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Original article

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

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Abstract

Dracocephalum kotschyi (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. kotschyi* 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 kotschyi*, 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. kotschyi* is growing wildly 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

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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 α- and β-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_2-C_3 (*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)-(+)-α-pinene, β-myrcene and traces of α-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 µL), amm primer (0.3 µL), degenerate primer (0.6 µL), dNTP (0.1 mM, 2 µL), DNA template (1 µL) 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 µL) was amplified with terminal deoxynucleotidyltransferase (1 µL), dCTP (10 mM, 5 µL) and 16 µL 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 µL) 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 µL), oligo (dT) anchor primer for 3'-RACE (0.3 µL), gene specific primer (10 µM, 0.3 µL), KOD Dash DNA polymerase (0.8 µL) 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 × 0.25 mm i.d., 0.25 µm 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_8-C_{25}) 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. kotschyi* 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.

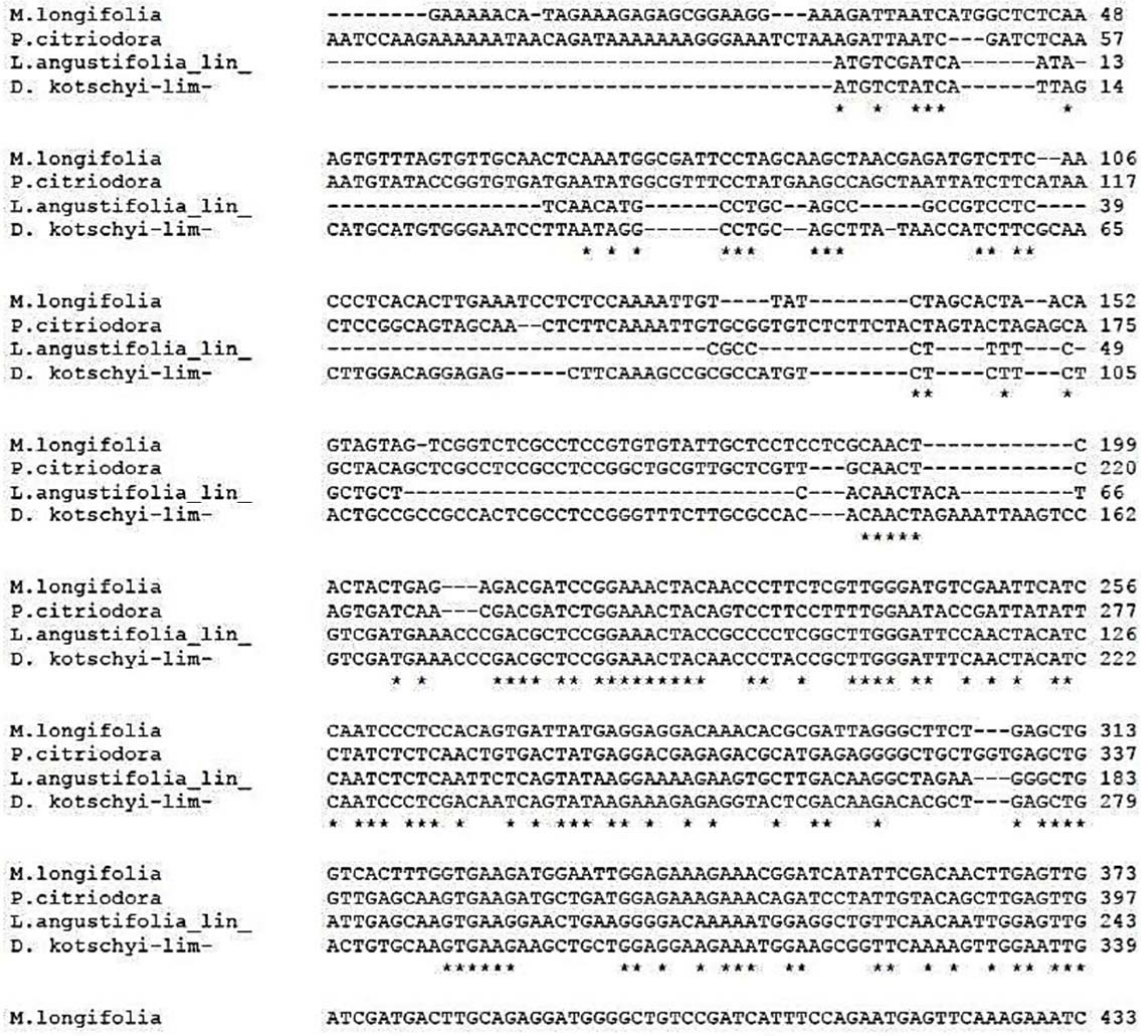

Figure 1. Alignment of nucleotide sequences (cDNA) of the isolated monoterpene synthase (*D. kotschyi*) with other limonene synthases (*M. longifolia and P. citriodora*) and linalool synthase of lavender (*L. angustifolia_lin*); the above mentioned alignment was performed by ClustalW software (http://www.ch.embnet.org/index.html).

