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Full Length Research Paper

Evaluation of essential oil composition and DNA diversity of mint resources from China

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Eight *Mentha spicata* accessions collected from China were characterized by both molecular and volatile analysis. Amplified fragment length polymorphism (AFLP) evaluation was performed to analyze the DNA polymorphism of the accessions. Essential oil from leaf was also detected by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The major components of the accessions are limonene (1.4 to 11.1%) and carvone contents (55.4 to 74.6%) for samples *M. spicata*1, *M. spicata*4, *M. spicata*5, *M. spicata*6 and *M. spicata*8, while other components are 1, 8-cineole (0.5 to 23.6%) and piperitenone oxide (30.8 to 38.5%) for samples *M. spicata*2 and *M. spicata*3, and *cis*-Sabinene hydrate (15%), menthol (16%) and pulegone (35.9%) for *M. spicata*7. Both AFLP and leaf volatile analysis were used to group the samples into two main groups, and similar dendrograms were obtained. However, the results shown in this study can be further applied in mint breeding.

Key words: *Mentha spicata*, essential oil, chemical diversity, AFLP, molecular diversity.

INTRODUCTION

Mint is the most important species in the genus *Mentha* (Lamiaceae family). This species is the third flavor used after vanilla and citrus (Maftei, 1992). The essential oil isolated from mint leaves has economical importance and is widely used in food, cosmetic, confectionary, chewing gum, toothpaste and pharmaceutical industries (Clark, 1988; Lawrence, 2006). Mint is widely cultivated from the commercial oil produced in many countries, such as America, India, China and Canada (Lawrence, 2006). Recently, in China, the presence of variety degeneration has created a big problem of decrease in oil quality and yield, and the rise in demand for mint oil has led to expansion in mint production areas, which in turn has put a lot of pressure on limited farmland resources. So, the selection of a mint cultivar with high growing rate and high content of essential oil may be a good solution to overcome those problems.

Knowledge of genetic diversity of a plant provides a basis in selection of superior parental combination (Schlotterer, 2004). Over the past decade, the genetic structure of *Mentha* had been estimated by several methods (Hadjiakhoondi, 2000; Edris, 2003; Bremnes, 2002; Najeh and Mohamed, 2004); although, wild mint resources were widely distributed in China, but to this day, its genetic background had not been studied.

Mentha spicata (native spearmint) is one of the best known hybrids in mint species. This species is widely cultivated for their essential oils. Essential oil composition from the leaves of *M. spicata* has been described previously, such as carvone and menthone-rich oils (Sticher and Flűck 1968), carvone and neodihydrocarveol-rich oils (Nagasawa et al., 1976a, b), dihydrocarveol and carvone-rich oils (Sivropulou, 1995), and carvone and linalool-rich oils (Hadjiakhoondi, 2000). Considering the diversity of the oil chemical composition in *M. spicata*, its accessions in China should be investigated in order to further evaluate its potential as a raw material for breeding.

The objective of this study was to use AFLP technique combined with GC/MS methods to evaluate the genetic structure of Chinese mint resources in order to facilitate breeding for high oil quality and yield in mint.

MATERIALS AND METHODS

Plant material and essential oil extraction

The experimental material comprised 8 *M. spicata* accessions

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Table 1. Geographical collection site and sample identification of *Mentha* individuals.

Species and code	State	Collection site	Longitude/Latitude	Abbreviation
M. spicata 1	Cultivar	Fukang, Xinjiang	44.26N/87.99E	M1
M. spicata 2	Wild	Minhang, Shanghai	31.03N/121.42E	M2
M. spicata 3	Wild	Minhang, Shanghai	31.03N/121.42E	M3
M. spicata 4	Wild	Yunnan, Kunming	25.04N/102.71E	M4
M. spicata 5	Wild	Five fingers mountain, Hainan	18.78N/109.54E	M5
M. spicata 6	Wild	Fangcheng, Henan	33.27N/113.02E	M6
M. spicata 7	Wild	Puyang, Henan	35.77N/115.03E	M7
M. spicata 8	Wild	Wujiang, Jiangsu	31.12N/120.67E	M8

