African Journal of Microbiology Research Vol. 4 (11) pp. 1122-1127, 4 June, 2010
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 © 2010 Academic Journals

Full Length Research Paper

Effect of *Mentha longifolia* L. ssp *longifolia* essential oil on the morphology of four pathogenic bacteria visualized by atomic force microscopy

Hajlaoui Hafedh[1]*, Ben Abdallah Fethi[1], Snoussi Mejdi[1,2], Noumi Emira[1] and Bakhrouf Amina[1]

[1]Laboratoire d'Analyse, Traitement et Valorisation des Polluants de l'Environnement et des Produits, Faculté de Pharmacie, Rue Avicenne 5000 Monastir, Tunisie.
[2]Laboratoire de Traitement des Eaux Usées, Centre de Recherches et des Technologies des Eaux (CERTE), Technopole de Borj-Cédria, BP 273- Soliman 8020, Tunisie.

Accepted 15 April, 2010

The chemical composition and the antibacterial activity of the essential oil of *Mentha longifolia* L. ssp *longifolia* are being investigated in the present work. The minimal inhibitory concentration (MIC) of this oil against four gram+ and gram- reference bacteria including *Salmonella typhimurium* LT2, *Escherichia coli* ATCC 35218, *Micrococcus luteus* NCIMB 8166 and *Staphylococcus aureus* ATCC 25923 was used to study the morphological alteration of bacterial wall cell visualized by atomic force microscopy (AFM). The chemical analysis of the essential oil showed the presence of 34 compounds. The most important ones were: menthol (32.51%), menthone (20.71%), pulegone (17.76%), 1,8-cineole (5.61%), terpineol-4 (4.87%) and piperitone (2.16%). The MIC for bacteria ranged from 0.19 to 1.56 mg/ml. We found that *M. longifolia* (Menthol chemotype) has a high antibacterial effect. Cell wall of the tested bacteria was damaged at MIC concentrations. This susceptibility is more accentuated in *S. typhimurium* and *E. coli* (rod bacteria), whereas the damage is less important in coccoid bacteria (*S. aureus* and *M. luteus*).

Key words: *Mentha longifolia*, essential oil, *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, *Salmonella typhimurium*, cell morphology, atomic force microscope.

INTRODUCTION

Resistance to antimicrobial agents by pathogenic bacteria accentuated by the ability of biofilm formation on clinical environment has emerged in recent years and is a major health problem (Colwell et al., 1992; Hausner et al., 1999). The increasing drug/chemical resistance of microorganisms (bacteria, viruses and fungi) and the insufficiency of the drugs commonly used in the treatment of infectious diseases led us to search for new sources of natural products with large spectre of biological activities(Abad et al., 2007).

In the light of these data, plant essential oils may be an alternative source of natural compounds for pathogenic bacteria because they constitute a rich source of bioactive chemicals and are commonly used as fragrances and as flavouring agents for food additives (Bauer et al., 2001; Mimica-Dukić et al., 2003; Hafedh et al., 2009). Indeed, the effectiveness of the activity of essential oils with respect to gram- and gram+ bacteria is largely documented in literature. These properties are due to the presence of active monoterpenes constituents (Morris et al., 1979; Knobloch et al., 1988).

The action mechanism of the essential oils is related to their chemical composition and their antimicrobial activity is not attributable to a unique mechanism but to a cascade of reaction in the entire bacterial cell (Burt et al., 2004). The hydrophobicity of the essential oils is the main properties responsible for the distribution of bacterial structures increasing their permeability due to their inability to partition the lipids from bacterial cell membrane. This permeability barrier role of cell membranes is integral to many cellular functions, including the maintenance of the energy status of the cell, other membrane-coupled energy-transducing processes,

*Corresponding author. E-mail: hajlaoui_htn@yahoo.fr. Tel: +216 73 461000. Fax: +216 73 461830.

solute transport and regulation of metabolism and control of turgor pressure (Poolman et al., 1987; Trumpower and Gennis, 1994). Toxic effects on membrane structure and function have generally been used to explain the antimicrobial action of essential oils and their monoterpenoid components (Andrews et al., 1980; Uribe et al., 1985; Knobloch et al., 1988).

