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The Chemo-Geographical Variation in Essential Oil Composition and the Antimicrobial Properties of "Wild Mint" – *Mentha longifolia* subsp. *polyadena* (Lamiaceae) in Southern Africa

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Abstract

Mentha longifolia (L.) L. subsp. *polyadena* Briq. was collected from eight localities in southern Africa for a study of the chemical composition and antimicrobial activity. The essential oils were obtained by hydrodistillation and analysed by GC and GC/MS and a cluster analysis was performed on the oil dataset. From eight samples (representing eight natural populations), two major chemotypes were identified: a menthofuran-rich type (51-62%); and a *cis*-piperitone oxide (15-36%) and piperitenone oxide-rich type (15-66%). The constituent analysis showed quantitative variation with higher amounts of oxygen-containing monoterpenes ranging from 57% to 90% whilst the sesquiterpene hydrocarbons ranged from 4% to 17%. The oil from the different geographical areas mostly showed moderate antimicrobial activity against *Staphylococcus aureus*, *S. epidermidis*, *Bacillus cereus*, *Moraxella catarrhalis*, *Yersinia enterocolitica* and *Enterococcus faecalis*. The oils were generally inactive against *Escherichia coli* and *Salmonella typhimurium*. *Candida albicans* and *Cryptococcus neoformans* indicated highest sensitivities for oil samples from Komukwane and Prins Albert. These results may in part provide scientific evidence for the extensive use of *Mentha longifolia* in traditional healing.

Key Word Index

Mentha longifolia subsp. *polyadena*, Lamiaceae, essential oil composition, 1,8-cineole, limonene, menthofuran, pulegone, *cis*-piperitone oxide, piperitenone oxide, antimicrobial activity.

Introduction

Mentha longifolia (L.) L., also known as wild mint, is widely distributed throughout southern Africa, occurring in most parts of South Africa, as well as in parts of Botswana, Namibia and Zimbabwe. The plant is a perennial herb common in wet places, with creeping rhizomes and erect flowering stems of up to 0.8 m in height. All parts are highly aromatic with a typical mint smell (1). In South Africa, three different subspecies of *Mentha longifolia* are recognized; *M. longifolia* subsp. *wissii* (Launert) Codd (Cape velvet mint) is known from only two localities, Brandberg (Namibia) and near Garies in Namaqualand. The long and thin, grey-green leaves are known to be unpleasantly aromatic.

Mentha longifolia subsp. *capensis* (Thunb.) Briq. is the most widespread taxon in South Africa and usually has a strong peppermint scent. *Mentha longifolia* subsp. *polyadena* Briq. (spearmint) has a disjunct distribution occurring in Gauteng, Swaziland, northern KwaZulu-Natal, eastern Free State and northern Lesotho and the southern Cape. It is also found again between Humansdorp and the Swartberg. The work reported here is restricted to the latter taxon.

Medicinally, milk or water decoctions of wild mint are mainly used for coughs, colds, asthma and other bronchial ailments by the Xhosa. It has also been used to treat headaches, fevers, indigestion, flatulence, hysteria, painful menstruation, delayed pregnancy and urinary tract

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Table I. Summary of some of the major components in the oils obtained from *Mentha longifolia* growing in different parts of the world

Country	Croatia	Yugoslavia	East Serbia	Russia	Kazhakstan	Lithuania	Greece	Iran	Morocco	Sudan*	South Africa	Botswana
						Main components (≥10%)						
Reference	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12, 13)	(14)	(15)		
piperitenone oxide	29					44			25		66	15
piperitone oxide									24			
piperitone		39										
carvone	34						58-66	62[12]		67		
limonene	10							20[12]		14		
menthone					19							
1,8-cineole						15	13	18[13]				
trans-dihydrocarvone							33					
menthone		11										
thymol			13									
p-cymene			14									
linalool				89								
β-caryophyllene				17								
menthol					47							
cis-carveol								54-78[13]				
menthofuran											51-62	
cis-piperitone oxide											15	36

* *M. longifolia* (L) Huds subsp. *schimperi*

infections. It is believed to be a diaphoretic and has mild spasmolytic action on the smooth muscle of the digestive tract hence useful for cramp-like complaints of the gastro-intestinal tract, gall bladder and the biliary tract. Externally, it has been used to treat wounds and swollen glands (2). Oral ingestion is reported to produce marked diuretic effects (3).

