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ORIGINAL RESEARCH ARTICLE

Chiral differentiation of terpenoids in bergamot mint (*Mentha citrata*) cultivar Kiran: A non-menthol mint essential oil

PRAGADHEESH VS • ANJU YADAV • CHANDAN S CHANOTIYA*

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ABSTRACT

Mentha citrata, popularly known as bergamot mint,is one of the mint species which does not possess menthol, a terpenoid chemical constituent that gives the name Mentha to the genus. CSIR-CIMAP has developed a cultivar cv. Kiran by induced mutagenesis. Gas chromatographic (GC) analysis of the essential oil of this cultivar shows linalool (44.9 %) and linalyl acetate (28.7%) as the major constituents. Further, the enantioselective GC analysis of the essential oil to determine the enantiomeric ratio of linalool and linalyl acetate indicate that (R)-(-)-linalool is in 78.8% excess over its enantiomer, whereas (R)-(-)-linalyl acetate is present as a single enantiomer. Other chiral terpenoids resolved in the enantioselective GC analysis are (1S)-(-)-β-pinene as the major exclusive enantiomer with 93.0% enantiomeric excess and (-)-sabinene with 60.2% enantiomeric excess than its enantiomers. The significance of enantiomer separation and enantiomeric excess of chiral terpenoids in the essential oil of M. citrata cv. Kiran is of great importance in ascertaining the authenticity of this essential oil.

INTRODUCTION

Terpenoids are the most structurally diverse group of secondary metabolites present in plants. Terpenoids contribute almost 30,000 compounds. Hundreds of monoterpenoids, sesquiterpenoids, diterpenoids and triterpenoids are identified till date (Degenhardt et al., 2009). The building block (smallest sub unit) of terpenoids is isoprene and terpenoid compounds are formed as multiples of $(C_5H_8)_n$, where n is the number of isoprene units linked together to form the terpenoid compounds (Paduch et al., 2007). Terpenes are found in nature as hydrocarbons and also with diverse functional groups like hydroxyl, carbonyl (ketone and aldehyde), ethers, epoxides etc. Essential oils constitute mainly monoterpenoids and sesquiterpenoids; however, some essential oils are reported to contain very low percentage of diterpenoids.

Mentha citrata cultivar cv. Kiran is developed by CSIR-CIMAP, Lucknow, India, as a high yielding variety of linalool and linalyl acetate by induced mutagenesis. *M. citrata* is mainly cultivated in the temperate, Mediterranean and subtropical regions

*Corresponding author; E-mail: chanotiya@gmail.com, cs.chanotiya@cimap.res.in
Laboratory of Aromatic Plants and Chiral Separation, Chemical Sciences Division, CSIR-Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow - 226 015, India
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(Padalia et al., 2013). The major composition of *M. citrata* essential oil is acyclic terpenoids which is completely different from the other species of the genus *Mentha*, as all other species produce cyclic terpenoids such as menthol, limonene, pulegone, menthone, menthofuran, menthyl acetate, carvone, piperitone, piperitenone oxide, etc (Bahl et al., 2000; Dwivedy et al., 2017; Goswami et al., 2015; Kokkini 1991; Pragadheesh et al., 2015, 2017; Singh et al., 2005). The presence of linalool and linalyl acetate imparts a citrusy smell to the essential oil of *M. citrata* (Murray and Lincoln, 1970).

The essential oil composition of *M. citrata* has been reported by many researchers. A report by Handa et al. (1964) shows that typical *M. citrata* essential oil comprises of α-pinene (0.6%), β-pinene (0.9%), limonene (1.1%), 1, 8-cineole (0.2%), piperitone (4.2%), piperitone oxide (3.0%), pulegone (8.1%), menthofuran (0.1%), linalool (32.4%) and linalyl acetate (45.0%). *M. citrata* plants grown in Argentina possess higher percentage of linalyl acetate at the full bloom stage (60.9%) to that of post-flowering stage (48.7%); whereas the percentage of linalool is less (23.8%) in full bloom stage as compared to the post flowering stage (35.4%). The other minor compound in both the oils is >2.0% limonene (Malizia et al., 1996). Essential oil of *M. citrata* cultivated in southern Croatia has 13.7% linalool and 21.5% linalyl acetate, while compounds such as β-myrcene, 1, 8-cineole, *cis*-β-ocimene, *trans*-β-ocimene, α-terpineol, neryl acetate, geranyl acetate and geraniol are present in significant amounts (Mastelic et al., 2000). *M. citrata* cv. Kiran developed at CSIR-CIMAP shows consistent essential oil production, at all the developmental stages. The quality of the essential oil is also not affected by the age of the crop (Bahl et al., 2000). The essential oil of *M. citrata* cv. Kiran cultivated at the foothills of western Himalayas constitutes 59.7% linalool, 18.4% linalyl acetate, other minor compounds such as limonene, α-terpineol, *trans*-*p*-menth-1-en-2-ol and nerol (Padalia et al., 2013). The chemical constituents present in *M. citrata* essential oil show diverse biological activities such as insecticidal activity against housefly larvae and pupae, antifungal activity against fungal pathogens of black gram (Kumar et al., 2012; Sharma et al., 2004).