(Table 1). *M. spicata*1 accession was offered by Shanghai Aromatic Agricultural Science and Technology Co. Ltd. *M. spicata*2 and *M. spicata*3 accessions were selected from the collection of the Aromatic Plant R&D Centre in School of Agriculture and Biology of Shanghai Jiao Tong University. *M. spicata*4, *M. spicata*5, *M. spicata*6, *M. spicata*7 and *M. spicata8* were collected by the members of the Aromatic Plant R&D Centre. These samples were identified by Dr YN-Wu, who is a botanist of the Pharmaceutical School in Shanghai Jiao Tong University. Voucher specimens were deposited at the herbarium of the Aromatic Plant R&D center at the School of Agriculture and Biology in Shanghai Jiaotong University.

The mature leaves of 8 accessions were collected in the flowering stage and subjected to hydrodistillation. The distillation process was conducted using a modified Clevenger-type apparatus. Distilled water (300 ml) was used for the distillation of fresh plant samples (100 g). Distillation time was approximately 1 h at boiling point. The oil phase was separated and dried over anhydrous sodium sulphate and kept in brown glass bottle at 4°C until GC-MS analyses were performed.

DNA extraction and AFLP reaction

Good quality genomic DNA of 8 accessions was extracted from 0.2 g of full expanded leaves using the cetyltrimethylammonium bromide (CTAB) method described by Doyle and Doyle (1978). The resulting DNA was measured in a 1% agarose-gel stained with ethidium bromide in comparison with a lambda DNA of known concentration; then the DNA was diluted with sterilized distilled water and subjected to amplification.

Primers used in this study were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. In this assay, six AFLP primer combinations (M-AAA/P- ACG, M-AAC/P-CAA, M-AAC/P-ACT, M-AAG/P-ACG, M-AAA/P-ACC and M-GAA/P-CAT) were used.

Genomic DNA was digested with *Pst*I and *Mse*I (New England Biolabs, USA), and the restricted fragments were ligated with *Pst*I and *Mse*I (New England Biolabs, USA) adaptators in the same reaction. In short, 2 µL (50 ng/µL) extracted DNA was added to a 23 µL digestion-ligation solution containing T4 Buffer (Progmega, USA), BSA100× (Progmega, USA), 50 pmol/µL *Mse*I adapator (Sangon, China), 50 pmol/µL *Pst*I (Sangon, China), 1.5U T4-ligase, 3U *Pst*I, 3U *Mse*I and 66 uM ATP, and was brought up to 23 µL with purified water. This resulting reaction mixture was then incubated at 37°C for 16 h. Preselective PCRs (20 µL) contained 10×PCR buffer (Progmega, USA), 5U *Taq* DNA polymerase, 25 mM $MgCl_2$, 25 mM of each dNTPs, 50 ng/µL *Pst*I-O primer (Sangon, China), 50 ng/µL *Mse*I-O primer (Sangon, China), and 2 µL of restriction-ligation reaction as template. The thermocycler conditions were: 94°C for 5 min, followed by 35 cycles of 94°C for 35 s, 56°C for 35 s, and 72°C for 1 min; and finally by 72°C for 7 min. Selective amplification reactions (20 µ L) contained 10×PCR buffer, 5 U *Taq* DNA polymerase, 25 mM $MgCl_2$, 25 mM of each dNTPs, 50 ng/µL of each selective primer, and 2 µ L of diluted (1 in 10) preselective PCR product as template. The PCR conditions were: 94°C for 2 min, followed by 12 cycles of 94°C for 35 s, 65°C decreasing by 0.7°C per cycle for 35 s, and 72°C for 1 min. This was then followed by 35 cycles of 94°C for 35 s, 56°C for 35 s and 72°C for 1 min; and finally by 72°C for 5 min. Subsequently, the PCR product was denaturated at 94°C for 5 min, prior to loading in 6% polyacrylamide gels with 45 W of electrophoresis meter for 2 h, and the silver staining was used to visualize the AFLP fragments.