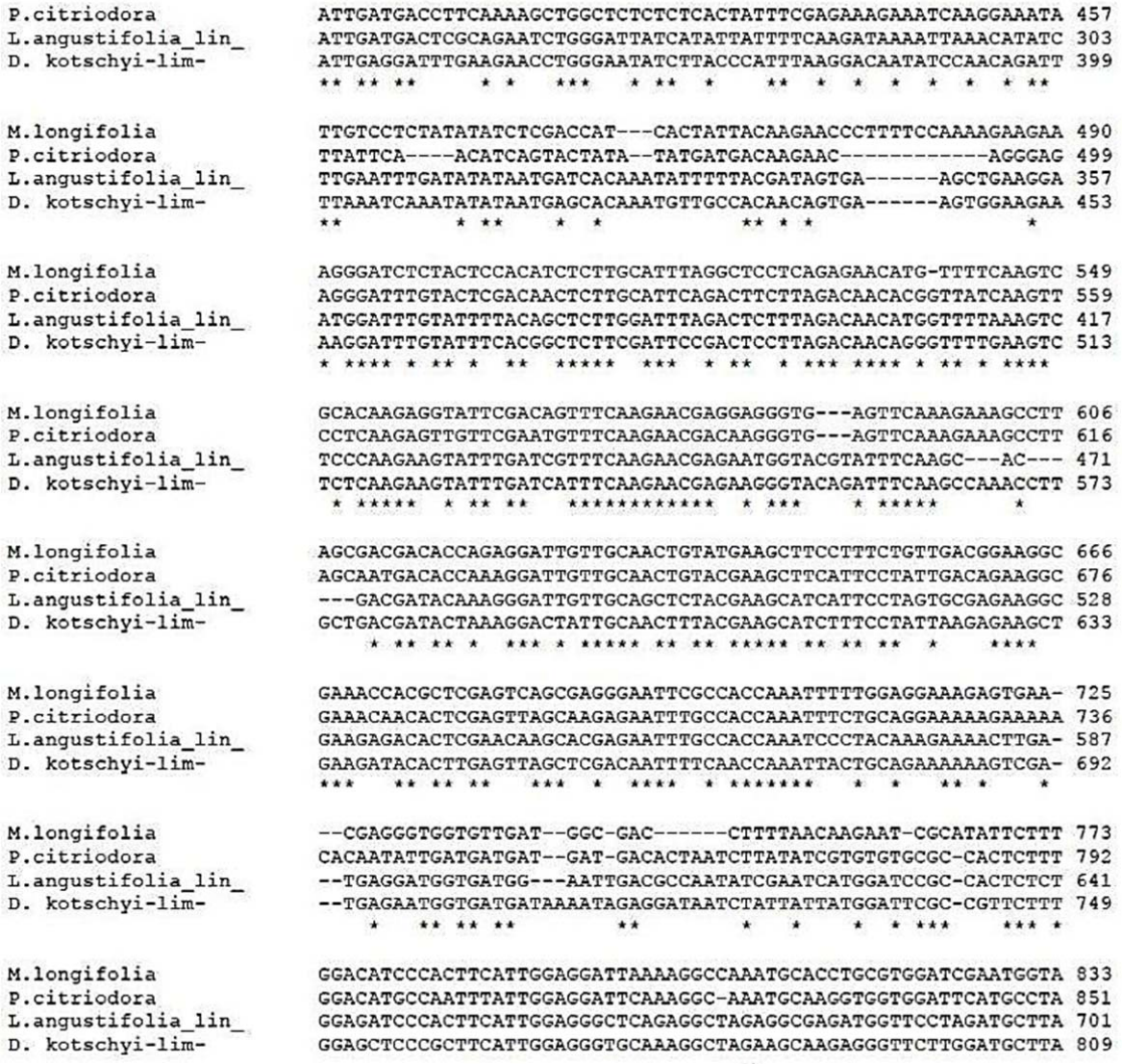

Figure 1. Continued

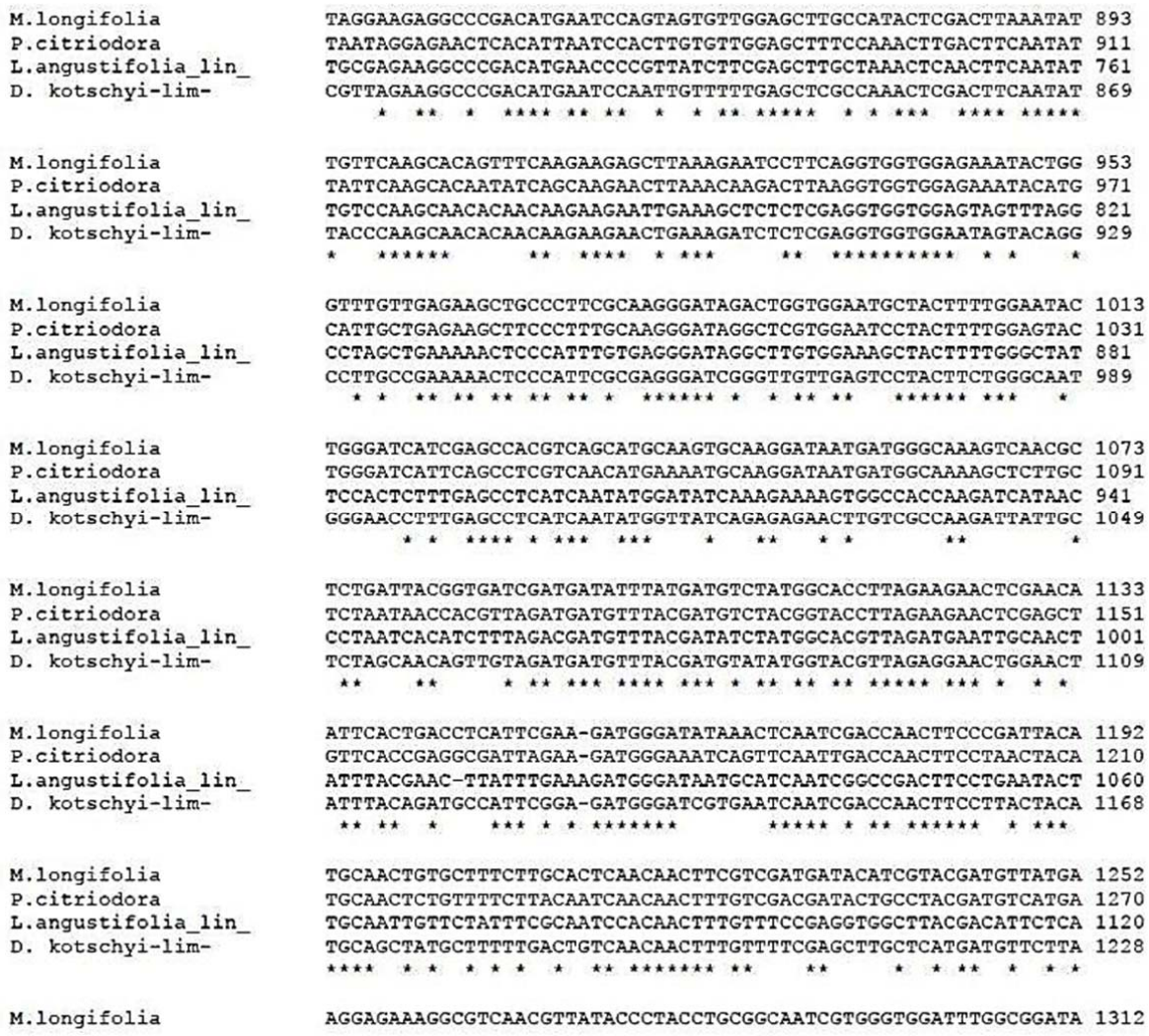

Figure 1. Continued

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P.citriodora            AAGAGAAAGATATCAACATCATCCCGTATCTACGAAAATCGTGGGTGGATTTGGCTGAGG 1330
L.angustifolia_lin_     AAGAAAAGGGTTTCACTAGTATTGTATATTTACAGAGATC-TGGGTGGATTTGCTAAAAG 1179
D. kotschyi-lim-        AGGATAAGAGTTTCAACTGCTTACCACATTTACAGAGATCGTGGCTAGACTTGGCTGAAG 1288
                        * ** **     ***      *     *  * *    *** *** * ** ***    *

M.longifolia            AGTATATGGTAGAGGCACGGTGGTTCTACGGCGGACACAAACCAAGTTTGGAAGAGTATT 1372
P.citriodora            CATATCTGGTAGAGGCGAAATGGTTCTATGGCGGATATAAACCAAATTTG---------- 1380
L.angustifolia_lin_     GATACCTAAAAGAGGCAAAG-GGTACAATAGTGGATACACGCCAAGCCTCGAGGAATATT 1238
D. kotschyi-lim-        CATATCTTGTCGAGGCTAAGTGGTACCACAGTAGATATACACCGAGCCTCGAGGAATATC 1348