In fact, the action mechanism of the essential oils includes: degradation of the cell wall (Helander et al., 1998, Gill et al., 2006), cytoplasmic-membrane damaging and cytoplasm coagulation (Gustafson et al., 1998; Ultee et al., 2000; 2002), damage to membrane proteins (Juven et al., 1994), leakage of cell contents (Lambert et al., 2001), depletion of the protive force motrice (Ultee and Smid, 2001), sharp reduction of the intracellular ATP pool through a reduction of ATP synthesis and increased hydrolysis, not obviously related to an increase in membrane permeability and reduction of the membrane potential due to an increase in the membrane permeability to protons (Burt et al., 2004). In yeast cells, α-pinene and β-pinene destroy cellular integrity; inhibit respiration and ion transport processes and increase membrane permeability (Uribe et al., 1985). More recently, Helander et al. (1998) have described effects of different essential components on outer membrane permeability in gram- bacteria. The fact that tea tree oil-induced damage to cell membrane structure accompanied the decline in viability for all three microorganisms included in this study, confirms it as the most likely cause of cell death.

The effectiveness of *Mentha longifolia* essential oils against gram+ and gram- bacteria has been well described by (Ghoulami et al., 2000; Rasooli et al., 2002; Mimica-Dukić et al., 2003; Hajlaoui et al., 2008; 2009). Therefore, the objectives of this study were: (1) to analyze the chemical composition of a hydrodistilled essential oil of *M. longifolia* plants collected from Sidi Bouzid (Centre of Tunisia) by a system in order to determine the essential oil chemotype; (2) to investigate the antimicrobial activity and the effect of *M. longifolia* essential oil on pathogenic bacteria morphology observed by atomic force microscopy.

MATERIALS AND METHODS

Microorganisms

Four reference strains chosen for this study include: two gram+ bacteria (*Micrococcus luteus* NCIMB 8166 and *Staphylococcus aureus* ATCC 25923) and two gram- bacteria (*Salmonella typhimurium* LT2 DT104, *Escherichia coli* ATCC 35218).

Essential oil analysis: Gas chromatograph/gas chromatograph-mass spectrometry

Plant of *M. longifolia* used in this study was collected from Sidi Bouzid (Centre of Tunisia). The leaves of the mint dried for seven days at room temperature and from buds were subjected to hydrodistillation using a Clevenger-type apparatus according to the European Pharmacopoeia (European Pharmacopoeia, 1975).

The gas chromatography analysis of the volatile oil was performed using a HP 5890-series II equipped with Flame ionization detectors (FID), HP-5 (BP-1) (5% phenyl+95% dimethylpolysiloxane) 30 m x 0.25 mm ID, 0.25 µm film thickness fused capillary column and HP Innowax (BP-20; polyethylenglycol) 30 m x 0.25 mm ID, 0.25 µm film thickness fused capillary column. The carrier gas was nitrogen (1.2 ml mn^{-1}). The oven temperature program was 1 min isothermal at 50°C, then 50 - 280°C (BP-1) and 50 - 220°C (BP-20) at rate of 5°C/min and held isothermal for 1 min. The injection port temperature was 250°C, detector: 280°C. Volume injected: 1 µl of 1% solution (diluted in hexane). Percentages of the constituents were calculated by electronic integration of FID peak areas without the use of response factor correction.

The analysis of the volatile constituents was run on a Hewlett-Packard GC-MS system (GC: 5890-series II; MSD 5972). The fused-silica HP-5 MS capillary column (30 m x 0.25 mm ID, film thickness of 0.25 µm) was directly coupled to the MS. The carrier gas was helium, with a flow rate of 1.2 ml mn-1. Oven temperature was programmed (50°C for 1 min, then 50 - 280°C at 5°C/min) and subsequently, held isothermal for 2 min. Injector port: 250°C, detector: 280°C, split ratio 1:50. Volume injected: 1 µl of 1% solution (diluted in hexane): HP 5972 recording at 70 e Volts; scan time 1.5 sec; mass Range 40 - 300 amu. Software adopted to handle mass spectra and chromatograms was a Chem Station. The components of the oils were identified by comparison of their mass spectra with those of a computer library (Wiley 275 library; Adams, 2001).