Apart from the essential oil composition, a few reports are available on the enantiomeric nature of linalool present in the *M. citrata* essential oils. The enantiomeric characterization of linalool in *M. citrata* shows that the (*R*)-(-)-linalool has an enantiomeric excess of 72.2%-75.7% over the (*S*)-(+)-linalool (Chanotiya and Yadav, 2009). A review on the enantiomeric distribution of linalool and linalyl acetate reports that the enantiomeric excess of (*R*)-(-)-linalool is more than 75.4% and that of (*R*)-linalyl acetate is more than 97.2% (Casabianca et al., 1998). To the best our knowledge, no report is available on the separation of linalool and linalyl acetate enantiomers (Fig. 1.) from India on a single enantioselective gas chromatographic analysis. The current article aims to characterize all the terpenoid enantiomers present in the *M.citrata* cv. Kiran essential oil using a direct analysis of essential oil using enantioselective gas chromatography.

MATERIALS AND METHODS

Plant material and extraction of essential Oil

Fresh plant material of *Mentha citrata* cv. Kiran was collected from the experimental farm at CSIR-CIMAP, Lucknow. To extract the essential oil, aerial parts of the plant was chopped into small pieces and hydrodistilled for 4 hours using a Clevenger type apparatus. The oil samples were dried over anhydrous Na_2SO_4 and stored at 4°C prior to analysis.

Gas chromatography (GC) and gas chromatography – mass spectrometry (GC-MS)

A PerkinElmer AutoSystem XL GC gas chromatography system was used with an Equity-5 column (60 m x 0.32 mm i.d., 0.25 µm) using hydrogen as carrier gas at a constant pressure of 10 psi. The column oven was programmed from 70°C (2 min hold) to 250°C (2 min hold) at the rate of 3°C/min and to 290°C (5 min hold) at 6°C/min. The split ratio was kept at 1:35. The injector temperature was 280°C and the FID detector was maintained at 290°C. GC-MS analysis was carried out on a PerkinElmer Clarus® 680 GC interfaced

with a Clarus® SQ 8 C Quadrupole mass spectrometry using a Elite-5 MS (30 m x 0.25 mm i.d., 0.25 µm) capillary column with helium as carrier gas at constant flow of 1 mL/min. Initially, the column oven was at 60°C and it was programmed at the rate of 3°C/min to 240°C and increased to 270°C at 5°C/min. Transfer line (between GC and MS) and source temperatures were kept at 220°C; ionization energy of electron impact ionization was 70 eV. Mass spectrometer scan range was kept as 40-450 amu. Characterization of terpenoid compounds were achieved using the retention time, elution order, relative retention index using a homologous series of *n*-alkanes (C_6-C_{28} hydrocarbons, Polyscience Corp. Niles IL), co-injection with standards in GC-FID and GC-MS (Aldrich and Fluka), and also comparing with mass spectral library such as NIST/EPA/NIH version 2.0g and Wiley Registry of Mass Spectral Data (9th edition) and confirming with the reported literature data (Adams, 1995).

Enantioselective gas chromatography

A Varian CP-3800 gas chromatograph was used with a chiral capillary column of Restek β-DEXse. The oven program was initially kept at 70°C (hold for 3 min) and increased to 120°C, at the rate of 3°C/min and to 230°C at 5°C/min. Hydrogen is used as a carrier gas at constant flow of 1.8 mL/min with split ratio of 1:140. The injector temperature was 220°C and the FID detector temperature was 230°C. Characterization of enantiomers was based on the retention time in chiral column, reported elution order of the enantiomers in the chiral phase, chiral relative retention index (cRRI) using *n*-alkanes (C_6-C_{28} hydrocarbons), and co-injection of pure enantiomers and compared with reported literature (Pragadheesh et al., 2013).

