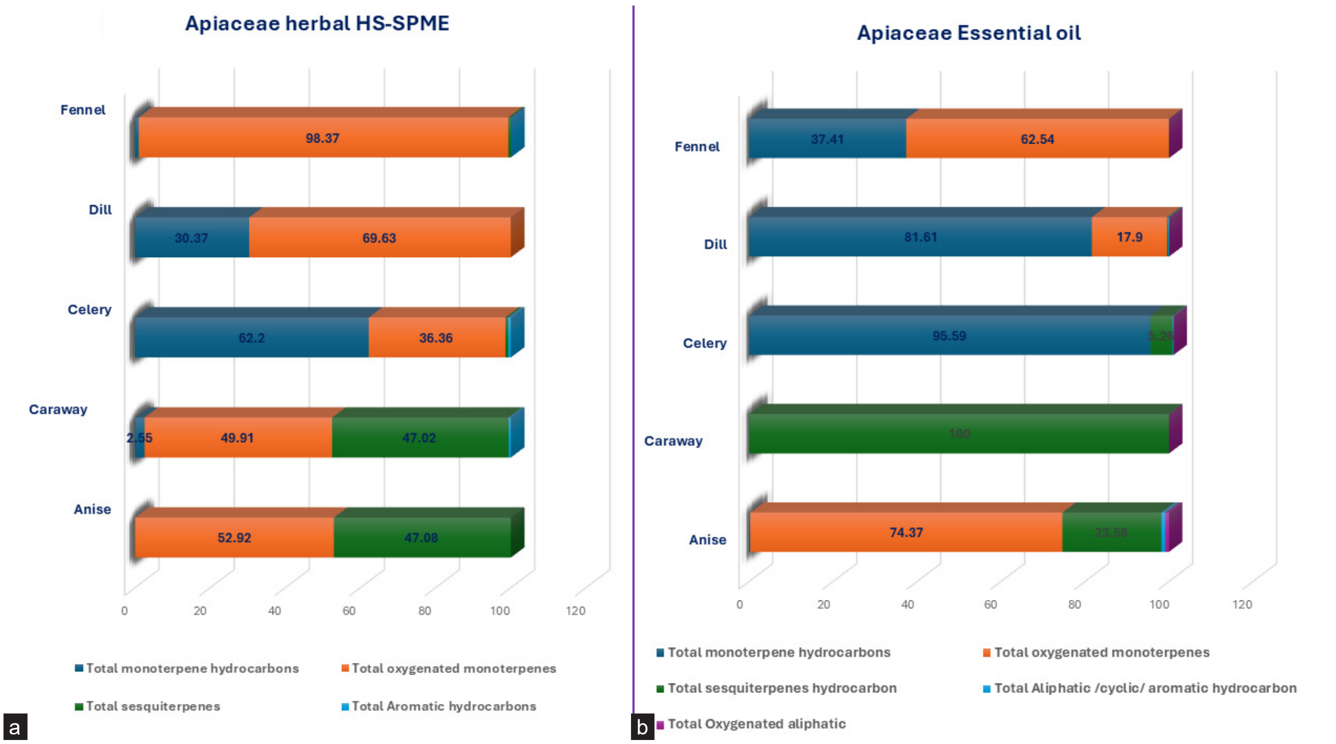

Figure 2: Representative bar charts of major metabolite classes identified in Apiaceae herbs analysed using (a) Head space solid phase micro extraction (HS-SPME)-GC-MS versus (b) extracted essential oil analysed using gas chromatography mass spectrometry (GC-MS).

Table 1: Relative percentage of volatile compounds detected in essential oil fractions of aerial parts of five *Apiaceae plants* using GC/MS measurements (n=3)

