Journal of Ecobiotechnology 2/4: 22-26, 2010
ISSN 2077-0464
Journal of Ecobiotechnology
http://journal-ecobiotechnology.com/

Quantitative and Rapid Antibacterial Assay of *Micromeria biflora* Benth. Leaf Essential Oil Against Dental Caries Causing Bacteria Using Phylogenetic Approach

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Keywords

Antibacterial activity
Dental caries
Essential oil
Micromeria biflora Benth
Phylogenetic Analysis
Streptococcus mutans

Abstract

The phylogenetic relationship of four dental caries causing bacterial pathogens has been studied using ITS1 sequences of the standard strains were aligned by using the ClustalW computer program. The essential oil obtained from the leaves of *Micromeria biflora* Benth., obtained by hydrodistillaton. The chemical compositions of the essential oil from *Micromeria biflora* Benth was analyzed by gas chromatography-mass spectrometry (GC-MS). The GC/MS analysis showed eight major active constituents in the leaf essential oil of *Micromeria biflora* Benth. The antibacterial activity of the oil was evaluated against four dental caries causing bacteria such as *Streptococcus mutans* (MTCC 890); *Lactobacillus acidophilus* (MTCC 447); *Streptococcus mitis* (MTCC 2695) and *Streptococcus salivarius* (MTCC 1938) using broth microdilution method recommended by Clinical Laboratory Standards Institute (CLSI) formely (NCCLS). It's showed excellent activity against *Streptococcus mutans* with their Minimum inhibition concentration (MIC) 0.15 mg/ml and (IC_{50}) 0.10 mg/ml and less effective against *Lactobacillus acidophilus*. The essential oil of *Micromeria biflora* Benth from leaf has played a significant role against dental caries causing bacteria. Relationships of the dental caries causing pathogens to the toxicity of the oil vis-à-vis phylogeny using molecular data of pathogens have also been discussed.

1. Introduction

Dental caries is a multifactorial infectious disease, usually associated with increased numbers of *Streptococcus mutans* at the site of the disease. In addition, other microflora like *Lactobacillus acidophilus, Streptococcus salivarius, Streptococcus mitis* and *Streptococcus sanguis* are also involved in the process of causing dental caries. Estimation of the salivary levels of this organism may be useful for assessing caries risk in patients and for monitoring their response to preventive measures (1). *Streptococcus mutans* is an important component of the biofilms on teeth (dental plaque) associated with many forms of dental caries. *Steptococcus mutans* adheres firmly to the smooth tooth surfaces and produces sticky water insoluble dextran from dietary sucrose, forming plaque, which facilitates the accumulation of microorganisms. *Streptococcus mutans* and other organisms in the plaque produce organic acids such as lactic acid that gradually destroy the enamel and form a cavity (2).

The *Micromeria biflora* Benth, known as Indian wild Thyme, belongs to family Lamiaceae found in tropical, temperate Himalayas and Western Ghats. It is hardy to zone 0, flowers from June to August, and the seeds ripened from August to September. The flowers are hermaphrodite (have both male and female organs) and are insect-pollinated. A paste of the root was pressed between the jaws to treat toothache (3). The plant was rubbed and the aroma inhaled to treat nose bleeds (3). A paste of the plant was used as a poultice to treat wounds (3). The juice of the plant is taken internally and also inhaled in the treatment of sinusitis. The objective of this study was to investigate toxicity of the *Micromeria biflora* Benth. leaf essential oil vis-à-vis phylogeny using molecular data of pathogens. In particular, 16S rDNA sequences have been widely used to construct bacterial phylogenetic relationships (4, 5).

2. Materials and Method

Collection of plant materials and extraction of essential oil

The essential oil was extracted from the fresh leaves of *Micromeria biflora* Benth. collected from the Himanchal Pradesh, India by hydro-distillatation using Clevenger's apparatus (6). A clear dark

reddish yellow coloured oily layer was separated and dried with anhydrous sodium sulphate.

Physio-chemical properties

The essential oils obtained from *Micromeria biflora* Benth was studied on various parameters of physio-chemical properties such as Plant height, Oil yield, Colour, Specific gravity, Optical rotation, Refractive index and Solubility in 90% alcohol. The results are given in the Table 2.