RESULTS AND DISCUSSION

For quality assessment, the physical properties of the *M. citrata* cv. Kiran essential oil were first determined. The oil has an optical rotation of – 9.792º, with a retention index of 1.4647 and a specific gravity of 0.8839. Gas chromatographic analysis of the essential oil depicts two major terpenoid compounds namely linalool (44.9%) and linalyl acetate (28.7%) as shown in Table 1. Minor terpenoid compounds in the essential oil include b-pinene (1.3%), *cis*-linalool oxide (1.5%), *trans*-linalool oxide (1.4%), *cis*-pinocamphone (2.3%), a-terpineol (3.2%) and geraniol acetate (1.7%). Lavender lactone (5-ethenyldihydro-5-methylfuran-2-one) is also present in low amount (0.1%) in *M. citrata* cv. Kiran essential oil. Previous reports suggests that lavender lactone may be formed by the oxidation of furanoid linalool oxide present in the essential oil, while linalool oxides are formed from linalool by enzymes or by auto-oxidation into epoxides (Ohloff, 1978; Onayade et al., 1998). The presence of linalool and linalyl acetate, which together contributes more than 83 % of the essential oil, makes this oil similar to the essential oils of *Lavandula angustifolia* (lavender), *Salvia sclarea* etc (Casabianca et al., 1998; Chanotiya and Yadav, 2009; Dzumayev et al., 1995; Verma, 2010) As shown by Murray and Lincoln, (1970) linalool is a precursor for a-terpineol, and the a-terpineol further leads to limonene and other cyclic compounds of 2-oxygenated-*p*-menthanes or 3-oxygenated-*p*-menthanes (Murray and Lincoln, 1970). Thus in *M. citrata*, the conversion of linalool to a-terpineol and to limonene is diverted and linalyl acetate is formed, which makes the essential oil composition of *M. citrata* different from other *Mentha* species. The presence of 44.9% of linalool and 28.7% of linalyl acetate falls well in the range of European Pharmacopeia for lavender essential oils which is 20-45 % for linalool and 25-46 % of linalyl acetate. This shows that *M. citrata* cv.Kiran essential oil can be a good alternative for lavender oil.

Enantioselctive analysis of the essential oil of *M. citrata* cv. Kiran using a chiral column, with 2,3-diethyl-6-*tert*-butyldimethylsilyl-β-cyclodextrin doped into 14% cyanopropylphenyl/86% dimethylpoly-siloxane as stationary phase, led to the resolution of six pairs of enantiomers namely, β-pinene, sabinene, limonene, linalool, linalyl acetate and α-terpineol in (Table 2). In the chiral analysis, (1*R*)-(+)-β-pinene exists around 93.0% more than its enantiomer (1*S*)-(-)-β-pinene. Similarly, (-)-sabinene and (+)-α-terpineol are present in 60.2% and 44.0% excess,respectively than their counter

Table 1: Relative percentage of major constituents of *M. citrata* essential oil

Compound	RI	% Composition
α-Pinene	932	0.1
Sabinene	971	0.2
β-Pinene	977	1.3
β-Myrcene	987	0.7
3-Octanol	992	t
Trans-hexenyl acetate	998	t
Hexyl acetate	1004	t
p-Cymene	1022	0.4
Limonene	1027	0.5
1,8-Cineole	1030	0.3
Lavender lactone	1033	0.1
Benzene acetaldehyde	1043	0.2
Cis-linalool oxide (Furanoid)	1071	1.5
Trans-linalool oxide (Furanoid)	1087	1.4
Linalool	1102	44.9
1-Octen-3-yl acetate	1108	0.4
3-Octanyl acetate	1120	0.2
Cis-pinocamphone	1176	2.3
α-Terpineol	1192	3.2
Sabinene hydrate acetate	1225	0.1
Linalyl acetate	1257	28.7
Geranial	1270	0.2
Geraniol acetate	1382	1.7
β-Caryophyllene	1425	0.2
Monoterpenoids		87.6
Sesquiterpenoids		0.2
Others		0.8
Total identified		88.6

RI: Retention Index

Table 2: Enantiomeric composition of terpenoid compounds present in the essential oil of *Mentha citrate cv.* Kiran

