



## Monoterpene synthase from *Dracocephalum kotschy* and SPME-GC-MS analysis of its aroma profile

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### Abstract

*Dracocephalum kotschy* (Lamiaceae), as one of the remarkable aromatic plants, widely grows and also is cultivated in various temperate regions of Iran. There are diverse reports about the composition of the oil of this plant representing limonene derivatives as its major compounds. There is no report on cloning of mono- or sesquiterpene synthases from this plant. In the present study, the aroma profile of *D. kotschy* has been extracted and analyzed via Headspace Solid-Phase Microextraction technique coupled with Gas Chromatography- Mass Spectroscopy. In order to determine the sequence of the active terpene synthase in this plant, first mRNA was prepared and cloning was performed by 3' and 5'-RACEs-PCR method, then cDNA was sequenced and finally aligned with other recognized terpene synthases. The results showed that the plant leaves mainly comprised geranial (37.2%), limonene-10-al (28.5%), limonene (20.1%) and 1,1-dimethoxy decane (14.5%). Sequencing the cDNA cloned from this plant revealed the presence of a monoterpene synthase absolutely similar to limonene synthase, responsible in formation of limonene, terpinolene, camphene and some other cyclic monoterpenes in its young leaves.

**Keywords:** *Dracocephalum kotschy*, essential oil, Headspace Solid-Phase Microextraction, Lamiaceae, monoterpene synthase

### Introduction

Most of the Lamiaceae species produce high amounts of essential oils. Aromatic plants develop a mixture of volatile compounds, which are widely employed in cosmetics as fragrance, in the food industry as flavoring and in the household products as scenting agents [1]. The genus *Dracocephalum* comprises eight known species, of which *D. kotschy* is growing wildy

in North and North-West areas of Iran. The constituents of the essential oil of this species have been frequently reported and limonene derivatives are mainly described as the most abundant compounds [2].

Terpenic compounds are classified among the hall mark bioactive natural compounds. They have mainly exhibited biological activity and

include sterols, saponins and volatile oils. Recent genetic approaches are capable of isolation and characterization of the biosynthetic enzymes especially by development of molecular biological techniques. For instance, sequence comparison of mono-, sesqui- and diterpene synthases have revealed that they have a similar structure [3,4]. Structural identification of a number of monoterpene synthases has indicated that they all have similar properties (like molecular mass, a divalent metal ion and neutral pH optimum requirements). Interestingly, a terpene synthase is able to form multiple products [3,5], e.g. the pinene synthases (from sage and grand fir) can catalyze the production of both  $\alpha$ - and  $\beta$ -pinenes [6].

Bibliography reveals that geranyl diphosphate (GDP) is a well-known natural substrate for monoterpene synthases. In fact, all the terpene synthases utilize GDP without formation of free intermediates [7]. Because GDP is not able to be directly cyclized, due to the C<sub>2</sub>-C<sub>3</sub> (*trans*) double bond, both isomerization and cyclization need to be caused in the biosynthetic pathway [3,7]. Regarding the above mentioned reasons, monoterpene cyclases have the capability of catalyzing both the isomerization and cyclization reactions. As it has been found so far, there are a few monoterpene synthases interfering in production of acyclic products like myrcene and linalool [6,8].

Literature review shows that two monoterpene synthases (LaLIMS and LaLINS) and one sesquiterpene synthase (LaBERS) have been cloned from lavender species. The enzyme LaLIMS can catalyze the formation of (R)-(+)-limonene, terpinolene, (1R, 5S)-(+)-camphene, (1R, 5R)-(+)- $\alpha$ -pinene,  $\beta$ -myrcene and traces of  $\alpha$ -phellandrene. The second enzyme LaLINS has a role in production of (R)-(-)-linalool, the main component of lavender essential oil [9]. Regarding that the volatile compounds of *D. kotschyi* are highly interesting for their biological activities such as trypanocidal effects and that there is no report on gene cloning of this medicinally valuable species, in the present

study, we have reported analyzing the aroma profile of a wild species of *D. kotschyi*, via Headspace Solid-Phase Microextraction technique coupled with gas chromatography-mass spectroscopy (SPME-GC-MS). Moreover, the cloning of a monoterpene synthase related to the flavor of its essential oil has been explained for the first time from the Iranian plant leaves.

## Experimental

### General procedure

Chemical reagents and solvents were purchased from Merck Co. (Germany). Gel and plasmid extraction kits were from Invitrogen Co. (UK). RNeasy Plant Mini Kit was prepared from Qiagen (USA), and vectors and *E. coli* competent cells were from Invitrogen. Polymerase chain reactions were performed on a Primus 25 (Peqlab, Germany) thermal cycler.

