Advances in Bioresearch
Volume 3 [2] June 2012: 36 - 39
ISSN 0976 – 4585

www.soeagra.com/abr/abr.htm

ORIGINAL ARTICLE

Genotype Comparison of Azarbaijan Native Citronellol Producing Thyme with *Thymus pubescens*

Elaheh Dalir Abdolahinia [1, 2]*, Hossein Nazemiyeh[1], Mohammad saeid Hejazi [1, 3]
[1] Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran
[2] Research Institutes of Physiology and Biotechnology, Zanjan University, Zanjan, Iran
[3] Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
* Author for correspondence: ElahehDalir@gmail.com

ABSTRACT
The compound of essential oil of thyme which was morphologically similar to Thymus pubescens (Lamiaceae) grown at Azarbijan region, was different from pervious reporters. This secondary metabolite extracted from this plant is citronellol which is the main essential oil in the rosa family. These findings showed clear chemical polymorphism with the Th. pubescens taxon that might lead us to new species. However, while non-coding internal transcribed spacer region (ITS) of ribosomal genes is the best region to determine new species molecular research in this project could not separate this chemotype from the others.
Key words: *thyme, Azarbijan region, chemotype*

INTRODUCTION

Thyme (*Thymus pubescens* Boiss. & Kotschy ex Celak) belonging to the Lamiaceae family is an important source both of medicines and foods (spices). Also, the genus is an important source of essential oil [1]. However, while, all those previous surveys reported thymol, carvacrol, p-cymen and g–terpinene as the major components in the oils [2-5], the recent report conducted by Nazemiyeh et.al., [6] citronellol-producing is the essential compound in *Th. pubescence* that grow in the area of Misho-Dagh, Payam village, Iran. In fact, citronellol is found in a floral, rose, and citrus scent as well as this chemical compound has never been found in thyme species [2-5]. Hence, this finding may be influenced through changes and mutation in genotype level and produce new species. In other word, based on the internal transcribed spacer (ITS) phylogenetic analysis and the genetic studies in various substances in other genera or families has been examined [7-9], and it has been proven that there are sets of principles for how chemical variation can be treated at the species level [10]. In the present study, our goals are twofold: (1) to assess whether molecular sequence data provide evidence for rare secondary material in thyme (2) to test whether chemotypes in *Th. pubescence* form separate groups.

MATERIAL AND METHOD

Plant samples

The leaves of *Thymus pubescens* Boiss. & Kotschy ex Celak were collected from the East Azarbaijan province, at the botany herbarium of The University of Tabriz and the Herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences. In total, this represented nine of *Th. pubescens* (that are preserved healthfully in the herbaria) were studied (Table 1).

Chemistry

These samples have been examined chemically before and shown in Table 1.

Genomic DNA isolation

DNA was extracted from samples listed in Table 1, using the methods of Doyle & Doyle, [11]. Briefly, 100 mg of dried leaves were ground into a fine powder in liquid nitrogen using a mortar and pistil, and then suspended into a lysis solution containing 10 mM Tris (pH 8),1 mM EDTA, 1.4 M NaCl, 2% CTAB and 1% PVP, and incubated at 65 °C for 20 min. Then, the extraction was carried out with an equal volume of chloroform-isoamyl alcohol (24:1) and precipitated from the aqueous phase with 0.6 volume of isopropanol and followed by washing with 70% ethanol. Finally, the resultant palette was dissolved in 50 L and stored at 70 °C.

PCR amplification

The whole ITS region including ITS1, 5.8S and ITS2 was amplified using forward ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and reverse ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers [12]. PCR reactions were performed in 50 µl volumes containing 10 x reaction buffer, 1.5 mM MgCl2, 200 µM of each dNTP, 0.2 µM of each primer, 50-100 ng of genomic DNA and 0.75 U of Taq DNA polymerase (Cinagen, Iran), with or without addition of 5% dimethylsulfoxide (DMSO). PCR amplifications were performed using the "hot start" protocol [21]. An initial denaturing step of 95 °C for 5 min was followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1min, and a final extension at 72 °C for 5 min time was applied. PCR amplified products were separated in 1% agarose gel electrophoresis. The amplified DNA products were extracted from the gel using the QIAquick purification kit (QIAGEN, Germany), then the forward and reverse strands were sequenced at least twice (Macrogen, S. Korea).