GC-FID analysis

Gas chromatography was carried out on an Agilent 6890N gas chromatograph fitted with a fused silica HP-innowax (30 m × 0.25 mm × 0.25 µm), in which nitrogen was the carrier gas (0.8 mL/min). Injector and detector (FID) temperatures were 250 and 270°C. The column temperature was programmed from 60 to 270°C at 4°C/min rate, and was finally raised to 270°C and held for 5 min, after which samples of 0.2 uL were injected in the split ratio of 1:50 mode.

GC–MS analysis

GC–MS analysis was performed on an Agilent Technology S6890/5973N mass spectrometer apparatus with a fused silica HP-innowax (30 m × 0.25 mm × 0.25 µm) coupled to a HP mass selective detector (MSD 5970 HP); ionization voltage (70eV) and electron multiplier energy (2000V). Gas chromatographic conditions were given, while the analysis was run by HP-5 MS (30 m ×0.25 mm× 0.25 µm) capillary column. In both cases, nitrogen was used as the carrier gas.

Identification analysis of components

Identification of the individual components was based on the retention indices (RI) in our laboratories. The retention indices were determined in relation to a homologous series of n-alkanes (C5–C26) under the same operating conditions. Further identification was made by matching with a mass spectral library and commercial libraries (WILLEY and NIST database/ChemStation data system).

Data analysis

Unambiguous bands between 150 and 1200 bp in AFLP analysis were scored. Afterwards the scoring results were transformed into a matrix with values present (1)/absen t (0) for each AFLP reaction.

