Presence of monoterpene synthase in four Labiatae species and Solid-Phase Microextraction- Gas chromatography-Mass Spectroscopy analysis of their aroma profiles

Soodabeh Saeidnia[1,2], Ahmad Reza Gohari[1,2], Azita Haddadi[2], Gholamreza Amin[1,3], Marjan Nikan[1], Abbass Hadjiakhoondi[1,3]

[1]Medicinal Plants Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, [2]Division of Pharmacy, College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, Canada, [3]Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

Submitted: 27-07-2013 Revised: 17-08-2013 Published: 18-03-2014

ABSTRACT

Background: The family Lamiaceae (Labiatae) has included some medicinal plants. some monoterpene synthases, including linalool and limonene synthases, have been cloned and functionally characterized from several plants of Labiatae family. **Materials and Methods:** In this study, presence of linalool and limonene synthases, in four species of Labiatae family including *Nepeta cataria*, *Lavandula angustifolia*, *Hyssopus officinalis* and *Salvia sclarea* has been determined by molecular biological techniques together with the Head space SPME – GC-MS analysis of the aroma profile of these species. **Results:** Indicated that none of the plant species produced distinguishable bands with primer pairs related to *d*-limonene synthase. Distinguishable bands around 1800 bp in cDNA samples of *L. angustifolia*, *H. officinalis* and *S. sclarea* were observed regarding to the presence of linalool synthase. Head space SPME-GC-MS analysis of the aroma profiles of the above-mentioned plants showed that linalool (31.0%), linalyl acetate (18.2%), were found as the major compounds of *L. angustifolia*, while geraniol (5.5%), nerol (34.0%) and α- citral (52.0%) were identified as the main compounds of the *N. cataria*. The major components of *H. officinalis* and *S. sclarea* oils were determined as *cis*-pinocamphone (57.3%), and linalool (19.0%), linalyl acetate (51.5%), respectively. **Conclusion:** *H. officinalis* was rich of cyclic monoterpenes, *L. angustifolia, N. cataria* and *S. sclarea* showed considerable amount of linear monoterpenes. The aroma profile of the above-mentioned plants contained low concentration of sesquiterpenes except *N. cataria,* which indicated no sesquiterpene. The profiles of the main components of these plants are in agreement with molecular assays.

Key words: Essential oil, headspace solid phase microextraction, labiatae, monoterpene synthase

Access this article online

Website: www.phcogres.com

DOI: 10.4103/0974-8490.129033

Quick Response Code:

INTRODUCTION

The family Labiateae (Lamiaceae) has 180 genera and about 3500 species. The aerial parts of these plants contain flavonoids, triterpenoids, and monoterpenes, particularly in the flowers and leaves.[1-3] Essential oils of these plants consist of various monoterpenes and sesquiterpenes, as well as their derivatives, such as alcohols, aldehydes, esters, and acetates.[4-6] Terpenoids are formed from the condensation of dimethylallyl pyrophosphate (DMAPP) and isopentyl pyrophosphate (IPP), to give geranyl diphosphate (GPP), farnesyl diphosphate (FPP), or geranyl geranyl diphosphate (GGPP).[7-9] Terpene synthases in plant kingdom may fall into two categories. First class (e.g., limonene synthase) involves in ionization of allylic diphosphate to initiate the reaction, but second class (e.g., copalyl diphosphate synthase (CPS)) acts in double-bond protonation of substrate.[10]

Among the terpene synthases, cyclases enzymes, such as limonene synthase (LMS), have been focused and studied

Address for correspondence:
Dr. Soodabeh Saeidnia, Medicinal Plants Research Center, Tehran University of Medical Sciences, P. O. Box 14155-6451, Tehran, Iran.
E-mail: saeidnia_s@sina.tums.ac.ir

in diverse plant species. LMS contains approximately 600 amino acids and is responsible for catalyzing the formation of limonene.[10] Limonene is a simple cyclic monoterpene, with a chiral center and two enantiomers (*d* and *l*). Although various *d*- and *l*-limonene synthase genes have been identified, there are a few reports about the relationship between gene transcription and biosynthesis during plant growth.[11] Linalool synthase (LIS), involves in biosynthesis of linalool as an acyclic monoterpene alcohol, is not simply classified on the basis of its amino acid sequence, because N-terminal part reveals higher sequence similarity to CPS but C-terminal exhibits higher similarity to LMS.[12,13] Intron positions in LMS-type terpene cyclase genes are very similar to each other.[14]

So far, some monoterpene synthases, including linalool and limonene synthases, have been cloned and functionally characterized from several plants of Labiatae family.[15,16] In the present study, we aimed to determine the presence of two monoterpene synthases, linalool and limonene synthases, in four species of Labiatae family including *Nepeta cataria*, *Lavandula angustifolia*, *Hyssopus officinalis* and *Salvia sclarea* together with the Head space Solid-phase Microextraction - Gas chromatography - Mass spectroscopy analysis of the aroma profile of these species.