GC-MS analysis

Gas Chromatography analysis of the oil was performed on a Perkin- Elmer GC 8500, using a fused capillary column (25m x 0.55 mm i.d., film thickness 0.25um), coated with dimethtyl siloxane (BP-1). The oven temperature was programmed at 60°C to 220°C at 5°C/min. then held isothermal at 220°C for 15 min. injector temperature, 250°C, detector temperature, 300°C, carrier gas, nitrogen at a linear velocity of 10 psi: split, 1:80

GC-MS data were obtained on a Shimazdu QP-2000 mass spectrometer at 70 ev and 250°C. GC column: Ulbon HR-1 equivalent to OV-1, fused silica capillary column 0.25 mm x 50m, film thickness 0.25 um. The initial temperature was 100°C for 7 min. and heated at 5°C/min to 250°C. Carrier gas helium at a flow rate of 2ml/min.The percentage composition of *Micromeria biflora* Benth leaves oil is given in the Table1.

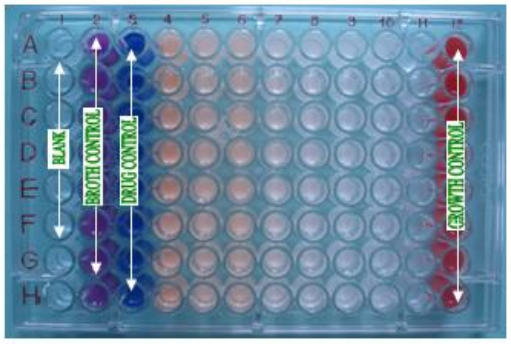
Dental caries causing bacterial pathogens

Four dental caries causing bacterial pathogens were selected for this study; *Streptococcus mutans* (MTCC 890); *Lactobacillus acidophilus* (MTCC 447); *Streptococcus mitis* (MTCC 2695) and *Streptococcus salivarius* (MTCC 1938). Cultures were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India. The cultures of bacteria were maintained on Nutrient agar slants at 4ºC throughout the study used as stock cultures.

Determination of minimum inhibitory concentration (MIC) and IC_{50} by broth micro dilution method

The antimicrobial activity of compounds was determined by broth micro dilution method recommended by Clinical Laboratory Standards Institute (CLSI) formerly NCCLS (7) using Mueller Hinton Broth. All the standard water borne bacterial cultures were maintained on Nutrient agar at 37°C. The 96-well tissue culture plates were used for twofold serial dilution. The proper growth control, drug control and the blank was adjusted onto the plate. Essential oil of *Micromeria biflora* Benth was dissolved in 5-10% DMSO at a concentration of 50mg/ml stock solution in case of natural antimicrobials, 20 µl of drug was added into 4th well of 96-well tissue culture plate horizontally having 180µl Mueller Hinton Broth (Fig. 1). So the maximum concentration of the test essential oil was 2.5mg/ml. From here the solution was serially diluted upto 4th well to 11th well resulting into the half of the concentration of test essential oil. The bacterial inoculum was prepared at 0.5 McFarland standards; the absorbance was equal to the inoculum suspension containing $1x10^7$ cells per ml for bacterial isolates. Then standard bacterial inoculums was added and kept for incubation at 37°C in a moist chamber. The Minimum Inhibitory Concentration (MIC) and Inhibitory Concentration at 50% (IC_{50}) was recorded spectrophotometrically at 492 nm using SpectraMaxplus 384 after 24 hrs incubation.

Figure 1. Format of drug testing by broth micro dilution method



Determination of minimum bactericidal concentration (MBC)

100 µl aliquot of inoculum was taken aseptically from incubated 96 well plate those well did not show turbidity and poured on to Nutrient agar plates then incubated for 24 hours at 37°C. MBC was defined as the lowest concentration of the essential oil at which 99.99% or more of the initial inoculum was killed. If there was no growth, it means the concentration was cidal. The number of surviving organisms was determined by viability counts. All tests were performed in triplicate.