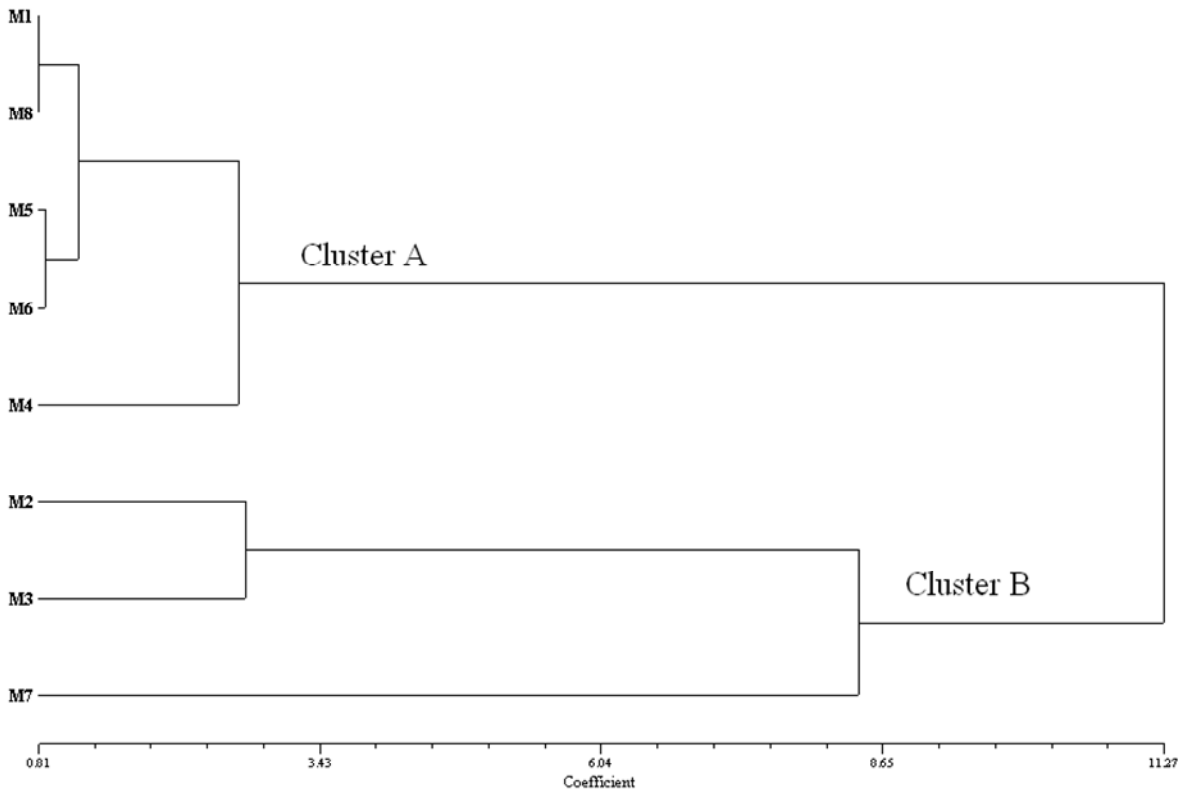

Figure 1. Dendrogram generated by the cluster analysis of phytochemical traits.

Cluster analysis of the essential oil composition and the AFLP analysis, using the NTSYS-pc version 2.01e software package (Rohlf, 2000), and the method of data analysis were the same with those of Fatma (2010).

RESULTS AND DISCUSSION

Chemical composition and diversity assessments of essential oil

A total of 51 components, representing 86.3 to 93.9% of the composition of the oil samples were identified (Table 2). These percentages were calculated using normalization of peak areas without application of the response correction factor. It was found that the chemical profiles of these oils were dissimilar. In the analysis of the chemical diversity, the 8 accessions were divided into two main clusters (Figure 1). Cluster A was grouped by M1, M4, M5, M6 and M8, which were mainly characterized by high limonene (1.4 to 11.1%) and carvone (55.4 to 74.6%) contents in their oil components. Cluster B included two populations: M2 and M3, and M7. The main oil con-stituents were 1, 8-cineole (0.5 to 23.6%) and piperitenone oxide (30.8 to 38.5%) in M2 and M3, and *cis*-sabinene hydrate (15%), menthol (16%) and pulegone (35.9%) in M7.

The assessment of the chemical variation is important to ensure that different chemotype resources may be used even more efficiently and sustainably in breeding programs (Lawrence, 1978). A direct comparison of the results shown in Table 1 with those found in the literature (Sticher and Flűck 1968; Nagasawa et al., 1976a, b; Sivropulou, 1995) confirmed the high variability in the chemical composition of *M. spicata* essential oils. It is known that the chemical composition of essential oils is affected by internal (for example, anatomical, biochemical and physiological characteristics of the plants) and external (environmental) factors (Barra, 2009). Chemotypes (or chemical phenol-types) are generally considered to be the phenotypical expression of a genotype (Rubiolo P, 2009). Our results from the chemotypes' analysis suggested that the two types of chemotypes may be derived from the different genotypes.

Molecular evaluation

To investigate the molecular diversity of wild *Mentha* from China, AFLP marker system was employed in this study. In AFLP analysis, the 6 selected AFLP primers amplified a total of 226 scorable bands. Scored fragments ranged from 150 to 1200 bp, with an average of 37.7 bands per primer, of which 142 (62.8%) were polymorphic. UPGMA cluster analysis (Figure 2) based on AFLP data showed