MATERIALS AND METHODS

Experimental

Chemical reagents and solvents were purchased from Merck Co. (Germany). Agarose and 1kb DNA size marker were prepared from Invitrogen Co. (UK). RNeasy Plant Mini Kit was prepared from Qiagen (USA). Polymerase chain reactions were performed on a Primus 25 (Peqlab, Germany) thermal cycler. Primers were produced in Cinnagene (Iran).

Plant material

All the plants mentioned here were grown in the Herboratum of Faculty of Pharmacy, Tehran University of Medical Sciences and identified by Dr. Gholamreza Amin (Department of Pharmacognosy) as *Nepeta cataria*, *Lavandula angustifolia*, *Hyssopus officinalis* and *Salvia sclarea*. Young leaves of each species were harvested (during June, 2010) from a plant grown outside in a mini-garden under natural conditions.

Primer designation

Protein sequences of linalool and limonene synthases from *Mentha spicata* (Gen-Bank Accession No. AAC37366), *Mentha citrate* (AAL99381), *Perilla frutescens* (AAL38029), *A. thaliana* (AAO85533) and *Lavandula angustifolia* (ABB73045, ABB73044) were aligned with free ClustalW software and revealed several conserved regions. Based on the DNA and peptide sequences the different primers have been designed and synthesized [Table 1].

cDNA Preparation and PCR

About 200 mg of each plant's leaves were frozen in liquid nitrogen and ground into a fine powder. Total RNA was extracted 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 TTT TTT TTT TTT TTT-3'] designed to have an adaptor sequence at the 5'-end to obtain the cDNA. The obtained cDNAs were employed as the templates in various PCRs with Taq and/or KOD Dash DNA polymerases. The temperature program was started at 94˚C (3 min), followed by 40 cycles: 94˚C for 30 s, 46˚C for 30 s (different annealing temperatures were used for each pair of primers) 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

Table 1: Primers designed according to different terpene synthases gene sequences

Primer	Sequence 5' to 3'	Reference
amm (FW)	GGC CAC GCG TCG ACT AC	[16]
ddmot2(REV)	TAG ATG ATA TTT ACG AT	-
ddmot3(REV)	TAG ATG ATG TTT ACG AT	-
ddmot4(REV)	GAT GAT GTT TAC GAT ATC TAT GGT AC	-
A (REV)	GGA (C/T) T (A/G) (C/T) TG (I) A (I)(C/T) T (I) TA (C/T) GAAGC (A/T) TC,	[16]
B (REV)	GA (C/T) GA (C/T) AT (A/C/T) TA (C/T) GA (C/T) GT (A/C/G/T) TA (C/T) GG (A/C/G/T)	[16]
B'(REV)	ACTGGATTCATGTCTGGTC	[16]
ann (FW)	GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG	[17] [18]
TerpDeg1(FW)	T (AC) C T (GC)(AC) G (AG) C A (AG) C A (GT) GG	[19]
TerpDeg2(FW)	GA (AG) G (AG)(AT) GAA (AG)(ACT) A (CT)(AT)(CT) TIG (plus I)	[19]
TerpDeg3(FW)	GA (CT) GA (CT) (AG) T (CT) T (AG)(CT) G AT (AG) T (GCT) T (AT) TG G	[19]
TerpDeg4(REV)	ACC A (CT) T (GCT)(AT) G C (CT) T C (CT)(AT) (GCT)(CT) A	[19]
GR3'(REV)	GCT GTC AAC GAT ACG CTA CGT AAC G	[19]
GR3'Nested (REV)	CGC TAC GTA ACG GCA TGA CAG TG	[19]
LaLIMS (FW)	AAA GTC GAT GAG AAT GGT GAT GAT	[19]
LaLINS (FW)	TGG CCA CCA AGA TCA TAA CCC TAA TCA C	[19]

the amplified fragment. The PCR was performed by DNA polymerase (0.2 μL), each degenerate forward and reverse primers (0.3 μL, 20 pmol), dNTP (0.1mM, 2 μL), DNA template (1 μL) and appropriate amounts of recommended buffer, DMSO and water. The partial size of monoterpene synthase sequences was estimated by gel electrophoresis. PCR products were run on a 1% (w/v) agarose gel along with a 1 kb DNA size marker, stained by ethidium bromide (0.5 μg/ml) and visualized in a gel documentation system.