Phylogenetic study

To find out the reason; why the essential oil is more effective against dental caries causing bacteria, phylogenetic relationship have been studied including *Streptococcus mutans*; *Lactobacillus acidophilus*; *Streptococcus mitis* and *Streptococcus salivarius* using the Clustal W computer program (8) and GENETYX-MAC 10.1 software (Software Development Co., Ltd., Tokyo, Japan). Phylogenetic trees were then constructed by the DNA maximum-likelihood (ML) method in the PHYLIP program (Phylogeny Inference Package), version 3.5c (9) and the neighbor-joining (NJ) (10), method in the NJPLOT program (11), Bootstrap analysis with the Clustal W program was performed.

Nucleotide sequence accession numbers

Data for the phylogenetic analysis were obtained from sequences contained in the GenBank nucleotide sequence database (12). The ITS1 sequences of the standard strains used in this study *Streptococcus mutans* (accession no. AF204255); *Lactobacillus acidophilus* (accession no. HM162411); *Streptococcus mitis* (accession no. NC013853) and *Streptococcus salivarius* (accession no. S41233) were aligned (4,5,13,14).

3. Results and Discussion

Plant essential oils and extracts have been used for many thousands of years (15) especially in food preservation, pharmaceuticals, alternative medicine and natural therapies (16).

In the present study, composition and relative percentages of essential oil of *Micromeria biflora* was determined. 4 major constituents were identified with high content of Thymol 54%, Iso thymol 9.9%, Gurjurene 3.3% and β-caryophyllene 6.6% with RetentionTime (RT) 8.06, 8.83, 10.20 and 12.86 respectively. However, earlier studies suggested that the *Micromeria biflora* sp. Arabica K. Walth, essential oil was analyzed by GC-MS (17), 30 components were identified representing 98.2% of the total oil. The major constituents were trans-caryophyllene (43.7%), caryophyllene oxide (18.0%), spathulenol (8.5%), α-humulene (4.6%), α-myrcene (3.1%), and germacrene-D (3.1%) The present investigation of GC-MS analysis of *Micromeria biflora* essential oil, chemical constituents were quite different due to different agroclimatic changes. The major components and their retention times are summarized in Table 1.

Table 1. Mass Spectoscopy analysis of *Micromeria biflora* Benth leaf essential oil

Peak No.	R. Time (Scan)	I. Scan-F. Scan	Area	Height	Major Compounds	%Total
1	8.06 (62)	59-79	603564	113551	Thymol	54.0
2	8.83 (85)	82-92	110841	40442	Iso-thymol	9.9
3	10.20 (126)	124-130	36581	8317	Gurjurene	3.3
4	12.86 (206)	204-210	74151	21178	β-caryophyllene	6.6

Physio-chemical properties of *Micromeria biflora* Benth leaf essential oil showed various parameters of such as Plant height, Oil yield, Colour, Specific gravity, Optical rotation, Refractive index and Solubility in 90% alcohol. The results are given in the Table 2.

Table 2. Physio-chemical properties of *Micromeria biflora* Benth leaf essential oil

S. No.	Parameter studies	*Micromeria biflora*
1.	Plant height	30 Cm
2.	Oil yield	0.03-0.07%
3.	Appearence	dark reddish yellow
4.	Specific gravity at 25°C	0.8913 to 0.91260
5.	Optical rotation	-3 to -25
6.	Refractive index at 20°C	1.468 to 1.488
7.	Solubility in 90% alcohal	Soluble

Filoche *et al.* (18) reported that essentail oil of *Cinnamon* showed antimicrobial potency (1.25–2.5 mg/ml) against *Streptococcus mutans* and *Lactobacillus plantarum*. However, in the present antibacterial activity of *Micromeria biflora* Benth leaf essential oil were assayed *in vitro* by a broth micro-dilution method against four dental caries causing bacteria such as *Streptococcus mutans* (MTCC 890); *Lactobacillus acidophilus* (MTCC 447); *Streptococcus mitis* (MTCC 2695) and *Streptococcus salivarius* (MTCC 1938) .