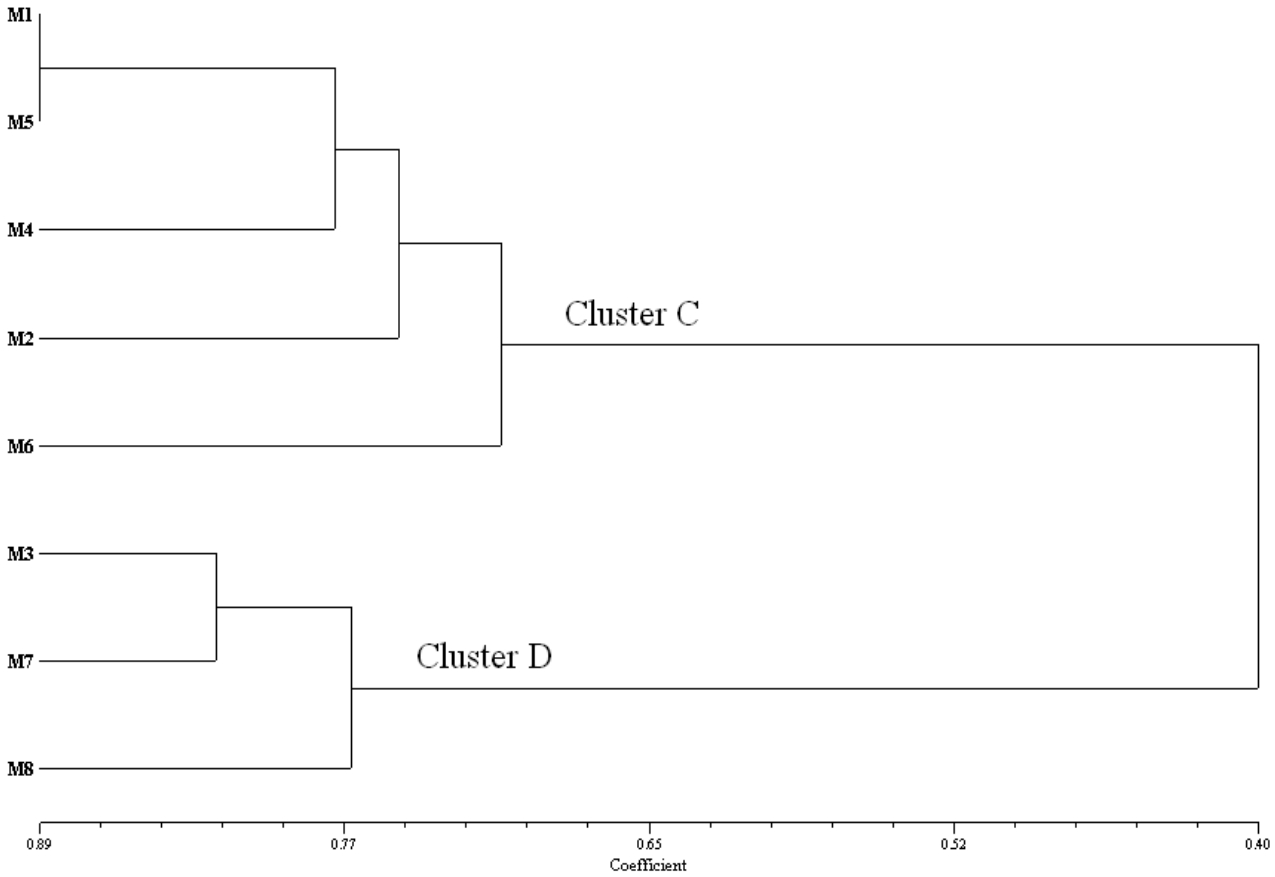

Figure 2. Dendrogram built by the hierarchical cluster analysis with all the variables using the UPGMA method and Euclidean squared distance.

that the 8 populations were divided into two main clusters. Cluster C was composed of the M1, M2, M4, M5 and M6 accessions, while cluster D included M3, M7 and M8 accessions.

This study on the genetic diversity of *M. spicata* populations from China reveals that the taxa maintained high levels of genetic polymorphism among populations. This high level of genetic diversity within populations is indicative of the genotype richness and gene flow (Fang, 2007). In this study, 8 *M. spicata* accessions were separated into two distinct groups. The results supported the findings that there were two genotypes in *M. spicata* accessions (Gobert, 2002; Ruttle, 1931; Morton, 1956). However, we did not know whether some possible correlations between chemotypes and genetic diversity existed or not in previous reports. Our cluster analysis obtained from both molecular studies and volatile compositions revealed a relationship between AFLP marker and volatile oils, except M2 and M8 accessions. The results suggest that the chemical polymorphism could be a result of genetic variability of individuals. Similar results were also drawn from other species, for example from *Santolina insularis* (Giorgio, 2010) and *A. umbelliformis* Lam. (Patriza, 2009). According to Barra (2009), the environment was also one of the factors that affected the chemical compositions of essential oil. Perhaps, this is one of the reasons why M2 and M8 accessions did not follow the correlation.