SPME- GC-MS analysis

Head space Solid-phase Microextraction (SPME) coupled to gas chromatography and mass spectrometry has been applied for analyzing the essential oil directly evaporated from young leaves of four species. 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 (C8-C25) 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.[20] Percentage of each component was calculated on the basis of the peak area.

RESULTS AND DISCUSSION

In this investigation, PCR method was employed with different primers designed regarding to the conserved amino acid sequence in various Labiatae plant terpene synthases, in order to reveal the presence of limonene and linalool synthases in four Labiatae species: *N. cataria*, *L. angustifolia*, *H. officinalis* and *S. sclarea*. As it is indicated in Table 2, none of the plant species produced distinguishable bands with primer pairs: amm-A, amm-B and ann-B' which were related to *d*-limonene synthase [Table 2]. Limonene is biologically formed from geranyl pyrophosphate (GPP) through linalyl pyrophosphate (LPP). Limonene synthases is thought to promote the cyclization of GPP into limonene and have been previously cloned from *Perilla, Mentha* and *Abies* and occurred *l*-enantiomer, while *d*-limonene synthase was cloned from *Schizonepeta tenuifolia* for the first time.[16] The degenerate forward primers TerpDeg1, TerpDeg2 and TerpDeg3 were designed on the basis of the conserved sequences F (RK)(LI) LRQ (HE) G, E (GD) E (DHS)(TI) L and DD (VI)(YF) D (VI)(YF) G. PCRs using these primers with reverse primer TerpDeg4 resulted in presence of the mentioned sequences in three Labiatae species employed in this study [Table 2]. The results were supported by further PCRs using LaLIMS (forward) and GR3' (reversed) primers, which revealed that limonene synthase should be expressed in all species except *N. cataria*. On the other hand, volatile oils of *L. angustifolia*, *H. officinalis* and *S. sclarea* contained limonene and its derivatives [Table 3].

Table 2: The results obtained from gel electrophoresis of various PCR products (cDNA of *N. cataria, L. angustifolia, H. officinalis and S. sclarea*) with different primer pairs

Primer pairs	*N. cataria*	*L. angustifolia*	*H. officinalis*	*S. sclarea*
amm-A	-	-	-	-
amm-B	-	-	-	-
ann- B'	-	-	-	-
amm-ddmot2	-	+	+	-
amm-ddmot3	+	+	+	-
amm-ddmot4	-	-	+	+
TerpDeg1- TerpDeg4	-	+	+	+
TerpDeg2- TerpDeg4	-	+	+	+
TerpDeg3- TerpDeg4	-	+	+	+
LaLIMS- GR3'	-	+	+	+
LaLINS- GR3'Nested	-	+	+	+

Table 3: Aroma profile composition of *L. angustifolia, N. cataria H. officinalis S. sclarea* young leaves obtained by HS/SPME

Compound names	KI DB-5	*L. angustifolia* (%)	*N. cataria* (%)	*H. officinalis* (%)	*S. sclarea* (%)
α-pinene	936	-	-	0.3	4.0
Sabinene	979	-	-	5.2	-
Myrcene	987	3.0	-	0.5	1.0
β-pinene	988	-	-	7.0	1.0
Limonene	1030	1.3	-	0.6	1.5
1,8-cineole	1034	0.9	-	-	2.3
Trans-ocimene	1057	2.2	-	-	0.7
Linalool	1099	31.0	-	1.2	19.0
Camphor	1140	0.9	-	-	0.3
Trans-pinocamphone	1160	-	-	2.5	-
Borneol	1165	1.2	-	-	0.7
Cis-pinocamphone	1175	-	-	57.3	-
Terpinen-4-ol	1180	2.9	-	7.0	-
α-terpineol	1190	6.3	-	-	4.0
β- citronellol	1227	-	8.0	-	-
Nerol	1229	-	34.0	-	0.3
Geraniol			5.5	-	-
Linalyl acetate	1260	18.2	-	-	51.5
α-citral	1270	-	52.0	-	-
Lavandulyl acetate	1289	10.7	-	-	-
Carvacrol	1311	-	-	3.1	-
β-bourbonene	1388	-	-	-	1.0
Caryophyllene	1404	5.2	-	0.4	2.0
Germacrene d	1480	-	-	1.0	1.0
Bicyclogermacrene	1500	-	-	-	0.2
Elemol	1548	-	-	0.5	0.5
Spathulenol	1577	-	-	0.6	0.2
Sclareole oxide	2220	-	-	-	0.1
Sclareol	2223	-	-	-	0.1
Cyclic monoterpenes	-	13.5	-	83.0	14.8
Linear monoterpenes	-	65.1	99.5	1.7	71.5
Sesquiterpenes	-	5.2	-	2.5	5.1
Total	-	83.8	99.5	87.2	91.4