### Plant material

The plant used in this study was gathered from Siah-Bishe in Chalus Road, northern parts of Iran, in July 2011. The wild plant specimen was identified by Mr. Yousef Ajani, from Institute of Medicinal Plants, ACECR, Karaj and a herbarium specimen is deposited in that Institute.

### cDNA preparation

Total RNA was extracted from the early growing stage of the *D. kotschyi* leaves using RNeasy Plant Mini Kit and reverse transcribed with oligo (dT) primer [ad: 5'-GCT GTC AAC GAT ACG CTA CGT AAC GGC ATG ACA GTG TTT TTTTTTTTTTT TTT-3'] designed to have an adaptor sequence at the 5'-end to obtain the cDNA for the 3'-RACE method. The resulting cDNA of the leaves was used as a template in subsequent PCR with Taqor KOD Dash DNA polymerases with various combinations of sense and antisense degenerate primers. The temperature program was designed on thermal cycler and started at 94 °C (3 min), followed by 33 cycles (94 °C for 30 s, 46 °C for 30 s and 72 °C for 1 min), then 72 °C for 2 min. Elongating times were different (30-60 s) based

on the expected length of the amplified fragment. The size of monoterpene synthase sequences (partial not complete) was estimated by gel electrophoresis. PCRs were repeated using the same primers to obtain more amounts of DNA, for cloning into the vector. The similarity of the cloned sequences to known sequences was checked with NCBI pBLAST.

#### *3' and 5'-RACEs-PCR method*

The 3'-RACE method was used to amplify the 3'-end of the monoterpene synthase. First, the polymerase chain reaction for 3'-RACE was carried out with degenerate primer [5'- TAG ATG ATG TTT ACG AT-3'] and an adaptor primer [amm: 5'- GGC CAC GCG TCG ACT AC-3']. The PCR was performed by KOD Dash DNA polymerase (0.2  $\mu$ L), amm primer (0.3  $\mu$ L), degenerate primer (0.6  $\mu$ L), dNTP (0.1 mM, 2  $\mu$ L), DNA template (1  $\mu$ L) and appropriate amounts of recommended buffer, DMSO and water by the temperature program (94 °C for 20 s, 40 °C for 15 s and 72 °C 30 s, 33 cycles). The subsequent PCR gave a 900 bp product, which was electrophoresed on 0.8% agarose gel and purified using the Gel-M™ Gel Extraction System (Viogene). The resulting DNA fragment was cloned into the plasmid vector pCR 2.1 using the TOPO TA cloning kit (Invitrogen). Specific antisense primer [LAV-I: 5'-ACC CCA TTC GTA GTT GTC GCA GAA CG-3'] designed on the basis of the sequence obtained from 3'-RACE. A poly C tail was appended to the cDNA for 5'-RACE by terminal dideoxynucleotidyltransferase, and the cDNA was purified on a PCR-M column (Viogene). To clone the 5'-end of the transcript (5'-RACE), cDNA was synthesized from mRNA with reverse transcriptase and gene specific reverse primers based on the known sequence parts (LAV-I). The purified product (64  $\mu$ L) was amplified with terminal deoxynucleotidyltransferase (1  $\mu$ L), dCTP (10 mM, 5  $\mu$ L) and 16  $\mu$ L of TdT buffer in PCR at 37 °C for 90 min, 70 °C for 10 min and 4 °C for 10 min to synthesize an oligo (dC)-tail. The cDNA (1  $\mu$ L) was used as a template in PCR

(temperature program: 94 °C for 30 s, 52 °C for 20 s, 74 °C for 50 s, 35 cycles) with dNTPs (10 mM, 2  $\mu$ L), oligo (dT) anchor primer for 3'-RACE (0.3  $\mu$ L), gene specific primer (10  $\mu$ M, 0.3  $\mu$ L), KOD Dash DNA polymerase (0.8  $\mu$ L) and appropriate amounts of buffer and water. Furthermore, a touch down protocol similar to that of used for 3'-RACE was applied.

#### *SPME- GC-MS analysis*

Headspace Solid-Phase Microextraction (SPME) coupled to gas chromatography and mass spectroscopy has been applied for analyzing the essential oil directly evaporated from the young leaves. GC-MS was performed on a cross-linked 5% methyl phenyl siloxane (HP-5, 30 m  $\times$  0.25 mm i.d., 0.25  $\mu$ m film thickness), carrier gas, He; split ratio, 1:15; quadruple mass spectrometer (Hewlett-Packard 6890) operating at 70 eV ionization energy. In order to obtain the retention index for each compound, normal alkanes (C<sub>8</sub>-C<sub>25</sub>) were injected at the same temperature and condition. The components were identified by comparison of their retention indices (RI, DB-5) and mass fragmentation with those reported in the literature [10]. Percentage of each component was calculated on the basis of the peak area.