Conclusions

This study reports the molecular and volatiles study of *M. spicata* naturalized in China. The molecular analysis using AFLP clearly showed that *M. spicata* samples are genetically highly polymorphic. *M. spicata* leaf volatiles were mainly limonene, carvone, 1,8-cineole, piperitenone oxide, *cis*-sabinene hydrate, menthol and pulegone-rich. However, a correlation could be drawn between the AFLP markers' data and the volatile analysis. Although, there was some evidence suggesting that the chemotypes were clustered predominantly according to their genetic structures, there were still some individuals that did not follow this rule, probably due to environmental factor.

Breeding program is a complex task that usually depends on detailed basic researches. Our results would be expected to provide some theoretical support in

Table 2. Chemical constituents of the essential oils of different accessions of *Mentha*.

RI[a]	RI[b]	Component	Method	Accession number							
				M1	M2	M3	M4	M5	M6	M7	M8
935	1022	α-Pinene	*RI*, MS	0.4	0.5	tr	0.4	0.1	0.4	0.6	0.5
976	1110	*β*-Pinene	*RI*, MS	0.6	1.3	3.5	0.6	0.2	0.5	0.7	0.6
970	1121	Sabinene	*RI*, MS	0.3	0.2	1.9	0.3	0.1	0.2	0.3	0.3
988	1165	Myrcene	*RI*, MS	0.2	0.2	7.8	0.5	0.1	0.2	0.4	0.2
1016	1180	*α*-Terpinene	*RI*, MS	-	0.2	-	tr	-	tr	-	-
1027	1205	Limonene	*RI*, MS	7.2	1.4	3.9	3.5	1.7	5.3	2.8	11.1
1034	1209	1,8-Cineole	*RI*, MS	3.6	21.9	23.6	3.5	2.6	1.2	0.5	0.8
1058	1240	*γ*-Terpinene	*RI*, MS	-	-	1.2	-	tr	-	-	-
1041	1248	*(Z)-β*-Ocimene	*RI*, MS	tr	0.2	0.3	-	-	tr	tr	-
1001	1299	Octanal	*RI*, MS	-	-	0.2	tr	-	-	-	tr
1101	1399	Nonanal	*RI*, MS	0.1	0.3	0.6	0.4	0.2	1.1	0.5	0.1
1105	1425	α-Thujone	*RI*, MS	-	0.1	0.1	-	-	tr	-	tr
1072	1449	*trans*-Linalool oxide (furanoid)	*RI*, MS	0.2	0.3	-	tr	tr	-	-	0.1
961	1457	1-Octen-3-ol	*RI*, MS	0.1	-	0.1	-	-	tr	tr	-
1352	1467	α-Cubebene	*RI*, MS	0.2	0.7	0.0	0.2	0.3	0.3	-	0.1
1154	1477	Menthone	*RI*, MS	-	tr	0.1	-	-	-	-	-
1370	1493	α-Ylangene	*RI*, MS	0.1	0.2	0.2	0.1	0.1	0.3	tr	0.1
1102	1553	Linalool	*RI*, MS	0.1	0.6	0.2	0.2	0.3	0.7	0.1	0.2
1068	1556	*cis*-Sabinene hydrate	*RI*, MS	0.2	0.3	1.3	2.3	0.2	0.1	15.0	-