Enantiomers	cRRI	Enantiomeric Composition (%)	Enantiomeric excess (*ee*)
(1*R*)-(+)-β-Pinene	957	3.5	
(1*S*)-(-)-β-Pinene	964	96.5	93.0
(+)-Sabinene	980	19.9	
(-)-Sabinene	992	80.1	60.2
(*S*)-(-)-Limonene	1059	50.0	0.0
(*R*)-(+)-Limonene	1074	50.0	
(*R*)-(-)-Linalool	1193	89.4	78.8
(*S*)-(+)-Linalool	1204	10.6	
(*R*)-(-)-Linalyl acetate	1276	99.2	98.4
(*S*)-(+)-Linalyl acetate	1279	0.8	
(-)-α-Terpineol	1318	28.0	
(+)-α-Terpineol	1328	72.0	44.0

cRRI : Chiral relative retention index on Restek α-DEXse chiral column (using *n*-alkane series); Enantiomeric composition (%): Ratio of enantiomers in percentage; Enantiomeric excess: *ee* = 100 x (EC_1 – EC_2)/(EC_1+EC_2), whereas EC_1 is the amount of major enantiomer and EC_2 is the amount of minor enantiomer

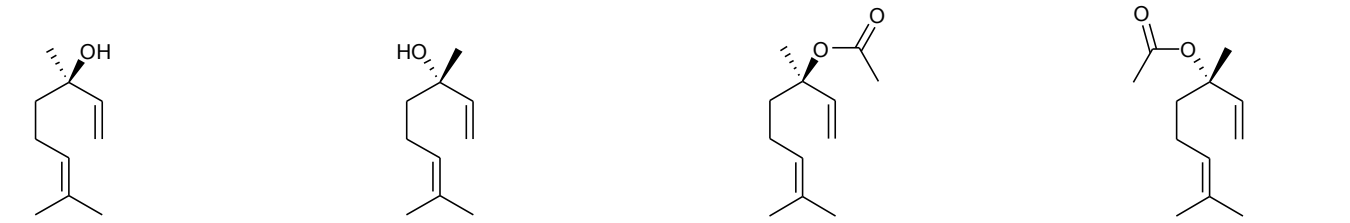

Figure 1: Enantiomers of linalool and linalyl acetate present in *M. citrata* cv. Kiran essential oil

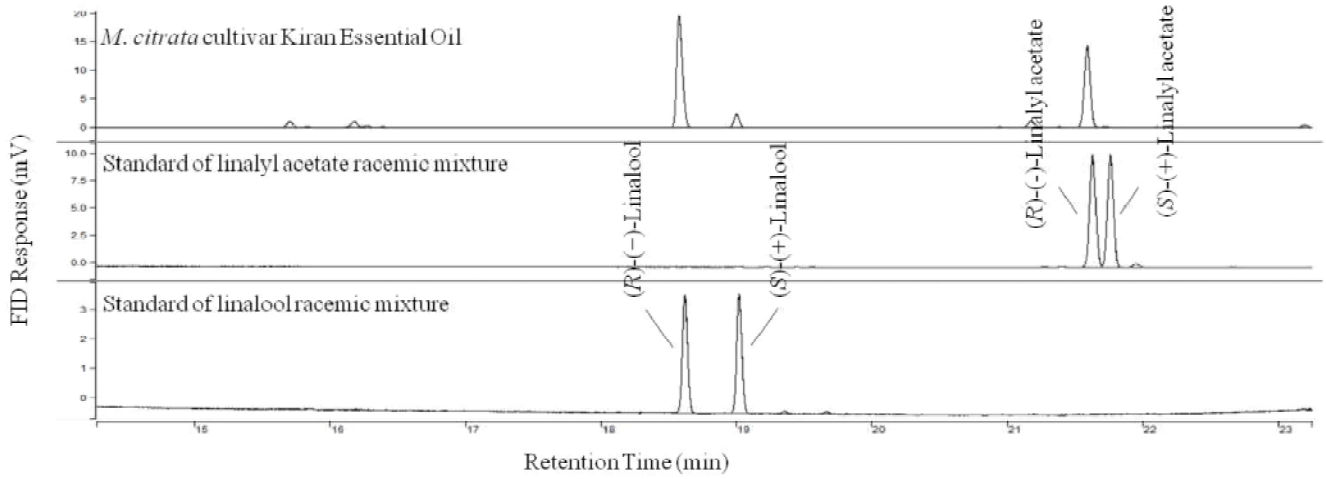

Figure 2: Chromatograms depicting the separation of linalool and linalyl acetate in enantioselective gas chromatography