No.	RT (min)	RI	Compound name	Average± S.D.				
				Anise	Caraway	Celery	Dill	Fennel
1	7.19	911	*α*-Thujene	-	-	-	0.38	
2	7.37	917	*α*-Pinene			0.09	3.72	1.03
3	7.8	933	Camphene	-	-	-	0.07	0.01
4	8.58	961	*β*-Thujene	0.01	-	0.15	-	0.02
5	8.65	964	*β*-Pinene	0.01	-	0.88	0.23	0.06
6	9.12	981	*β*-Myrcene	0.03	-	33.04	1.17	0.22
7	9.52	995	*α*-Phellandrene	0.02	-	0.08	39.62	0.02
8	9.88	1007	*α*-Terpinene	-	-	-	0.89	-
9	10.14	1016	Cymene	0.01	-	4.57	10.47	0.06
10	10.25	1019	Limonene	0.17	-	39.79	10.25	31.92
11	10.28	1021	*β*-Phellandrene	-	-	-	14.52	-
12	10.562	1029	*β*-Ocimene	-	-	13.94	-	3.13
13	11.23	1051	*γ*-Terpinene	0.02	-	3.05	0.1	0.1
14	12.14	1081	*δ*-2-Carene	-	-	-	0.19	.84
Total monoterpene hydrocarbons				**0.25**	**-**	**95.59**	**81.61**	**37.41**
15	14.647	1161	Carvenone	-	-	-	0.26	-
16	15.17	1177	2-Caren-4-ol				12.81	-
17	15.23	1179	Cymen-8-ol					0.02
18	15.69	1194	Sabinol				1.27	-

(*Contd...*)

(*Contd...*)

No.	RT (min)	RI	Compound name	Average± S.D.				
				Anise	Caraway	Celery	Dill	Fennel
19	15.69	1194	Estragole	3.68	-	-	-	46.68
20	16.59	1225	Fenchyl acetate	-	-	-	-	.15
21	17.17	1245	Limonene diepoxide	-	-	-	3.56	-
22	17.27	1249	Anethole	0.13	-	-	-	15.69
23	18.5	1292	Anethole isomer	70.56	-	-	-	-
Total oxygenated monoterpenes				**74.37**	**--**	**--**	**17.9**	**62.54**
24	19.6	1330	*δ*-EIemene	0.09	0.02	-	-	-
25	20.7	1368	*α*-Cubebene	0.02	0.1	-	-	0.01
26	20.95	1378	*β*-Bourbonene	0.04	-	-	-	-
27	21.12	1383	*β*-Elemene	0.11	9.61	-	-	-
28	21.88	1411	*β*- Humulene	0.15	2.21	2.14	-	0.04
29	22.14	1422	*β*-Cubebene	0.03	0.2	-	-	-
30	22.22	1425	*γ*-Elemene	-	0.17	-	-	-
31	22.27	1426	*α*-Bergamotene	0.03	-	-	-	-
32	22.55	1474	Germacrene-D	0.02	0.07	-	-	-
33	22.69	1443	*α*-Himachalene	0.01	-	-	-	-
34	22.8	1447	*α*- Humulene	-	0.47	-	-	-
35	22.78	1447	*β*-Famesene	0.08	-	-	-	-
36	22.8	1447	*α*-Bisabolene	0.14	-	-	-	-
37	23.04	1457	muurola-4(14),5-diene	-	0.02	-	-	-
38	23.37	1470	α-Muurolene	-	0.49	-	-	-
39	23.37	1470	Germacrene-D	-	-	-	0.07	-
40	23.45	1473	Longifolene	0.15	-	-	-	-
41	23.56	1477	*ε*-Muurolene	7.63	22.81	-	-	-
42	23.57	1478	Germacrene D	10.45	60.03	-	-	-
43	23.82	1487	*α*-Zingiberene	2.03	-	-	-	-
44	23.67	1481	*β*-Eudesmene	-	0.68	3.12	-	-
45	23.93	1492	Elixene	0.18	2.1	-	-	-
46	24.15	1500	*β*-Bisabolene	1.27	-	-	-	-
47	24.15	1501	Famesene	0.84	-	-	-	-
48	24.56	1516	*β*-Sesquiphellandrene	0.29	-	-	-	-
49	24.57	1516	*δ*-Cadinene	-	0.25	-	0.21	0.01
50	25.49	1552	Germacrene-B	-	0.71	-	-	-
Total sesquiterpenes hydrocarbon				23.56	100	5.26	.28	.06
51	6.89	900	1,2,3,4,5-Pentamethylcyclopentane	0.01	-	0.22	0.04	-
52	9.23	985	Mesitylene	0.02	-	-	-	-
53	12.47	1091	Undecane	-	-	-	0.18	-
54	13.55	1125	Cyclohexene, 3,4-diethenyl-3-methyl-	0.91	-	-	-	-
Total Aliphatic /cyclic/ aromatic hydrocarbon				0.94	-	0.22	0.22	0
55	9.63	999	(*E*)-4-Hexenyl acetate	0.84 0.001 0.02	-	-	-	-
56	16.56	1224	3-Hexenyl valerate	0.02	-	-	-	-
Total oxygenated aliphatic				0.86	-	-	-	-