The essential oils of *Mentha* species contain numerous monoterpenes, amongst others, carvone, limonene, menthone and menthol (4). The composition is known to vary considerably at different localities, but no information appears to be available for the South African counterparts of this species and its various subspecies. Table I shows the variation of the major components present in the oils of *M. longifolia* growing in the countries indicated. Piperitenone oxide has been identified as the major compound in three previous studies (5,10,14). Carvone and limonene are common components in the oils of the plant collected in Croatia, Iran and Sudan (5,12,15). The plant collected from Greece contained carvone as one of its major components (11). 1,8-Cineole was a common component in the oils of plants collected from Lithuania, Greece and Iran (10,11,13). East Serbia produced the only chemotype rich in both thymol and p-cymene (7). It must be noted however that the *M. longifolia* from Sudan was of a different subspecies. Morocco produced the only chemotype rich in both piperitenone oxide and piperitone (14). The Russian chemotype was characterized by high levels (89%) of (+)-linalool (8).

Experimental

Plant material and isolation of oil: The aerial parts of *Mentha longifolia* subsp. *polyadena* were collected in the 2004/2005 flowering season from eight different localities in the wild. The fresh plant material was air-dried and the oils collected after hydrodistillation in 500 mL of de-ionized water for approximately 3 h using a modified Clevenger-type apparatus. Voucher specimens (Table III) are retained in the School of Pharmacy, Tshwane University of Technology.

***Analysis*:** The oil compositions were analyzed by GC and GC/MS. The GC analyses were performed using a Perkin Elmer 8700 gas chromatograph equipped with two FIDs, a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (30 m x 0.25 mm, 0.25 µm film thickness; J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica column (30 m x 0.25 mm, film thickness 0.15 µm; J & W Scientific Inc.). The oven temperature was programmed 45°-175°C, at 3°C/min, subsequently at 15°C/min up to 300°C, and then held isothermally for 10 min; injector and detector temperatures, 280°C and 290°C, respectively;

carrier gas, hydrogen, adjusted to a linear velocity of 30 cm/s. The samples were injected using the split sampling technique, ratio 1:50. The percentage composition of the oil samples was computed from the GC peak areas using the normalization method; the data were calculated as mean values of two injections from each oil sample without using response factors.

The GC/MS unit consisted on a Perkin Elmer Autosystem XL gas chromatograph, equipped with DB-1 fused-silica column (30 m x 0.25 mm, 0.25 µm film thickness; J & W Scientific, Inc.), and interfaced with a Perkin-Elmer Turbomass mass spectrometer (software version 4.1). Oven temperature was as above; transfer line temperature, 280°C; ion trap temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30cm/s; split ratio, 1:40; ionization energy, 70 eV; ionization current, 60 µA; scan range, 40-300 u; scan time, 1 s.

The identity of the components was assigned by comparison of their retention indices, relative to C_8-C_{17} n-alkanes, and their mass spectra with corresponding data of components of reference oils, laboratory-synthesized components and commercially available standards from an in-house library.

***Statistical analysis*:** The percentage composition of the oil samples was used to determine the relationship between the different samples of *Mentha* species by cluster analysis using the NTSYS-pc software (version 2.02, Exeter Software, Setauket, New York) developed by Rohlf (16). Correlation was selected as a measure of similarity, and the unweighted pair-group method with arithmetic average (UPGMA) was used for cluster definition. The degree of correlation was evaluated according to Pestana and Gageiro (17) ie: very high if correlation ranged between 0.9 and 1, high, between 0.7 and 0.89, moderate, between 0.4 and 0.69, low, between 0.2 and 0.3 and very low if <0.2.