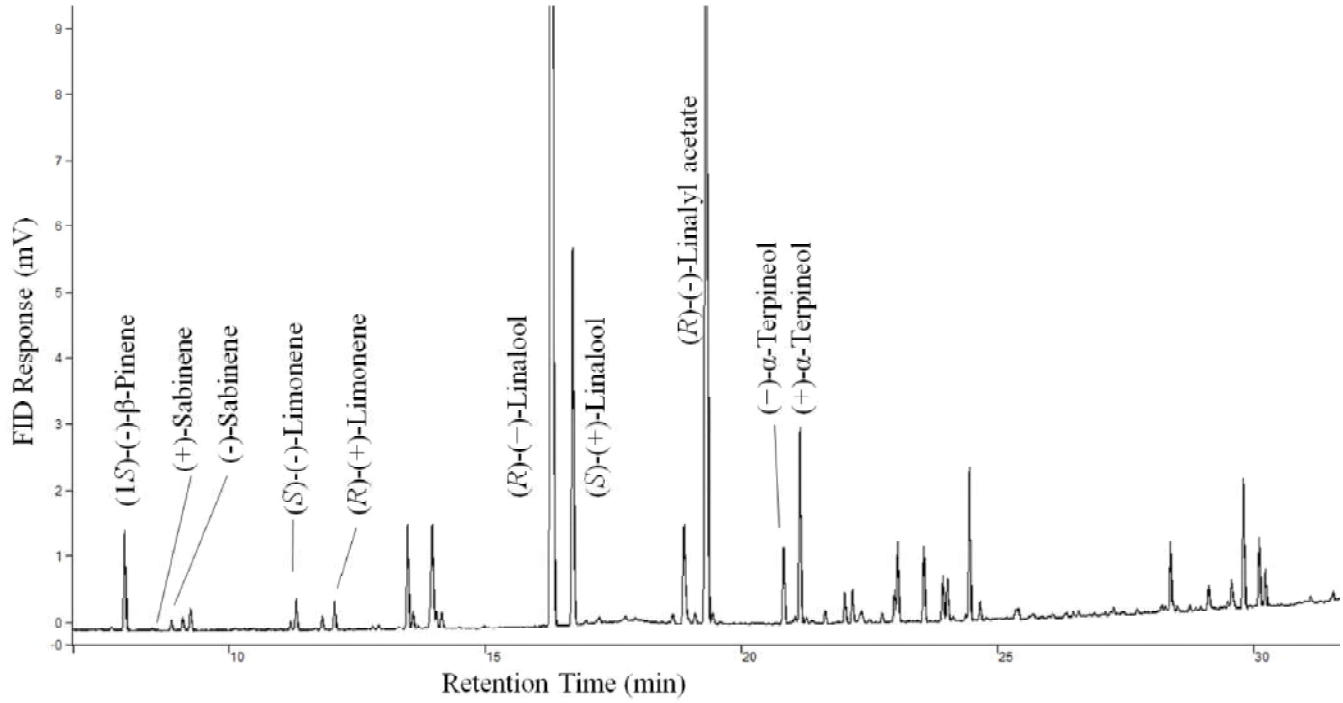

Figure 3: Enantioselective GC chromatogram showing the separation of terpenoid enantiomers in *M. citrata* cv. Kiran essential oil

enatiomers. Limonene is found as racemic mixture. Even though (*S*)-(+)-linalool is present upto 10.6%, (*S*)-(+)-linalyl acetate is only 0.8%, suggesting that the conversion of linalool to linalyl acetate is highly enantiospecific with only (*R*)-(-)-linalool converting into (*R*)-(-)-linalyl acetate, leaving behind (*S*)-(+)-linalool.The *M. citrata* cv. Kiran essential oil has 10.6% of (*S*)-(+)-linalool and 0.8% of (*S*)-(+)-linalyl acetate which falls under the limits set by the European Pharmacopoeia (EP5) standards of lavender essential oil as (*S*)-(+)-linalool upto maximum of 12% and (*S*)-(+)-linalyl acetate upto maximum of 1%. Therefore we propose *M. citrata* cv. Kiran as an alternative of lavender essential oil. Further, the chemical composition including enantiomeric differentiation studies (Table 2 and Figs. 2 and 3) can be used for ascertaining the authenticity of essential oil derived from *M. citrata* cv. Kiran. (*S*)-linalyl acetate is very rare in nature with the exception of *Elettaria cardamomum* essential oil, where (*S*)-linalyl acetate is reported with more than 99% enantiomeric excess (*ee*) (Casabianca et al., 1998). The search for other plants having high *ee* of (*S*)-linalyl acetate is under progress.

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