Interestingly, PCRs with LaLINS (forward) and GR3'Nested (reversed) primers resulted in distinguishable bands around 1800 bp in three cDNA samples, which could be related to linalool synthase, well known as a "dead end product" in the general monoterpene biosynthetic pathway.[21] Because linalool is found in the essential oils [Table 3] of three species (*L. angustifolia*, *H. officinalis* and *S. sclarea*), linalool synthase should be expressed simultaneously in these plants whose main constituents harbor linalool structure.

Among the selected Labiatae plants, *N. cataria* did not show neither limonene nor linalool synthases. The essential oil of this plant was also enriched of geraniol derivatives supporting positive bands with ddmot3 and amm primers.

The results of the head space SPME-GC-MS analysis of the aroma profiles of the above-mentioned plants have been summarized in Table 3 and showed that linalool (31.0%), linalyl acetate (18.2%), lavandulyl acetate (10.7%) and alpha-terpineol (6.3%) were found as the major compounds of *L. angustifolia*, while β- citronellol (8.0%), geraniol (5.5%), nerol (34.0%) and α- citral (52.0%) were identified as the main compounds of the *N. cataria* [Table 1]. The major components of *H. officinalis* and *S. sclarea* oils were determined as *cis*-pinocamphone (57.3%), β-pinene (7.0%), terpinen-4-ol (7.0%), and linalool (19.0%), linalyl acetate (51.5%), α-pinene (4.0%), respectively.

The profiles of the main components of these plants are in agreement with those reported in the literature.[6,22-24] Although *H. officinalis* was rich of cyclic monoterpenes, *L. angustifolia, N. cataria* and *S. sclarea* showed considerable amount of linear monoterpenes. The aroma profile of the above mentioned plants contained low concentration of sesquiterpenes except *N. cataria,* which indicated no sesquiterpene.

CONCLUSIONS

Plants of Lamiaceae family are well-known for application in traditional medicine and phytotherapy.[25] In this study, presence of linalool and limonene synthases, in four species of Labiatae family including *N. cataria, L. angustifolia, H. officinalis* and *S. sclarea* has been determined by molecular biological techniques together with the Head space SPME - GC-MS analysis of the aroma profile of these species. Taking together, *H. officinalis* was rich of cyclic monoterpenes, *L. angustifolia, N. cataria* and *S. sclarea* showed considerable amount of linear monoterpenes. The aroma profile of the above mentioned plants contained low concentration of sesquiterpenes except *N. cataria,* which indicated no sesquiterpene. The profiles of the main components of these plants are in agreement with molecular assays.

ACKNOWLEDGMENT

This research has been supported by Tehran University of Medical Sciences and health Services grant No. 15246.