                          **  *    *****     *** * *  *  ** * *  ** *   *

M.longifolia            TGGAGAACTCATGGCAGTCGATAAGTGGGCCCTGTA-TGTTAACGCACATATTCTTCCGA 1431
P.citriodora            ------------------------------------------------------------
L.angustifolia_lin_     TCGACAACGCATTCATGACAAT-AGGGGCCCCTCCGGTACTATCGCAAGCTTATTTCACA 1297
D. kotschyi-lim-        TCAATATTGCAAGAGTTTCAGTTACGTGTCCCACTA-TAGTTTCACAAATGTACTTTGCA 1407

M.longifolia            GTAACAGATTCGTTCACAAAGGAGACCGTCGACAGTTTGTACAAATACCACGATTTAGTT 1491
P.citriodora            ------------------------------------------------------------
L.angustifolia_lin_     TTA-------------GGAAGCTCATCATCGAGAGCATGTACGAATATGACAACATACTT 1344
D. kotschyi-lim-        TTACCAATTCCGATAGAGAAACCGGTCATCGAGATCATGTACAAATACCACGACATACTT 1467

M.longifolia            CGTTGGTCATCCTTCGTTCTGCGGCTTGCTGATGATCTGGGAACCTCGGTGGAAGAGGTG 1551
P.citriodora            ------------------------------------------------------------