Antimicrobial assays: Antimicrobial assays were performed on the essential oil samples at least in duplicate and where sufficient sample would allow, in triplicate. Antimicrobial activity was conducted using selected yeasts, Gram-positive and Gram-negative bacteria based on the traditional uses of this plant. The traditional use and associated test pathogens are as follows: for the treatment of infected external wounds, *Staphylococcus aureus* (ATCC 12600) and *Staphylococcus epidermidis* (ATCC 2223); urinary tract infections, *Escherichia coli* (ATCC 8739), *Enterococcus faecalis* (ATCC 29212) and *Candida albicans* (ATCC 10231); bronchial ailments, *Klebsiella pneumoniae* (NCTC 9633), *Moraxella catarrhalis* (clinical strain) and *Cryptococcus neoformans* (ATCC 90112) and gastro-intestinal complaints, *Salmonella typhimurium* (ATCC 14028), *Bacillus cereus* (ATCC 11778) and *Yersinia enterocolitica* (ATCC 23715). The microdilution assay was used to determine the minimum inhibitory concentration (MIC) (18). Each well of a 96 well micro-titre plate was initially filled with 100 µL of sterile water. Oil solutions were prepared at a starting concentration of 128 mg/mL in acetone and serially diluted. Thereafter, each well was filled with 100 µL of the culture medium comprising of an inoculum (1 x 10^6 colony forming units, CFU/mL). After

Table II. Percentage composition of the oils obtained from eight different populations of *Mentha longifolia* subsp. *polyadena* from Southern Africa

Compound	RI[a]	Population / Locality							
		Potchefstroom	Wakkerstroom	Komukwane	Prins Albert	Clocolan	Lydenburg	Dullstroom	Pretoria
α-thujene	924	0.2	0.1	0.1	t	t	0.1	t	t
α-pinene	930	1.8	1.1	2.0	t	0.9	0.5	1.2	1.4
camphene	938	1.6	0.1	0.9	t	t	0.4	0.7	0.5
sabinene	958	0.8	0.7	0.9	t	0.9	0.3	0.7	0.6
1-octen-3-ol	961				t		t		
β-pinene	963	2.1	1.8	2.0	t	1.7	0.8	1.6	1.7
3-octanol	974			t	0.1				
myrcene	975	1.2	0.8	2.3	t	0.2	0.6	0.3	0.4
phenylacetaldehyde	1002	t	t	t	t				
α-terpinene	1002	0.1	t	0.1	t	t	0.2	t	t
p-cymene	1003	0.2	t	0.1	t	t	0.2	t	t
1,8-cineole	1005	7.2	4.1	4.5	0.3	6.3	1.4	8.9	3.3
limonene	1009	7.2	4.1	4.5	0.3	6.3	1.4	3.8	3.3
γ-terpinene	1035	0.2	t	0.1	t	t	0.3	0.1	t
(E)-β-ocimene	1027								t
trans-sabinene hydrate	1037	0.2	t	0.1	t		0.8	0.2	t
terpinolene	1064	0.1	t	0.1	t		0.1	t	t
cis-sabinene hydrate	1066						0.1		
linalol	1074	0.2	0.1	1.8	0.7	t	1.5	0.3	t
trans-p-menth-2-en-1-ol	1074						0.1		