RT: Retention time; RI: Retention Index; SD: Standard deviation; GC/MS: Gas chromatography mass spectrometry

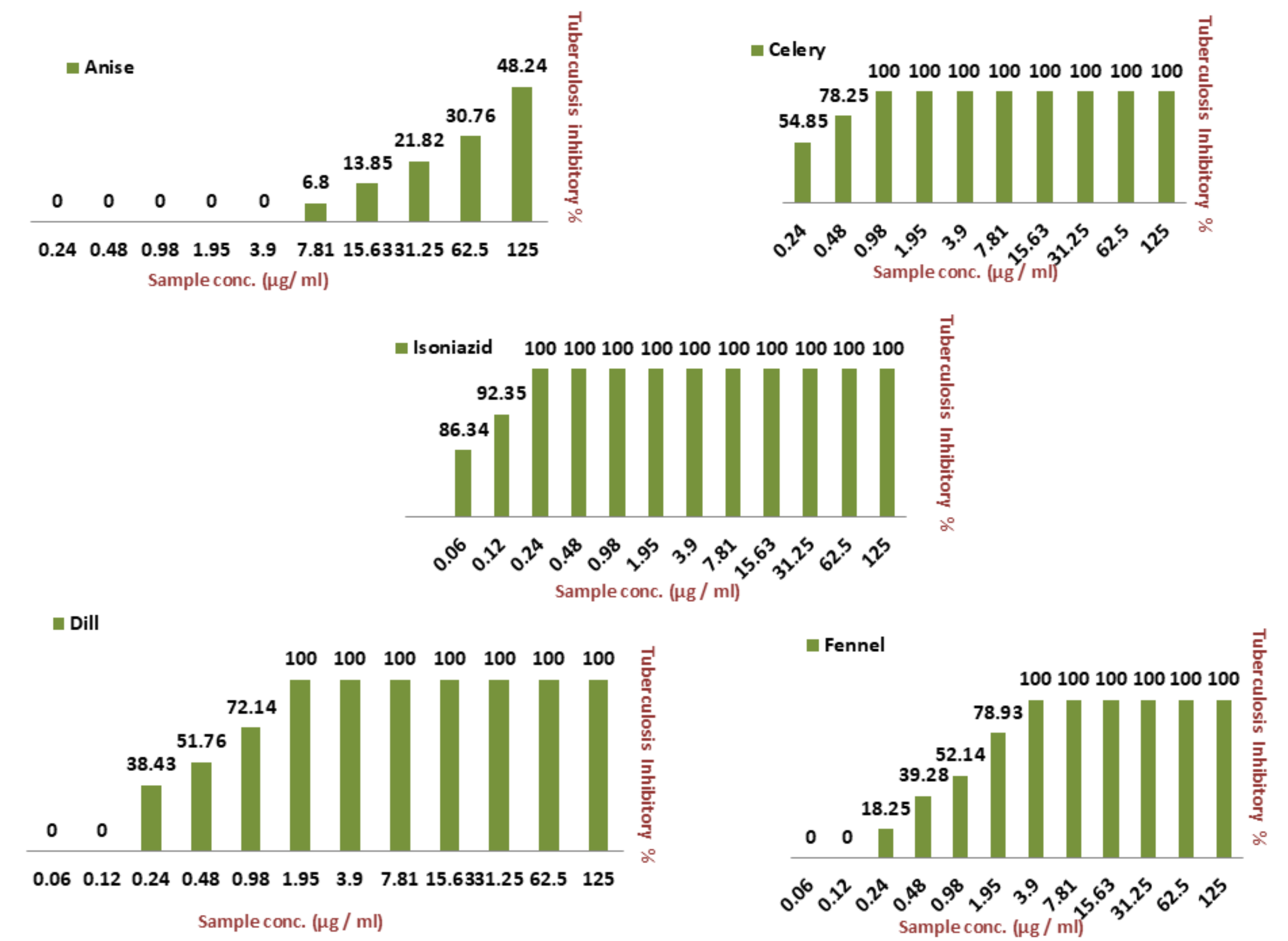

Figure 3: Anti-tuberculosis activity of Apiaceae Essential oils (EOs) compared to isoniazid as standard drug.