#### **Results and Discussion**

The young leaves of the plant were gathered from a plant in the natural growth area and immediately put in the liquid nitrogen and transferred to the laboratory for further investigations as well. Then after the RNA extraction and cDNA preparation, PCR procedures were down with degenerate primers designed according to the conserved amino acid sequence in various plant terpene synthases. Sequence analysis was performed by using the cDNA provided from the young leaves of *D. kotschy* as a template. As a matter of fact, the obtained data from partial cloning revealed the core sequence containing the 3'-flanking region of limonene synthase. From one side, the partial sequence was similar to the monoterpene synthase genes reported in Gen Bank in advance.

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M.longifolia      -----GAAAAACA-TAGAAAGAGAGCGGAAGG---AAAGATTAATCATGGCTCTCAA 48
P.citriodora     AATCCAAGAAAAATAACAGATAAAAAAAGGGAAATCTAAAGATTAATC---GATCTCAA 57
L.angustifolia_lin_ -----ATGTCGATCA-----ATA- 13
D. kotschyi-lim- -----ATGCTATCA-----TTAG 14
                      * * *** *

M.longifolia      AGTGTTTAGTGTGCAACTCAAATGGCGATTCTAGCAAGCTAACGAGATGTCTTC--AA 106
P.citriodora     AATGTATACCGGTGTGATGAATATGGCGTTTCCTATGAAGCCAGCTAATTATCTTCATAA 117
L.angustifolia_lin_ -----TCAACATG-----CCTGC--AGCC----GCCGTCCTC---- 39
D. kotschyi-lim-  CATGCATGTGGGAATCCTTAATAGG-----CCTGC--AGCTTA-TAACCATCTTCGCAA 65
                      * * *   ***   ***   * * *

M.longifolia      CCCTCACACTTGAARATCCTCTCCAAAATTGT---TAT-----CTAGCACTA--ACA 152
P.citriodora     CTCCGGCAGTAGCAA--CTCTTCAAAATGTGCGGTGTCTCTTCTACTAGTACTAGAGCA 175
L.angustifolia_lin_ -----CGCC-----CT---TTT---C- 49
D. kotschyi-lim-  CTTGGACAGGAGAG-----CTTCAAAGCCGCGCCATGT-----CT---CTT---CT 105
                      **   * *

M.longifolia      GTAGTAG-TCGGTCTCGCCTCCGCTGTGTATTGCTCCTCCTCGCAACT-----C 199
P.citriodora     GCTACAGCTCGCCTCCGCTCCGGCTGCGTTGCTCGTT---GCAACT-----C 220
L.angustifolia_lin_ GCTGCT-----C---ACAACATACA-----T 66
D. kotschyi-lim-  ACTGCCCGCCCACTCGCCTCCGGGTTCTTGCGCCAC---ACAACTAGAAATTAAGTCC 162
                      *****

M.longifolia      ACTACTGAG---AGACGATCCGGAACTACAACCCTTCTCGTTGGGATGTGCAATTCATC 256
P.citriodora     AGTGATCAA---CGACGATCTGGAACTACAGTCCTTCTTTTGGAATACCGATTATATT 277
L.angustifolia_lin_ GTCGATGAAACCCGACGCTCCGGAACTACCGCCCTCGGCTGGGATTCGAACACTACATC 126
D. kotschyi-lim-  GTCGATGAAACCCGACGCTCCGGAACTACAACCCTACCGCTGGGATTTCAACTACATC 222
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M.longifolia      CAATCCCTCCACAGTGATTATGAGGAGGACAAACACGCGATTAGGGCTTCT---GAGCTG 313
P.citriodora     CTATCTCTCAACTGTGACTATGAGGACGAGAGACGCATGAGAGGGGCTGCTGGTGAGCTG 337
L.angustifolia_lin_ CAATCTCTCAATCTCAGTATAAGGAAAAGAAGTCTGACAAAGGCTAGAA---GGGCTG 183
D. kotschyi-lim-  CAATCCCTCGACAATCAGTATAAGGAAAAGAGAGGTACTCGACAAGACACGCT---GAGCTG 279
                      * * * * * * * * * * * * * * * * * * * *