Table II. continued

Compound	RI[a]	Population / Locality							
		Potchefstroom	Wakkerstroom	Komukwane	Prins Albert	Clocolan	Lydenburg	Dullstroom	Pretoria
menthone	1120	0.4	0.6	0.3		1.7	5.0	0.3	t
isomenthone	1126	0.1	t	0.9		0.5	0.2	t	t
menthofuran	1134	55.3	59.7	0.9		61.6	51.4	58.0	52.8
borneol	1134	2.0	t	1.5	5.1		0.8	t	
p-cymen-8-ol	1148				0.2				
terpinen-4-ol	1148	0.4	0.3	0.2		0.1	1.1	0.2	t
myrtenal	1153				t		0.1		
α-terpineol	1159	0.1	0.1	0.2	0.8	t	0.4	0.1	t
myrtenol	1168				t				
pulegone	1210	0.4	t	0.1		0.2	12.0	0.2	t
cis-piperitone oxide	1236			35.7	14.7			t	
bornyl acetate	1265	1.0	0.2	0.1	0.5	t	0.2	1.0	0.4
6-hydroxy-carvotanacetone*	1269			0.2					
thymol	1275			0.3					
piperitenone	1289			2.9	1.6				
piperitenone oxide	1332			14.6	65.7				
δ-elemene	1332				0.2		t		
β-bourbonene	1379	0.2	0.1	0.6	0.1	0.1	0.4	0.4	0.5
β-elemene	1388	0.1	0.3	0.4	0.2	1.2	0.2	1.3	0.6
β-caryophyllene	1414	4.9	5.3	8.0	2.5	3.6	5.3	3.9	5.7
β-copaene*	1422	0.1	0.2	0.2		t	0.1	t	t
α-humulene	1447	0.6	0.7	1.3	0.2	0.5	0.7	1.9	0.8
germacrene D	1474	1.6	3.8	4.5	1.2	1.5	2.4	0.9	1.2
bicyclogermacrene	1487	0.7	2.6	1.0	t	0.7	1.0	1.1	0.5
γ-cadinene	1500	0.3	0.6	0.4		0.9	0.2	3.4	0.8
δ-cadinene	1505	0.5	0.8	0.3		0.6	0.3	t	1.8
(E)-nerolidol	1549				0.2		0.3	0.4	
spathulenol	1551	0.3	0.9	0.3		0.5	0.4	0.5	1.3
caryophyllene oxide	1561	1.1	1.1	0.9	0.2	1.1	0.2	0.3	0.4
T-cadinol	1616	0.2	0.3	t	t		0.2	0.1	0.4
α-muurolol	1618	t	0.3	t	0.2	0.2	t	t	0.5
α-cadinol	1626	0.4	0.4	0.7	t	0.5	0.3	0.1	0.9
% Identification		93.8	91.2	96.0	95.0	91.8	92.0	91.9	79.8
Grouped components									
Monoterpene hydrocarbons		15.5	8.7	13.1	0.3	10.0	4.9	8.4	7.9
Oxygen containing monoterpenes		67.3	65.1	64.3	89.6	70.4	75.1	69.2	56.5
Sesquiterpene hydrocarbons		9.0	14.4	16.7	4.4	9.1	10.6	12.9	11.9
Oxygen containing sesquiterpenes		2.0	3.0	1.9	0.6	2.3	1.4	1.4	3.5
Others		t	t	t	0.1	t	t	t	t
Oil Yield (%), w/w (dry wt.)		1.09	0.59	0.61	0.36	1.46	0.88	0.72	1.01

[a]RI = retention index relative to C_8-C_{17} n-alkanes on the DB-1 column; *based on mass spectra only; t = trace (<0.05%); Potchefstroom - North West Province; Wakkerstroom - Mpumalanga Province; Komukwane – Botswana; Prins Albert - Eastern Cape Province; Clocolan - Free State Province; Lydenburg - Mpumalanga Province; Dullstroom - Mpumalanga Province; Pretoria - Gauteng Province

sealing with a sterile adhesive, the micro-titre plate was incubated at 37°C for 24-48 h, depending on the pathogen studied. A 0.02 mg/mL ρ-iodonitrotetrazolium violet (INT) aqueous solution was prepared and 40 µL added to each well. The micro-titre plates were allowed to stand for 6 h for bacterial strains and 24 h for the yeast strains. Tetrazolium salts like INT, are used to indicate the biological activity because the colorless compound acts as an electron acceptor and is reduced to a colored product by biologically active organisms as reported by Eloff (18). The contents of the well turned red if any microbial growth was present.