41.6 and 21.7%, respectively. Next to germacrene D, elemene was detected in caraway, represented by β- and γ-elemene at 19.1 and 1.7 %, respectively. Sesquiterpene hydrocarbons were detected as the major class in caraway EO at 100%, followed by 23.56% and 5.26% in anise and celery, compared to traces in dill and fennel EOs, respectively. EOs enriched in germacrene-D possess antibacterial activity against human pathogens.[35] Next to germacrene-D, muurolene (23%) and elemene (10%) were detected at considerable levels in caraway EOs and in accordance with previous reports.[36]

3.1.4. Aliphatic/aromatic/oxygenated hydrocarbons

Aromatic monoterpene hydrocarbons were detected at trace levels in caraway, celery, and fennel aerial parts, represented by elemicin, safrole, and thymol methyl ether. Likewise, aliphatic and aromatic hydrocarbons were detected at trace levels in anise, celery, and dill EOs, ranging from 0.2 – 0.9%. Mesitylene is an aromatic hydrocarbon with a sweet aromatic odor,[37] was detected only in anise, though at a trace level of 0.02%. Oxygenated aliphatic hydrocarbons represented by hexenyl acetate and hexenyl valerate were detected only in anise at trace level ≤1% and this suggests that in Apiaceae aerial parts, aliphatic hydrocarbons do not constitute a major form in their EOs.

3.2. Anti-tuberculosis activity of apiaceous aerial parts EOs

Tuberculosis (TB) is one of the epidemics that threatens global public health, causing a lethal airborne infection by *Mycobacterium tuberculosis*.[38] Treatment of TB has become more difficult and challenging because of drug-resistant variants.[39] Consequently, the anti-tuberculosis activity of the 5 derived EOs was tested using an *in vitro* assay, especially considering their richness in antimicrobial aroma compounds. Results of the anti-TB assay of EOs against *M. tuberculosis* are shown in Figure 3, revealing that celery EO was the most active, as evident by its lowest MIC value of 0.98 μg/mL [Figure S2], followed by dill and fennel with MIC ranging from 1.95 - 3.9 μg/mL, whereas the positive drug control (isoniazid) at 0.06 μg/mL. Such promising activity of celery and dill might be attributed to their enrichment in monoterpene hydrocarbons exemplified by limonene, β-myrcene, and α-phellandrene. Limonene and myrcene detected at high levels in celery and fennel interact with the cytoplasmic membranes of bacteria, making loss of the tuberculosis membrane integrity.[40] Furthermore, dill encompassed a high level of α-phellandrene known to exhibit strong anti-tuberculosis activity.[41] Interestingly, EOs enriched in oxygenated monoterpenes such as anethole in anise oil showed low efficacy and suggesting that in *M. tuberculosis,* monoterpene hydrocarbons are more active than their oxygenated derivatives. An analysis of individual volatiles against *M. tuberculosis* can provide a conclusive role for each volatile compound.

4. CONCLUSION

The present study provides the most comprehensive aroma profiling of apiaceous aerial parts. Volatiles heterogeneity among the 5 examined apiaceous plants was revealed in the context of aroma metabolites of fresh aerial parts compared to extracted EOs. A total of 56 volatiles were detected in EO compared to 47 volatile compounds in fresh aerial parts via HS-SPME. Aroma compounds detected in EOs belonged to monoterpenes, oxygenated monoterpenes, sesquiterpenes, aliphatic, aromatic, and oxygenated aliphatic hydrocarbons. Monoterpene hydrocarbons, viz., myrcene, phellandrene, and limonene, were the major class in celery and dill, whereas oxygenated monoterpenes, viz., (E)-anethole and estragole, were the most abundant class in anise and fennel. Sesquiterpenes were most abundant in caraway, represented by germacrene D (60%), muurolene (22.82%). Finally, anti-tuberculosis assay for isolated EOs revealed potential activity in the case of celery and dill versus anise EO, showing the least effect. The presence of monoterpene hydrocarbons in these plants, especially limonene, myrcene, and phellandrene is likely to account for their anti-tuberculosis activity. Further testing of these isolates against *M. tuberculosis* using in vitro and ideally in vivo animal models shall provide conclusive evidence on their potential as antibiotics against that pathogen.

Ethical approval: Institutional Review Board approval is not required.

Declaration of patient consent: Patient's consent not required as there are no patients in this study.

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