M.longifolia      GTCACCTTGGTGAAGATGGAATTGGAGAAGAAACGGATCATATTGACAACCTTGAGTTG 373
P.citriodora     GTTGAGCAAGTGAAGATGCTGATGGAGAAAGAAACAGATCCTATTGTACAGCTTGAGTTG 397
L.angustifolia_lin_ ATTGAGCAAGTGAAGGAACTGAAGGGGACAAAATGGAGGCTGTTCAACAATTGGAGTTG 243
D. kotschyi-lim-  ACTGTGCAAGTGAAGAAGCTGCTGGAGGAAGAAATGGAAGCGGTTCAAAGTTGGAATTG 339
                      *****   * * * * * * * * * *

M.longifolia      ATCGATGACTTGACAGAGGATGGGGCTGTCCGATCATTTCAGAATGAGTTCAAAGAAATC 433

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**Figure 1.** Alignment of nucleotide sequences (cDNA) of the isolated monoterpene synthase (*D. kotschyi*) with other limonene synthases (*M. longifolia* and *P. citriodora*) and linalool synthase of lavender (*L. angustifolia\_lin*); the above mentioned alignment was performed by ClustalW software (<http://www.ch.embnet.org/index.html>).

P.citriodora	ATTGATGACCTTCAAAGCTGGCTCTCTCTCACTATTTTCGAGAAAGAAATCAAGGAAATA	457
L.angustifolia_lin_	ATTGATGACTCGCAGAATCTGGGATTATCATATATTTTCAAGATAAAATTAACATATC	303
D. kotschy-lim-	ATTGAGGATTTGAAGAACCTGGGAATATCTTACCATTTAAGGACAATATCCAACAGAT	399
	** ** ** * * ** * ** * * ** * * ** * * **	
M.longifolia	TTGTCCTCTATATATCTCGACCAT---CACTATTACAAGAACCCTTTTCCAAAAGAAGAA	490
P.citriodora	TTATTCA---ACATCAGTACTATA--TATGATGACAAGAAC-----AGGGAG	499
L.angustifolia_lin_	TTGAATTTGATATATAATGATCACAATATTTTACGATAGTGA-----AGCTGAAGGA	357
D. kotschy-lim-	TAAATCAAATATATAATGAGCACAAATGTTGCCACAACAGTGA-----AGTGAAGAA	453
	** * ** * * ** * * ** * * ** * *	
M.longifolia	AGGGATCTCTACTCCACATCTCTTGCAATTTAGGCTCCTCAGAGAACATG-TTTTCAAGTC	549
P.citriodora	AGGGATTTGACTCGACAACCTTGCACTCAGACTTCTTAGACAACACGGTTATCAAGTT	559
L.angustifolia_lin_	ATGGATTTGATATTTTACAGCTCTTGCAATTTAGACTCTTTAGACAACATGGTTTTAAAGTC	417
D. kotschy-lim-	AAGGATTTGATTTTACGGCTCTTCGATTCCGACTCCTTAGACAACAGGGTTTTGAAGTC	513
	* ** * * * * * ** * ** * * ** * ** * ** * ** * ** * ** * ** *	
M.longifolia	GCACAAGAGGTATTCGACAGTTTCAAGAACGAGGAGGGTG---AGTTCAAAGAAAGCCTT	606
P.citriodora	CCTCAAGAGTTGTTTCGAATGTTTCAAGAACGACAAGGGTG---AGTTCAAAGAAAGCCTT	616
L.angustifolia_lin_	TCCAAGAAGTATTTGATCGTTTCAAGAACGAGAATGGTACGATTTTCAAGC---AC---	471
D. kotschy-lim-	TCTCAAGAAGTATTTGATCATTTCAGAACGAGAAGGGTACAGATTTCAAGCCAAACCTT	573
	* ** * ** *	
M.longifolia	AGCGACGACACCAGAGGATTTGTTGCAACTGTATGAAGCTTCCTTTCTGTTGACGGAAGGC	666
P.citriodora	AGCAATGACACCAAAGGATTTGTTGCAACTGTACGAAGCTTCATTCCTATTGACAGAAGGC	676
L.angustifolia_lin_	---GACGATACAAAGGGATTGTTGCAAGCTCTACGAAGCATCATTCCCTAGTGCCGAGAAGGC	528
D. kotschy-lim-	GCTGACGATACTAAAGGACTATTGCAACTTACGAAGCATCTTTCTATTAGAGAAGCT	633
	* *	
M.longifolia	GAAACCACGCTCGAGTACGCGAGGGAATTCGCCACCAAATTTTGGAGGAAAGAGTGAA-	725
P.citriodora	GAAACAACACTCGAGTTAGCAAGAGAATTTGCCACCAAATTTCTGCAGGAAAAAGAAAA	736
L.angustifolia_lin_	GAAGAGACACTCGAACACGACGAGAATTTGCCACCAAATCCCTACAAAGAAAAGTGA-	587
D. kotschy-lim-	GAAGATACACTTGAGTTAGCTCGACAATTTCAACCAAATTAAGTGAAGGAAAAAGTGA-	692
	** *	
M.longifolia	--CGAGGGTGGTGTGAT--GGC-GAC-----CTTTAACAAGAAT-CGCATATCTTT	773
P.citriodora	CACAATATTTGATGATGAT--GAT-GACACTAATCTTATATCGTGTGTGCGC-CACTCTTT	792
L.angustifolia_lin_	--TGAGGATGGTGATGG---AATTGACGCCAATATCGAATCATGGATCCGC-CACTCTCT	641
D. kotschy-lim-	--TGAGAATGGTGATGATAAATAGAGGATAATCTATTATTATGGATTGCG-CGTCTCTT	749
	* *	
M.longifolia	GGACATCCCCTTCATTGGAGGATTAAGAGCCAAATGCACCTGCGTGGATCGAATGGTA	833
P.citriodora	GGACATGCCAATTTATTGGAGGATTCARAGGC-AAATGCAAGGTGGTGGATTTCATGCCTA	851
L.angustifolia_lin_	GGAGATCCCCTTCATTGGAGGGCTCAGAGGCTAGAGGCGAGATGGTTCTAGATGCTTA	701
D. kotschy-lim-	GGAGCTCCCCTTCATTGGAGGGTGCAARAGGCTAGAAGCAAGAGGGTCTTGATGCTTA	809