Sequence analysis

The ITS sequences were aligned with ITS sequences of *Thymus pubescens* by the use of ClustalX [13].

RESULT

Chemistry

There is significant difference among chemical compounds in these plants studied. Samples of Tabriz University indicated thymol, carvacrol, p-cymen and g–terpinene as the dominant components in the oils [14], which are similar to previous reports [4, 15,16], while citronellol and acetate are major compounds of secondary materials in samples of Tabriz University of Medical Sciences [6]. The higher percentage of citronellol in thyme was surprising because it is important secondary material in components of rose oil [17] as well as this compound is not reported in Lamiaceae family till now.

ITS analysis

The ranges of the length of the ITS region are 750 bp in all plants which was submitted in GenBank with EU374715 accession number (fig 1). After aligning sequences, there is not an insert/ delete (indel) nucleotide among investigated sequences.

DISCUSSION

The same sequence in thyme means that the variation in the ITS region is insufficient to discriminate the inner structures of *Th. pubescens* populations on East Azarbijan because there was no meaningful correlation between ITS sequences and chemotype. On the other hand that phenomenon such as clonal Interference may cause reproduction in these small populations. In other word, in this event, the genotype of all individuals would be responsible for their chemotypes [18,19].

Table1: Plant material studied in this research and the accession number for ITS1-5.8S-ITS2 sequence in GenBank

***Thymus* spp.**	**Code style**	**Collection area(**Azerbaijan**)**	**Voucher No.**	**Herbarium Place**	**The percentage of compounds**	**GenBank Accession**
Th. pubescens	ARS	Arshadchaman	1233	University of Tabriz	44-57.6% Thymol 10.3-12.9% γ-terpinene 6.8-7.2% p- cymene 3.8-10.8% Geraniol (Yavari *et. al.* 2011)	EU374715
	K	Arasbaran	8878			
	MIS	Moghan	5938			
	M	Mishginshahr	8877			
	MISH-1	Misho-Dagh Mountain	Tbz-FPh-152	Tabriz University of Medical Sciences	42-42.6% Citronellol 14% Acetate 13-13.1% Geraniol (Nazemiyeh *et. al.* 2011)	
	Mish-2		Tbz-FPh-153			

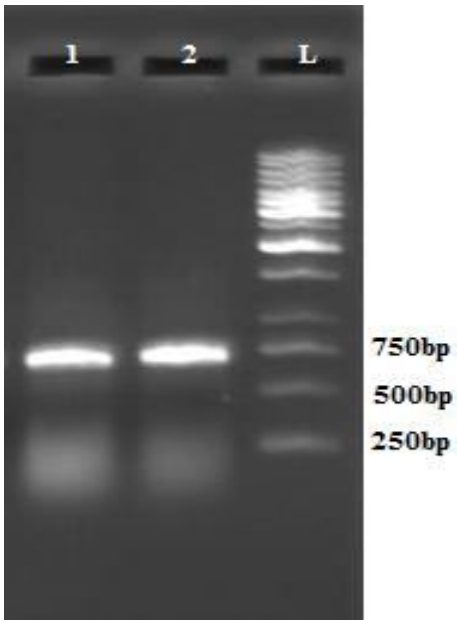

Figure 1: Separation in agarose of PCR products

It is noteworthy that different secondary metabolites of thyme with the same ITS sequence may be produced affecting some kind of relationship with their environment although this was not examined in this study. Thus we suggest that research on secondary metabolite variation could be a vital key in understanding thyme ecology, reproduction, behavioral differences, and classification. We consider it possible that variation in secondary metabolites may be related to environmental adaptation and adaptive evolution in thyme [20].