1257	1569	Linalyl acetate	*RI*, MS	-	0.2	-	-	-	-	0.6	tr
1158	1584	Pinocarvone	*RI*, MS	tr	0.2	-	-	-	tr	tr	-
1288	1598	Bornyl acetate	*RI*, MS	-	0.4	1.2	-	0.2	tr	0.1	-
1187	1604	Neoisomenthol	*RI*, MS	0.1	-	tr	0.9	-	1.6	0.9	0.2
1178	1608	Terpinen-4-ol	*RI*, MS	0.1	0.4	0.5	0.5	0.1	0.1	0.3	-
1418	1617	β-Caryophyllene	*RI*, MS	0.6	0.4	0.1	5.0	0.5	0.9	0.1	0.5
1436	1630	Aromadendrene	*RI*, MS	-	tr	-	tr	0.1	-	tr	-
1194	1646	Myrtenal	*RI*, MS	0.1	2.0	0.1	0.3	0.4	1.0	-	0.2
1182	1656	Menthol	*RI*, MS	tr	tr	-	-	tr	tr	16.0	-
1237	1663	Pulegone	*RI*, MS	-	0.6	0.1	-	-	-	35.9	-
1124	1678	*trans-p*-Mentha-2,8-dien-1-ol	*RI*, MS	0.9	2.1	1.5	8.3	-	1.1	0.1	0.1
1196	1685	γ-Terpineol	*RI*, MS	-	0.2	0.1	-	0.3	1.2	0.6	0.5
1240	1694	Neral	*RI*, MS	tr	0.1	0.2	tr	0.1	tr	-	-
1190	1704	α-Terpineol	*RI*, MS	0.1	0.6	0.2	0.4	-	-	0.1	-
1169	1717	Borneol	*RI*, MS	-	0.2	0.2	0.6	-	0.7	0.7	tr
1481	1729	Germacrane D	*RI*, MS	tr	0.1	0.1	tr	tr	tr	0.1	tr
1250	1739	Geranial	*RI*, MS	tr	0.2	0.2	2.8	-	1.2	0.3	-
1337	1746	Piperitone	*RI*, MS	-	16.7	1.2	-	-	-	7.2	tr
1244	1758	Carvone	*RI*, MS	74.6	0.2	0.2	55.4	70.7	66.8	tr	71.6
1512	1772	δ-Cadinene	*RI*,MS	0.1	-	tr	0.1	-	0.1	tr	-
1515	1779	γ-Cadinene	*RI*, MS	0.3	0.1	-	1.7	0.3	0.2	-	tr
1196	1800	Myrtenol	*RI*, MS	0.1	0.4	0.2	-	0.1	0.1	tr	tr
1230	1810	Nerol	*RI*, MS	0.2	0.3	-	0.1	0.6	0.2	-	0.2
1217	1842	*trans*-Carveol	*RI*, MS	0.2	0.3	0.3	0.9	0.3	0.3	tr	0.1
1540	1847	Calamenene	*RI*, MS	0.9	0.2	-	0.1	1.7	1.9	-	1.2
1245	1853	Carvone oxide	*RI*, MS	0.5	0.2	0.2	-	0.1	0.1	0.2	0.5
1475	1975	Dodecanol	*RI*, MS	0.8	tr	-	1.5	1.4	0.5	9.8	0.1
1363	1983	Piperitenone oxide	*RI*, MS	tr	30.8	38.5	tr	0.2	-	tr	tr
1583	2011	Caryophyllene oxide	*RI*, MS	0.1	0.5	-	-	0.2	tr	-	tr
1565	2056	Ledol	*RI*, MS	0.1	-	-	-	0.1	tr	tr	-

Table 2. Continued

1588	2098	Globulol	*RI*, MS	-	0.3	-	0.8	0.1	-	tr	tr
1593	2104	Viridiflorol	*RI*, MS	0.1	0.2	-	tr	0.1	tr	tr	0.1
		Total identification (%)		93.2	86.3	90.1	91.4	83.5	88.3	93.9	89.4

RI^a- retention index relative to C5-C26 n-alkanes on the HP- 5 column; RI^b- retention index relative to C5-C26 n-alkanes on the HP-innowax column; tr- traces (<0.1).

improving the quality and yield of the essential oil.