**Figure 1. Continued**

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M.longifolia TAGGAAGAGGCCCGACATGAATCCAGTAGTGTGGAGCTTGCCATACTCGACTTAAATAT 893
P.citriodora TAATAGGAGAACTCACATTAATCCACTTGTGTGGAGCTTCCAAACTGACTTCAATAT 911
L.angustifolia_lin_ TGCGAGAAGGCCCGACATGAACCCCGTTATCTTCGAGCTTGCTAAACTCAACTTCAATAT 761
D. kotschyi-lim- CGTTAGAAGGCCCGACATGAATCCAATGTTTTTGGAGCTCGCCAAACTCGACTTCAATAT 869
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M.longifolia TGTTCAAGCACAGTTTCAAGAAGAGCTTAAAGAATCCTTCAGGTGGTGGAGAAATACTGG 953
P.citriodora TATTCAAGCACAAATATCAGCAAGAACTTAAACAAGACTTAAGGTGGTGGAGAAATACATG 971
L.angustifolia_lin_ TGTTCAAGCACAAACACAAGAAGAAATGAAAGCTCTCTCGAGGTGGTGGAGTAGTTAGG 821
D. kotschyi-lim- TACCAAGCAACACAACAAGAAGAAGAACTGAAAGATCTCTCGAGGTGGTGGAAATAGTACAGG 929
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M.longifolia GTTGTGTTGAGAAGCTGCCTTCGCAAGGGATAGACTGGTGGAAATGCTACTTTTGGGAATAC 1013
P.citriodora CATTGCTGAGAAGCTTCCCTTTGCAAGGGATAGGCTCGTGGAAATCCTACTTTTGGAGTAC 1031
L.angustifolia_lin_ CCTAGCTGAAAACTCCCATTTGTGAGGGATAGGCTTGTGGAAGCTACTTTTGGGCTAT 881
D. kotschyi-lim- CCTTGCCGAAAACTCCCATTCGCGAGGGATCGGGTGTGTGAGTCTACTTCTGGGCAAT 989
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M.longifolia TGGGATCATCGAGCCACGTCAGCATGCAAGTGAAGGATAATGATGGGCAAAGTCAACGC 1073
P.citriodora TGGGATCATTCAGCCTCGTCAACATGAAATGCAAGGATAATGATGGCAAAGCTCTTGC 1091
L.angustifolia_lin_ TCCACTCTTTGAGCCTCATCAATATGGATATCAAGAAAAGTGGCCACCAAGATCATTAAC 941
D. kotschyi-lim- GGGAACTTTGAGCCTCATCAATATGGTTATCAGAGAGAAGTGTGCGCAAGATTATTGC 1049
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M.longifolia TCTGATTACGGTGATCGATGATATTTATGATGCTATGGCACCTTAGAAGAAGTCAACAA 1133
P.citriodora TCTAATAACCACGTTAGATGATGTTTACGATGCTACGGTACCTTAGAAGAAGTCAAGCT 1151
L.angustifolia_lin_ CCTAATCACATCTTTAGACGATGTTTACGATATCTATGGCACGTTAGATGAATGGCAACT 1001
D. kotschyi-lim- TCTAGCAACAGTTGTAGATGATGTTTACGATGATATGGTACGTTAGAGGAAGTGGAACT 1109
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M.longifolia ATTCACTGACCTCATTCGAA-GATGGGATATAAACTCAATCGACCAACTTCCCGATTACA 1192
P.citriodora GTTCACCGAGGCGATTAGAA-GATGGGAAATCAGTTCAAATGACCAACTTCCCTAACTACA 1210
L.angustifolia_lin_ ATTTACGAAC-TTATTTGAAAGATGGGATAATGCATCAATCGGCCGACTTCCCTGAATACT 1060
D. kotschyi-lim- ATTTACAGATGCCATTCGGA-GATGGGATCGTGAATCAATCGACCAACTTCCCTACTACA 1168
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M.longifolia TGCAACTGTGCTTTCTTGCACTCAACAAGTTCGTCGATGATAACATCGTACGATGTTATGA 1252
P.citriodora TGCAACTGTGCTTTCTTACAATCAACAAGTTCGTCGACGATACTGCCTACGATGTCATGA 1270
L.angustifolia_lin_ TGCAATTGTTCTATTTGCAATCCACAAGTTCGTTTCCGAGGTGGCTACGACATTCTCA 1120
D. kotschyi-lim- TGCAGCTATGCTTTTGTGACTGTCAACAAGTTCGTTTTCGAGCTGCTCATGATGTTCTTA 1228
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M.longifolia AGGAGAAAGGCGTCAACGTTATACCCTACCTGCGCAATCGTGGGTGGATTGGCGGATA 1312