REFERENCES

1. Rechinger K. H. Flora Iranica (1982). 154. Graz: *Akademische Druckund Verlagsanstalt*, 531–553.
2. Stahl-Biskup E. (1991). The chemical composition of Thymus oils: a review of literature 1960-1989, *J. Essen. Oil Res.*, 3: 61-62.
3. Rustaiyan, A.Q., Masoudi, S., Monfared A. (2000). Volatile constituents of *Thymus* species grown wild in Iran, *Planta Med*, 66: 197-198.
4. Abousaber, M., Hadjakhoondi, A., Shafiee, (2002). A. Composition of the essential oil of Thymus pubescens Boiss. et Kotschy ex Celak and Thymus fedtschenkoi Roniger from Iran, *J. Essent. Oil Res.*14 (**3**):154-155.
5. Askari, F., Sefidkon, F., Mirza M. (2003). Essential oil composition of Thymus pubescens Boiss. et Kotschy ex Celak from different locality of Tehran, Iranian, *Med. Arom. Plants Res.*, 19 (**4**):125-137.
6. Nazemiyeh, H., Lotfipoor, F., Delazar, A., Razavi, S. M. Asnaashari, S., Kasebi, N., Talebpour, A.H., Nahar, L. & Sarker, S.D. (2011) Chemical Composition, and Antibacterial and Free-Radical- Scavenging Activities of the Essential Oils of a Citronellol Producing New Chemotype of Thymus pubescens Boiss. & Kotschy ex Celak, *Rec. Nat. Prod.* 5 (**3**): 184-192
7. Blanco, O., Crespo, A. Ree, R. H., Lumbsch, H. T. (2006). Major clades of parmelioid lichens (Parmeliaceae, Ascomycota) and the evolution of their morphological and chemical diversity, *Molecular Phylogenetics and Evolution*, 39 (**1**): 52–69.
8. Helms, G., Friedl, T., Rambold, G. (2003) Phylogenetic relationships of the Physciaceae inferred from rDNA sequence data and selected phenotypic characters. *Mycologia*, 95 (**6**): 1078–1099.
9. Ihlen, P. G., Ekman S. (2002). Outline of phylogeny and character evolution in Rhizocarpon (Rhizocarpaceae, lichenized Ascomycota) based on nuclear ITS and mitochondrial SSU ribosomal DNA sequences, *Biological Journal of the Linnean Society*, 77 (**4**): 535–546.
10. Brodo, I. M. (1986). Interpreting chemical variation in lichens for systematic purposes. *The Bryologist.* 89 (**2**): 132–138.
11. Doyle, J.J., Doyle, J.L. (1990). A rapid total DNA preparation procedure for fresh plant tissue. Focus. 12: 13–15.
12. White, T.J., Bruns, T., Lee, S., Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols a guide to methods and applications.* 315–322.
13. Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F. & Higgins D.G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*, 25: 4876–4882.
14. Yavari, A.R., Nazeri, V., Sefidkon F. & Hassani. M.E. (2010). Study on some ecological factors, morphological traits, ploidy levels and essential oil composition of Thymus pubescens Boiss. & Kotschy ex Celak in two natural regions of East Azerbaijan province, *Iranian Journal of Medicinal and Aromatic Plants,* 26(**4**): 500-512.
15. Mehrgan, H., Mojab, F., Pakdaman,S. and Poursaeed M. (2008). Antibacterial Activity of Thymus pubescens Methanolic Extract, *Iranian Journal of Pharmaceutical Research* 7 (**4**): 291-295.

16. Mahmoudi, M., Morteza-Semnani, K., Mojra, E. (2008). Anti-inflammatory and antinociceptive activity of Thymus pubescens extract, *Fitoterapia*, 79 (**5**): 361-365.
17. Moeina, M., Karamib, F., Tavallalib, H., Ghasemia, Y., (2010). Composition of the Essential Oil of Rosa damascena Mill. From South of Iran. *Iranian Journal of Pharmaceutical Sciences.* 6 (**1**): 59-62.
18. Zhou, Q., Guo, S., Huang, M., Wei, J. (2006). A study of the genetic variability of Rhizoplaca chrysoleuca using DNA sequences and secondary metabolic substances. *Mycologia,* 98(**1**): 57–67
19. Nelsen, M., Gargas, A. (2009). Assessing clonality and chemotype monophyly in Thamnolia (Icmadophilaceae), *The Bryologist,* 112(**1**): 42–53.
20. Abdolahinia E.D., Bashir N.S., Hagnazari, A., Nazemiyeh, H. and Hejazi, M.S. (2011). A Comparative Phenotypic And ITS Based Genotypic Study in Thyme species (*Thymus* L. Lamiaceae), *Vegetos*, 24 (**2**):102-113.
21. Chou, Q., Russell, M., Birch, D.E., Raymond, J., Bloch, W. (1992). Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications, *Nucleic Acids Res*. 20 (**7**): 1717–1723.