L.angustifolia_lin_     CGCGTTTCGGGAATGCTCGTGAGGCTTCCCGATGACCTAGGAACATCATCGTTCGAGATG 1404
D. kotschyi-lim-        TACCTCTCAGGAATGCTTCTAAGGCTTCCTGATGATCTAGGAACAGCATCGTTTGAGTTG 1527

M.longifolia            AGCAGAGGCGATGTGCCGAAATCACTTCAGTGCTACATGAGTGACTACAATGCATCGGAG 1611
P.citriodora            ------------------------------------------------------------
L.angustifolia_lin_     GAGAGAGGCGACGTGCCGAAATCGGTCCAGCTATACATGAAGGAAACAAATGCTACGGAG 1464
D. kotschyi-lim-        AAGAGAGGTGATGTGCAAAAAGCAGTCCAGTGTTATATGAAGGAAAGAAATGTTCCTGAA 1587

M.longifolia            GCGGAGGCGCGGAAGCACGTGAAATGGCTGATAGCGGAGGTGTGGAAGAAGATGAATGCG 1671
P.citriodora            ------------------------------------------------------------
L.angustifolia_lin_     GAGGAGGCGGTGGAGCACGTGAGGTTTTTGAATCGGGAGGCGTGGAAGAAGATGAACACG 1524
D. kotschyi-lim-        AATGAGGCACGAGAACATGTGAAGTTTCTGATTCGGGAGGCGTCGAAGCAGATAAACACC 1647