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Figure 1. Continued

P.citriodora	AAGAGAAAGATATCAACATCATCCCCTATCTACGAAAATCGTGGGTGGATTGGCTGAGG	1330
L.angustifolia_lin_	AAGAAAAGGGTTTCACTAGTATTGTATATTTACAGAGATC-TGGGTGGATTGGCTAAAAG	1179
D. kotschy-lim-	AGGATAAGAGTTTCAACTGCTTACCACATTTACAGAGATCGTGGCTAGACTGGCTGAAG	1288
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M.longifolia	AGTATATGGTAGAGGCACGGTGGTCTACGGCGGACACAAACCAAGTTTGGAAAGAGTATT	1372
P.citriodora	CATATCTGGTAGAGGCAAAATGGTTCATGGCGGATATAAACCAAATTTG-----	1380
L.angustifolia_lin_	GATACCTAAAAGAGGCCAAG-GGTACAATAGTGGATACACGCCAAGCCTCGAGGAATATT	1238
D. kotschy-lim-	CATATCTGTGCGAGGCTAAGTGGTACCACAGTAGATATACACCGAGCCTCGAGGAATATC	1348
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M.longifolia	TGGAGAACTCATGGCAGTCGATAAGTGGGCCCTGTA-TGTTAACGCACATATTCTTCCGA	1431
P.citriodora	-----	
L.angustifolia_lin_	TCGACAACGCATTTCATGACAAT-AGGGGCCCTCCGGTACTATCGCAAGCTTATTTTACA	1297
D. kotschy-lim-	TCAATATGCAAGAGTTTCAGTTACGTGCCACTA-TAGTTTCACAAATGTAAGTTTGC	1407
M.longifolia	GTAACAGATTCGTTCCAAAGGAGACCGTCGACAGTTTGTACAAATACCACGATTTAGTT	1491
P.citriodora	-----	
L.angustifolia_lin_	TTA-----GGAAGCTCATCATCGAGAGCATGTACGAATATGACAACATACTT	1344
D. kotschy-lim-	TTACCAATTCGATAGAGAAAACCGGTCATCGAGATCATGTACAAATACCACGACATACTT	1467
M.longifolia	CGTTGGTCATCCTTCGTTCTGCGGCTTGCTGATGATCTGGGAACCTCGGTGGAAGAGGTG	1551
P.citriodora	-----	
L.angustifolia_lin_	CGCGTTTCGGGAATGCTCGTGAGGCTTCCCGATGACCTAGGAACATCATCGTTCCGAGATG	1404
D. kotschy-lim-	TACCTCTCAGGAATGCTTCTAAGGCTTCTGTGATGATCTAGGAACAGCATCGTTTGAGTTG	1527
M.longifolia	AGCAGAGGCGATGTGCCAAATCACTTCAGTGTACATGAGTGACTACAATGCATCGGAG	1611
P.citriodora	-----	
L.angustifolia_lin_	GAGAGAGGCGACGTGCCGAAATCGGTCCAGCTATACATGAAGGAAACAAATGCTACGGAG	1464
D. kotschy-lim-	AAGAGAGGTGATGTGCAAAAAGCAGTCCAGTGTATATGAAGGAAAGAAATGTTCTCGAA	1587
M.longifolia	GCGGAGGCGCGGAAGCACGTGAAATGGCTGATAGCGGAGGTGTGGAAGAAGATGAATGCG	1671
P.citriodora	-----	
L.angustifolia_lin_	GAGGAGGCGGTGGAGCACGTGAGGTTTTTGAATCGGGAGGCGTGAAGAAGATGAACACG	1524
D. kotschy-lim-	AATGAGGCACGAGAACATGTGAAGTTTCTGATTCGGGAGGCGTGAAGCAGATAAACACC	1647
M.longifolia	GAGAGGGTGTGGAAGGATTCTCCATTCGGCAA--GATTTTATAGGATGTGCAGCTGATTT	1730
P.citriodora	-----	
L.angustifolia_lin_	GCGGAGGCGCGGTTGATTCTCCGTT-AGTGAGTGACGTGGTGGCGGTGGCGGCGAATCT	1583
D. kotschy-lim-	GCGATGGCGACCG---ATTGTCCATTTACTGAA-GATTTTGTGTGGCTGCAGCGAATCT	1703