Results and Discussion

Oil yields: The samples were variable in oil yield ranging from 0.36% to 1.46% (w/w dry weight) as shown in Table II.

Oil composition: All the populations studied afforded oils dominated by the oxygen-containing monoterpenes, ranging from 57-90% (Table II). The identified oil components are listed in Table II in order of their elution on the DB-1 column. Despite the fact that this fraction dominated in all the oils, some chemical variability was clear in all samples studied, which was confirmed by the cluster analysis, with a correlation coefficient varying between 0.0 and 0.99 (Figure 1).

Two groups of samples could be defined based on the degree of correlation in the oil composition. One group of samples showed a very high degree of correlation (S_{corr}>0.9) in the oil composition. Five of the six oil samples of this group (Potchefstroom, Clocolan Dullstroom, Wakkerstroom and Pretoria, S_{corr}>0.95) were dominated by menthofuran (53-62%) (Figure 1, Table II). Lydenburg oil showed a high degree of correlation with this group, S_{corr}=0.95, mainly due to the high amount of menthofuran (51%), but showed a high percentage of pulegone (12%), which ranged from traces to 0.4% in the other five samples.

A second group of oils, those of Komukwane and Prins Albert, showed only a moderate correlation among each other (S_{corr} between 0.4 and 0.69) and a very low correlation (S_{corr} <0.2) with the former group. These oils were dominated by *cis*-piperitone oxide and piperitenone oxide in both cases, but with opposite degree of importance (36% and 15%, respectively for Komukwane and 15% and 66%, respectively for Prins Albert).

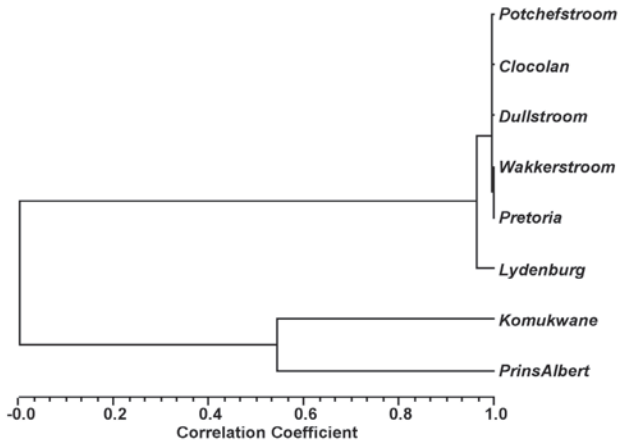

Figure 1. Dendrogram obtained by cluster analysis of the percentage composition of the oils from the *Mentha longifolia* subsp. *polyadena* populations examined, based on correlation and using the unweighted pair-group method with arithmetic average (UPGMA)

Table III. Microdilution assay results (mg/mL) for the eight samples of *Mentha longifolia* ssp. *polyadena* oil tested on 11 different pathogens

Sample	Yeast		Gram-positive				Gram-negative				
(and voucher number)	Ca	Cn	Sa	Se	Bc	Ef	Kp	St	Ec	Ye	Mc
Potchefstroom (AV 1096)	32.0	8.0	12.0	12.0	8.0	12.0	32.0	32.0	32.0	12.0	8.0
Lydenburg (AV 1097)	24.0	8.0	8.0	4.0	8.0	8.0	6.0	32.0	32.0	6.0	8.0
Dullstroom (AV 1094)	32.0	24.0	8.0	1.0	8.0	8.0	32.0	24.0	32.0	6.0	4.0
Komukwane (AV 1095)	3.0	0.5	6.0	4.0	8.0	8.0	24.0	8.0	16.0	4.0	4.0
Prins Albert (AV 1135)	0.5	1.6	6.0	*	*	*	4.0	*	*	*	*
Wakkerstroom (AV 1132)	32.0	8.0	8.0	4.0	12.0	8.0	8.0	32.0	32.0	8.0	4.0
Clocolan (AV 1133)	32.0	24.0	12.0	2.0	6.0	12.0	8.0	32.0	32.0	8.0	4.0
Pretoria (AV1134)	24.0	6.0	8.0	3.0	4.0	16.0	8.0	*	*	4.0	*
+ Control **											
Amphotericin B	3.1	6.3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
+ Control **											
Ciprofloxacin	N/A	N/A	3.1	0.8	0.4	6.3	25.0	0.4	12.5	1.6	1.5
- Control	-	-	-	-	-	-	-	-	-	-	-