According to the results, *Micromeria biflora* Benth leaf essential oil was found to be active against all dental caries causing bacteria. The strongest antibacterial activity was seen against *Streptococcus mutans* with a Minimum inhibitory concentration (MIC) value 0.15 mg/ml and IC_{50} value 0.10 mg/ml. While Minimum inhibitory concentration (MIC) value of *Lactobacillus acidophilus*; *Streptococcus mitis* and *Streptococcus salivarius was* 0.35 mg/ml, 0.20 mg/ml and 0.19 mg/ml respectively. These results are shown in Table 3. Nascimento *et al.,* (19) also

reported that the *Hyptis pectinata* essential oil exhibited considerable inhibitory effect against either all the clinical isolates obtained from patients' saliva or the ATCC strains tested, with minimum inhibitory and bactericidal concentrations of 200µg/mL.

Table 3. Anticaries activity of *Micromeria biflora* Benth leaf essential oil

S. No.	Caries causing bacteria	Antibacterial activity in mg/ml		
		MIC	IC_{50}	MBC
1.	*Streptococcus mutans*	0.15	0.10	0.19
2.	*Lactobacillus acidophilus*	0.35	0.18	0.38
3.	*Streptococcus mitis*	0. 20	0. 16	0.38
4.	*Streptococcus salivarius*	0. 19	0. 15	0.38

The phylogenetic relationships of dental caries causing bacteria were demonstrated by using internal transcribed spacer 1 (ITS1) obtained from GenBank. Alignment of the 16S rRNA nucleotide sequence, adjusted to 1,435 bases, was performed by the computer program MegAlign (DNASTAR Inc.). On the Phylogenetic analysis a clear picture can be drawn as shown in Fig. 2 and 3 *Streptococcus mutans and Streptococcus salvarius* belong to same genetical stock so; they were close to each other. That is the reason the Minimum Inhibitory Concentration (MIC) of test essential oil against *S. mutans* (0.15mg/ml) and *S. salvarius* (0.19mg/ml) reflect almost similar toxicity.

Figure 2. Alignment of ITS1, 16S rDNA sequences of dental caries causing bacterial pathogens

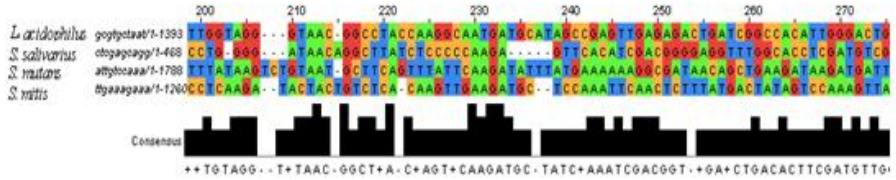


Figure 3. Results of cladogram (Neigbour Joining Tree plot) of dental caries causing bacteria using standard ITS1 sequences

However, *Lactobacillus acidophilus* ***and*** *Streptococcus mitis* found to be closer to each other with their closed MIC *0.35 mg/ml, 0.20*mg/ml. Results are showed in Fig. 2 and 3. An important characteristic of essential oils and their components is their hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable (20,21). Extensive leakage from bacterial cells or the exit of critical molecules and ions will lead to death (22).

4. Conclusions

In the present study clearly demonstrates that the leaf essential oil of *M. biflora* Benth was exhibited potent bactericidal action and as a therapeutic remedy against dental caries causing bacteria. The effectiveness of the oil was equal to those caries causing bacteria which are close in phylogenetic tree. As such, in future the oil can be used as a potential source of effective and cheap herbal formulation after undergoing successful multicentral topical testing.

Acknowledgements

The authors are very grateful to Department of Science and Technology for financial support and Head, Department of Botany, University of Allahabad for giving me this opportunity to perform experimental work successfully.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the manuscript.

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Please Cite This Article As: Rohit Kumar Mishra, Awadhesh Kumar, Amritesh Chandra Shukla, Pravin Tiwari and Anupam Dikshit. 2010. Quantitative and Rapid Antibacterial Assay of *Micromeria biflora* Benth. Leaf Essential Oil Against Dental Caries Causing Bacteria Using Phylogenetic Approach. J. Ecobiotechnol. 2(4):22-26.