REFERENCES

Barra A (2009). Factors affecting chemical variability of essential oils: a review of recent developments. Nat. Prod. Commun. 4: 1147-1154.

Bremnes L (2002). Herbs. Eyewitness-Handbooks; DK Publishing: New York, USA. Clark GS Menthol. Perfum Flavor. 13: 37-46.

Doyle J, Doyle JL (1978). Isolation of plant DNA from fresh tissue. Am. J. Bot. 75: 12-38.

Edris E, Shalaby AS, Fadel HM, Abdel Wahab MA (2003). Evaluation of a chemotype of spearmint (*Mentha spicata* L.) grown in Siwa Oasis, Egypt. Eur. Food. Res. Technol. 218: 74-78.

Fang H (2007). Numerical classification of *Mentha* L. J. Anhui Agric. Sci. 35: 8181-8183.

Giorgio G (2010). Comparative characterization of *Santolina insularis* chemotypes by essential oil composition, 5S-rRNA-NTS sequencing and EcoRV RFLP-PCR. Phytochemistry, 71: 930-936.

Gobert V (2002). Hybridization in section *Mentha* (Lamiaceae) inferred from AFLP markers. Am. J. Bot. 89(12): 2017-2023.

Hadjiakhoondi A, Aghel N, Zamanizadeh-Nadgar N, Vatandoost H (2000). Chemical and biological study of Mentha spicata L. essential oil from Iran. DARU, 8: 19-21.

Lawrence BM (1978). A study of the monoterpene interrelationships in the genus Mentha with special reference to the origin of pulegone and menthofuran. Ph.D. Thesis. State University.

Lawrence BM (2006). Mint: The genus *Mentha.* CRC Press. Boca Raton, FL.

Maftei M (1992). Dry culinary herbs: an overview of selected Western European Markets. In: Verlet N, Ed., Compete Rendu des 3 e´mes Rencontres Techniques et Economiques Plantes Aromatiques et Me´dicinales. C.F.P.P.A. Nyons. pp. 249-292.

Morton JK (1956).The chromosome numbers of the British Menthae. Watsonia, 3: 244–252.

Najeh BF, Mohamed B (2004). Genetic diversity in wild Tunisian populations of *Mentha pulegium* L.(Lamiaceae). Gene. Resour . Crop Environ. 51: 309-321.

Nagasawa T, Umemoto K, Tsuneya T, Shiga M (1976a). Studies on essential oil *Mentha spicata L.* var. crispa Benth. Part IV. Essential oil of *Mentha spicata L. var. crispa* Benth. grown in winter season. Nippon Nogei Kagaku Kaishi. 50: 287-289.

Nagasawa T, Umemoto K, Tsuneya T, Shiga M (1976b). Studies on the wild mints of Tokai districts. Part VII. Essential oils of wild mints containing (t)-menthofuran as a major constituent. Nippon Nogei Kagaku Kaish. 50: 291-293.

Rubiolo P, Matteodo M, Bicchi C, Appendino G, Gnavi G, Bertea C, Maffei M (2009). Chemical and biomolecular characterization of artemisia umbelliformis Lam., an important ingredient of the alpine liqueur Genepı. J. Agric. Food Chem. 57: 3436-3443.

Rohlf FJ (2000). NTSYS-pc: Numerical taxonomy and multivariate analysis system, Exeter Software, Setauket, New York, USA. 2: p. 1.

Ruttle ML (1931). Cytological and embryological studies of the genus *Mentha*. Gartenbauwissenschaft. 44: 428-468.

Sivropulou A, Kokkini S, Lanaras T, Arsenakis M (1995). Antimicrobial activity of mint essential oils. J. Agric. Food Chem. 43: 2384-2388.

Schlotterer C (2004). The evolution of molecular markers-just a matter of fashion? Nat. Rev. Gene. 5: 63-69.

Sticher O, Flűck H (1968). Die Zusmmensetzung von genuinen, extrahierten und destillierten ätherischenö len einiger Mentha-Arten. Pharm. Acta Helv. 3: 411-446.