* not tested due to insufficient sample; ** all MIC values ("x") for the + controls are recorded as "x" to the power 10^{-4}; N/A – Not Applicable; Ca - *Candida albicans* (ATCC 10231); Cn - *Cryptococcus neoformans* (ATCC 90112); Sa - *Staphylococcus aureus* (ATCC 12600); Se - *Staphylococcus epidermidis* (ATCC 2223); Bc - *Bacillus cereus* (ATCC 11778); Ef - *Enterococcus faecalis* (ATCC 29212); Kp - *Klebsiella pneumoniae* (NCTC 9633); St - *Salmonella typhimurium* (ATCC 14028); Ec - *Escherichia coli* (ATCC 8739); Ye - *Yersinia enterocolitica* (ATCC 23715); Mc - *Moraxella catarrhalis* (clinical strain)

Based on this data two different chemotypes can be proposed for *M. longifolia* subsp. *polyadena*, the menthofuran and the *cis*-piperitone oxide/piperitenone oxide chemotypes.

Antimicrobial assays: The Komukwane and Prins Albert samples showed good activity against *C. albicans* (MIC values of 3 mg/mL and 0.5 mg/mL, respectively), whereas the other samples showed poor to no activity (Table III). Antimicrobial activity for the yeasts *C. neoformans* and *C. albicans* indicated variable efficacy depending on the locality. The Komukwane and Prins Albert populations showed the highest activity against *C. neoformans* (MIC values of 0.5 mg/mL and 1.6 mg/mL, respectively) and *C. albicans* (MIC values of 3 mg/mL and 0.5 mg/mL, respectively).

In general, activity against the Gram-positive pathogens (*Staphylococcus aureus, S. epidermidis, Bacillus cereus* and *Enterococcus faecalis*) was moderate to good. The strongest activity among the Gram-positive pathogens was for *S. epidermidis* where activity ranged from 1-12 mg/mL depending on the locality (Table III). This could possibly confirm the rationale for the traditional use of this plant as a treatment for wounds and skin infections.

The Gram-negative pathogens (*Escherichia coli, Klebsiella pneumoniae*, *Moraxella catarrhalis, Salmonella typhimurium* and *Yersinia enterocolitica*) were less susceptible to the oil samples. The MIC values (4-8 mg/mL) for *Morexella catarrhalis* indicated the least variability of all the pathogens studied.

Mentha longifolia displays quantitative and qualitative variations between natural populations which seem to be random and are not correlated to the geographic distribution of the plant. Furthermore, from the eight natural plant populations assessed, two distinct major chemotypes were noted. The study also documented a menthofuran chemotype in *M. longifolia* for the first time. The plant also displays moderate to good antibacterial activity, in particular against Gram-positive bacteria, which justifies its wide use in African traditional medicine in the treatment of respiratory disorders, skin infections and gastrointestinal disorders. There was considerable antimicrobial variability between the geographical populations. For example, the MIC values for *C. albicans* ranged from 0.5 mg/mL to 32 mg/mL, whereas for *C. neoformans*, the MIC values ranged between 0.5 mg/mL and 24 mg/mL. Some populations having similar chemical profiles (especially the major compounds) displayed varying results in the antimicrobial assays. This suggests that the presence of trace components, even those as yet unidentified, can possibly influence the biological activity of the oil to a significant extent.

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