Figure 1. Continued

M.longifolia	AGGAAGGA-GGCGCAGTTGATGTACCATAATGGAGATGGGCACGGCACACAACATCCTAT	1789
P.citriodora	-----	
L.angustifolia_lin_	TGGAAGGGCGCGCAGTTTATGTATTTTCGACGGAGATGGTA-----ACCAGTCTAG	1634
D. kotschyi-lim-	TGGAAGAGTGGCGAATTTTGTGTACGTCGACGGAGATGGTTTTGGCGTGCAACACTCAA	1763
M.longifolia	AATACATCAACAAATGACCAGAACCTTATTCGAGCCCTTTCATGAGAGGTGATGATGAT	1849
P.citriodora	-----	
L.angustifolia_lin_	TTTGCAGCAGTGGATTGTGAGCATGCTGTTCGAGCCGTACGCATGA-----	1680
D. kotschyi-lim-	AATATATGAACAGATTGGAACCCCTGATGTTCGAGCCATATCCCTAA-----	1809
M.longifolia	GAGCCATCGTTTACTTACTTAAATCTACCAAAGTTTTTCGAAGGCATAGTTTGTAAATC	1909
P.citriodora	-----	
L.angustifolia_lin_	-----	
D. kotschyi-lim-	-----	
M.longifolia	TTCAAGCACCAATGGAATAAGGAGAATCGGCTCAAACAACGTGGCATTGGCCACCACGT	1969
P.citriodora	-----	
L.angustifolia_lin_	-----	
L.angustifolia_lim_	-----	
M.longifolia	GAGCACAAGGAGAGTCTGTCGTCGTTTATGGATGAACTATTCAATTTTATGCATGTAATA	2029
P.citriodora	-----	
L.angustifolia_lin_	-----	
L.angustifolia_lim_	-----	
M.longifolia	ATTAAGTTCAAGTTCAAGAGCCTTCTGCATATTTAACTATGTACTTG	2076
P.citriodora	-----	
L.angustifolia_lin_	-----	
L.angustifolia_lim_	-----	

Figure 1. Continued

On the other side, the 5'- and 3'-ends were cloned by RACE-PCR to get the full-length sequences. To reach the full length of limonene synthase, the 5'-RACE method was employed to complete the remained 5'-region (figure 1). The bibliography and required sequence alignments using NCBI finally revealed that the transcript from mRNA may identical as LaLIMS, which was already reported from *Lavandula* species [9]. The open reading frame of the above mentioned limonene synthase consists of 1809 bp, coding for protein with 602 amino acids and the predicted molecular mass of 70.3 kDa. Literature review shows two characteristic motives for monoterpene synthases as DDxxD and

(N,D)D(L,I,V)x(S,T)xxxE, which are reported to be completely conserved in limonene synthase sequences [9]. These motives are essential for substrate (*e.g.* Mn<sup>2+</sup>, Mg<sup>2+</sup>) binding and ionization [11]. A distinguished part of the active site of such enzymes, which are frequently found in monoterpene synthases, is LQLYEASFLL and well conserved in LaLIMS [12].