REFERENCES

1. Abu-Dahab R, Afifi F, Kasabri V, Majdalawi L, Naffa R. Comparison of the antiproliferative activity of crude ethanol extracts of nine *salvia* species grown in Jordan against breast cancer cell line models. Pharmacogn Mag 2012;8:319-24.
2. Kumar PM, Sasmal D, Mazumder PM. The anti hyperglycemic effect of aerial parts of *Salvia splendens* (scarlet sage) in streptozotocin-induced diabetic-rat. Pharmacogn Res 2010;2:190-4.
3. Shuge T, Xiaoying Z, Fan Z, Dongqing A, Tao Y. Essential oil composition of the *Dracocephalum moldavica* L from Xinjiang in China. Pharmacogn Res 2009;1:172-4.
4. Gohari AR, Hadjiakhoondi A, Sadat-Ebrahimi SE, Saeidnia S, Shafiee A. Composition of volatile oils of *Satureja spicigera* and *Satureja macrantha* from Iran. Flavour Fragr J 2006;21:510-2.
5. Saeidnia S, Gohari AR, Hadjiakhoondi A, Shafiee A. Bioactive compounds of the volatile oil of *Dracocephalum kotschyi*. Z Naturforsch C 2007;62:793-6.
6. Fakhari AR, Salehi P, Heydari R, Ebrahimi SN, Haddad PR. Hydrodistillation-headspace solvent microextraction, a new method for analysis of the essential oil components of *Lavandula angustifolia* Mill. J Chromatogr A 2005;1098:14-8.
7. Gershenzon J, Croteau R. Terpenoid biosynthesis: the basic pathway and formation of monoterpenes, sesquiterpenes, and diterpenes. In: Moore TS, editor. Lipid metabolism in plants. Florida: CRC Press; 1993. p. 340-88.
8. Yamaguchi S, Saito T, Abe H, Yamane H, Murofushi N, Kamiya Y. Molecular cloning and characterization of a cDNA encoding the gibberellin biosynthetic enzyme ent-kaurene synthase B from pumpkin (*Cucurbita maxima* L.). Plant J 1996;10:203-13.
9. Wen W, Yu R. Artemisinin biosynthesis and its regulatory enzymes: Progress and perspective. Pharmacogn Rev 2011;5:189-94.
10. Cseke L, Dudareva N, Pichersky E. Structure and evolution of linalool synthase. Mol Biol Evol 1998;15:1491-8.
11. Shimada T, Endo T, Fujii H, Omura M. Isolation and characterization of a new *d*-limonene synthase gene with a different expression pattern in *Citrus unshiu* Marc. Scientia Horticulturae 2005;105:507-12.
12. Pichersky E, Lewinsohn E, Croteau R. Purification and characterization of S-linalool synthase, an enzyme involved in the production of floral scent in *Clarkia breweri*. Arch Biochem Biophys 1995;316:803-7.
13. Bohlmann J, Steele CL, Croteau R. Monoterpene synthases from grand fir (*Abies grandis*). J Biol Chem 1997;272:21784-92.
14. Back K, Chappell J. Cloning and bacterial expression of a sesquiterpene cyclase from *Hyoscyamus muticus* and its molecular comparison to related terpene cyclases. J Biol Chem 1995;270:7375-81.
15. Colby SM, Alonso WR, Katahira EJ, McGarvey DJ, Croteau R. 4S-limonene synthase from the oil glands of spearmint (*Mentha spicata*). cDNA isolation, characterization, and bacterial expression of the catalytically active monoterpene cyclase. J Biol Chem 1993;268:23016-24.
16. Maruyama T, Ito M, Kiuchi F, Honda G. Molecular cloning, functional expression and characterization of d-limonene synthase from *Schizonepeta tenuifolia*. Biol Pharm Bull 2001;24:373-7.

17. Russell S, Meadows LA, Russell RR. Microarray Technology in Practice. USA: Academic Press; 2009.
18. Hosoi M, Ito M, Yagura T, Adams RP, Honda G. cDNA isolation and functional expression of myrcene synthase from *Perilla frutescens.* Biol Pharm Bull 2004;27:1979-85.
19. Landmann C, Fink B, Festner M, Dregus M, Engel KH, Schwab W. Cloning and functional characterization of three terpene synthases from *lavender* (*Lavandula angustifolia*). Arch Biochem Biophys 2007;465:417-29.
20. Adams RP. Identification of essential oil components by gas chromatography/Quadruple Mass Spectroscopy. Carol Stream llinois: Allured; 2001.
21. Masumoto N, Korin M, Ito M. Geraniol and linalool synthases from wild species of *perilla.* Phytochemistry 2010;71:1068-75.
22. Saeidnia S, Gohari AR, Hadjiakhoondi A. Trypanocidal activity of oil of the young leaves of *Nepeta cataria* L. obtained by solvent extraction. J Med Plants 2008;7:54-7.
23. Yousefzadi M, Sonboli A, Karimi F, Ebrahimi SN, Asghari B, Zeinali A. Antimicrobial activity of some *Salvia* species essential oils from Iran. Z Naturforsch C 2007;62:514-8.
24. Kazazi H, Rezaei K. Effect of various parameters on the selective extraction of main components from *Hyssop* using supercritical fluid extraction (SFE). Food Sci Technol Res 2009;15:645-52.
25. Shekarchi M, Hajimehdipoor H, Saeidnia S, Gohari AR, Hamedani MP. Comparative study of rosmarinic acid content in some plants of Labiatae family. Pharmacogn Mag 2012;8:37-41.

Cite this article as: Saeidnia S, Gohari AR, Haddadi A, Amin G, Nikan M, Hadjiakhoondi A. Presence of monoterpene synthase in four Labiatae species and Solid-Phase Microextraction- Gas chromatography-Mass Spectroscopy analysis of their aroma profiles. Phcog Res 2014;6:138-42.

Source of Support: Nil, **Conflict of Interest:** None declared.