M.longifolia            GAGAGGGTGTCGAAGGATTCTCCATTCGGCAAA-GATTTTATAGGATGTGCAGCTGATTT 1730
P.citriodora            ------------------------------------------------------------
L.angustifolia_lin_     GCGGAGGCGGCCGGTGATTCTCCGTT-AGTGAGTGACGTGGTGGCGGTGGCGGCGAATCT 1583
D. kotschyi-lim-        GCGATGGCGACCG---ATTGTCCATTTACTGAA-GATTTTGCTGTGGCTGCAGCGAATCT 1703
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Figure 1. Continued

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M.longifolia            AGGAAGGA-GGCGCAGTTGATGTACCATAATGGAGATGGGCACGGCACACAACATCCTAT 1789
P.citriodora            ------------------------------------------------------------
L.angustifolia_lin_     TGGAAGGGCGGCGCAGTTTATGTATTTCGACGGAGATGGTA---------ACCAGTCTAG 1634
D. kotschyi-lim-        TGGAAGAGTGGCGAATTTTGTGTACGTCGACGGAGATGGTTTTGGCGTGCAACACTCAAA 1763

M.longifolia            AATACATCAACAAATGACCAGAACCTTATTCGAGCCCTTTGCATGAGAGGTGATGATGAT 1849
P.citriodora            ------------------------------------------------------------
L.angustifolia_lin_     TTTGCAGCAGTGGATTGTGAGCATGCTGTTCGAGCCGTACGCATGA-------------- 1680
D. kotschyi-lim-        AATATATGAACAGATTGGAACCCTGATGTTCGAGCCATATCCCTAA-------------- 1809

M.longifolia            GAGCCATCGTTTACTTACTTAAATTCTACCAAAGTTTTTCGAAGGCATAGTTTGTAATTC 1909
P.citriodora            ------------------------------------------------------------
L.angustifolia_lin_     ------------------------------------------------------------
D. kotschyi-lim-        ------------------------------------------------------------

M.longifolia            TTCAAGCACCAAATGGAATAAGGAGAATCGGCTCAAACAACGTGGCATTTGCCACCACGT 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_     -----------------------------------------------
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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^{2+}, Mg^{2+}) 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. Limonen 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).

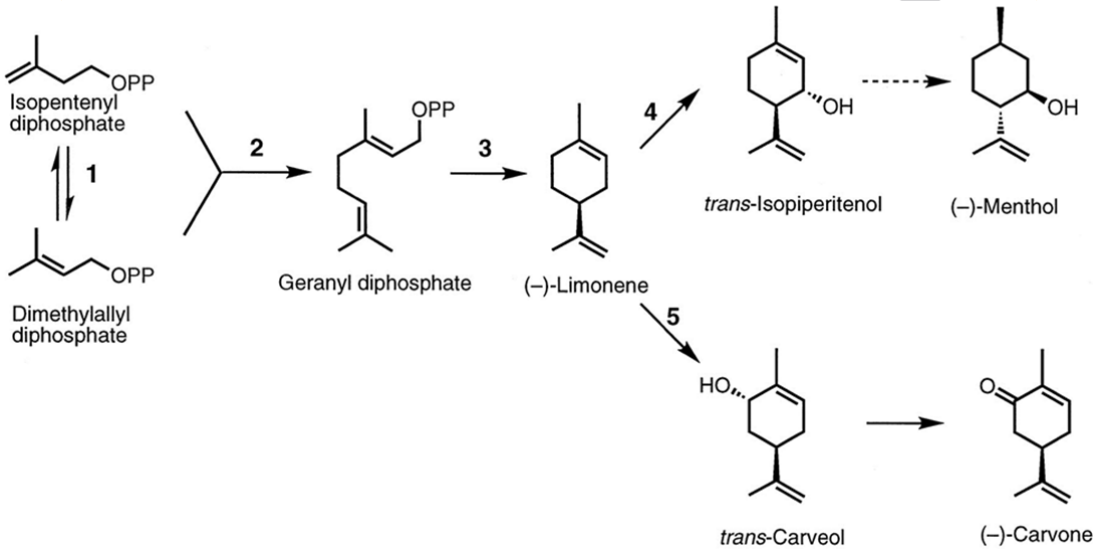

Figure 2. Biosynthetic pathway for limonene and its derivatives in the plants

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. kotschyi* 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. kotschyi* (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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