To the best of our knowledge, limonene is a cyclic monoterpene distributed in the plants. Limonene is going through the cyclization of terpenoids. Its biosynthesis is getting started from geranyl pyrophosphate (GPP) that is a usual precursor for all monoterpenes (figure 2). Furthermore, limonene synthases can progress



the cyclization of GPP into limonene and have been found in *Perilla*, *Mentha* and *Abies* genus of Lamiaceae [6,13,14]. Actually, terpene synthases have been cloned from different species and also the phylogenetic distances among them have been well documented. As a matter of fact, they are classified into six groups TPS a-f. Nevertheless, there are a few differences between these groups, some conserved motifs are also

identified in all TPSs such as mTPSs signature arginine-rich N-terminal RR(x8) W motif [15,16].

On the other side, head space SPME-GC-MS analysis of the aroma profile of *D. kotschyi* exhibits that geranial (37.2%), limonene-10-al (28.5%), limonene (20.1%) and 1,1-dimethoxy decane (14.5%) were found as the major compounds of this plant (table 1).

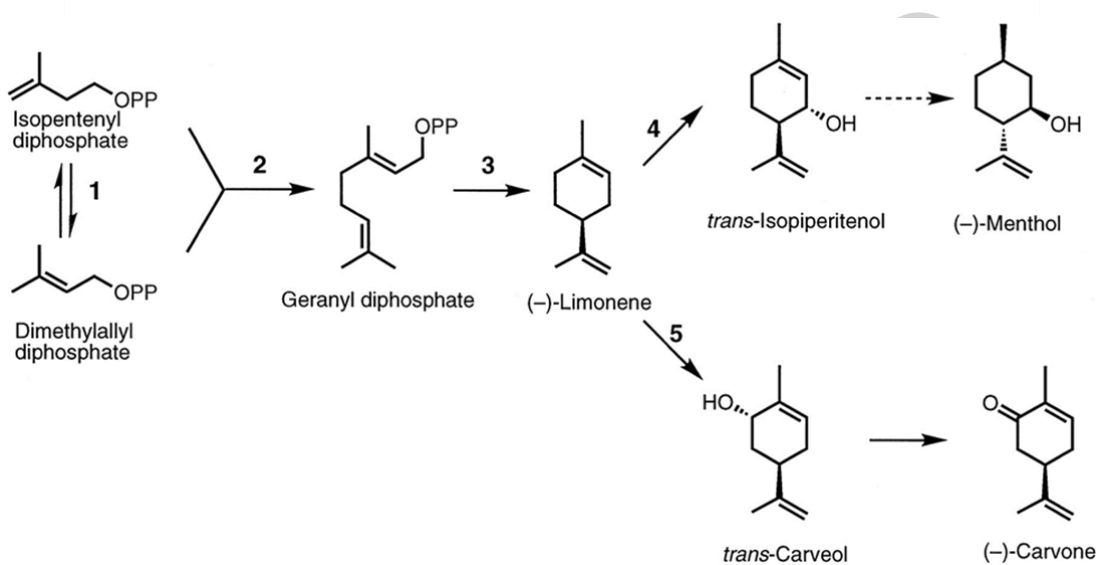


Figure 2. Biosynthetic pathway for limonene and its derivatives in the plants



Figure 3. *Dracocephalum kotschyi*, Touchal, Alborz Mounts, Tehran (altitude 3000 m). The picture is obtained from: <http://www.gloria.ac.at/?l=430>

On the other hand, the aroma profile of the major compounds is in agreement with those reported in the literature for this plant [2, 17, 18], however there are some variations in the quantities.

**Table 1.** Chemical composition of the aroma of *D. kotschy* young leaves obtained by SPME.

Compounds	Retention Indices	
	HP-5	Percentage (%)
geraniol	987	37.2
limonene	1030	20.1
limonene-10-al	1034	28.5
1,1-dimethoxy decane	-	14.5
cyclic monoterpenes	-	48.6
total	-	90.3

Obviously, different factors like genotype, altitude, microclimate and cultivation condition may influence on the composition of the essential oils for the same species [19,20]. In conclusion, *D. kotschy* (figure 3) is one of the important sources of limonene and its related cyclic monoterpenes with considerable bioactivities and can be used for biosynthetic pathways studies.

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