Micro-well determination of minimal inhibition concentration

The minimal inhibition concentration (MIC) value was determined for all bacterial strains used in this study as described by Gulluce et al. (2007). The inoculums of the bacterial strains were prepared from 12 h broth cultures (medium LB, pH: 7.4) and suspensions were adjusted to 0.5 McFarland standard turbidity. Each essential oil that was dissolved in 10% dimethylsulfoxide (DMSO) was first diluted to the highest concentration (50 mg/ml) to be tested, and then serial two-fold dilutions were made in a concentration range from 0.0488 – 50 mg/ml for spearmint oil in 5 ml sterile test tubes containing nutrient broth. The 96-well plates were prepared by dispensing into each well 95 µl of nutrient broth and 5 µl of the inoculum. A 100 µl aliquot from the stock solutions of essential oil was added into the first wells. Then, 100 µl from the serial dilutions were transferred into eleven consecutive wells. The last well containing 195 µl of nutrient broth without essential oil and 5 µl of the inoculum on each strip was used as the negative control. The final volume in each well was 200 µl. The plates were incubated at 37°C for 18 - 24h. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of the microorganisms. The test was performed in triplicate.

Determination of morphological changes by atomic force microscopy (AFM)

The test microorganisms included the following gram+ bacteria: *S. aureus*, *M. luteus* and gram- bacteria: *E. coli*, *S. typhimurium*, pathogenic human strains were used to evaluate the effects of the *M. longifolia* essential oil. For the experiments, each tested strain was cultured in Tryptic Soy broth (TSB, Pronadisa, Spain) overnight at 37°C, the suspensions were adjusted to 0.5 McFarland standard turbidity and 1 ml of the cultures was transferred into new tube containing 4 ml TSB with MIC for each strain and incubated for 24 h at 37°C with shaking.

In order to visualize the effect of the *M. longifolia* essential oil on

Table 1. The major compounds of *M. longifolia* essential oil.

	Components	Kovats index (KI)		Percentage (%)
		HP-5	HP-20M	
1	1,8 Cineole	1021	1208	5.61
2	Menthone	1139	1456	20.71
3	Terpineol-4	1178	1581	4.87
4	Menthol	1171	1612	32.51
5	Pulegone	1228	1645	17.76
6	Piperitone	1286	1730	2.16

The components and their percentages are listed in order of their elution on apolar column (HP-5) and polar column (HP-20M).

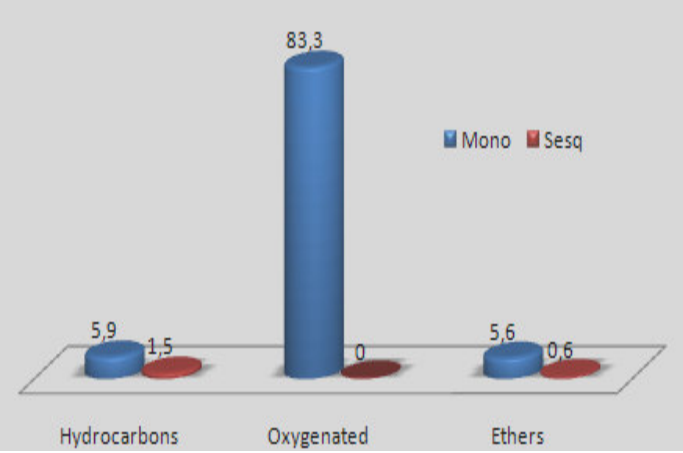

Figure 1. Percentage (%) of identified components of *M. longifolia* essential oil classified by organic family compound. (Mono: monoterpenes; Sesq: sesquiterpenes).

tested bacteria morphology, the bacterial cells were examined in triplicate by AFM (Nanoscope IIIA, Digital Instrument, VEECO). For the experiments, the cells were collected after treatment, washed three times with phosphate-buffered saline (PBS) and centrifuged. The final pellet was resuspended in PBS, placed on a round microscope cover slide and was simply dried in air according to the method previously described (Braga and Ricci, 1998).

RESULTS AND DISCUSSION

Major compounds found in essential oil of *M. longifolia*

The extraction of essential oil was made by hydrodistillation and its analysis was studied by GC and GC-MS. This study allowed identification of 34 composed representing 97.2% of the totality of the components of the oil of *M. longifolia*. The oil is particularly rich in menthol (32.51%), menthone (20.71%), pulegone (17.76%), 1,8-cineole (5.61%), terpineol-4 (4.87%) and piperitone (2.16%). These results are summarized in Table 1. The major part of the identified volatile compounds is oxygenated monoterpenes (83.3%) followed by hydrocarbons and ethers (Figure 1).

Table 2. The MIC values *M. longifolia* of the essential oil against the bacterial strains tested in microdilution assay.

Strains	Essential oil (mg/ml)
S. typhimurium LT2 DT104	1.56
E. coli ATCC 35218	0.78
M. luteus NCIMB 8166	0.19
S. aureus ATCC 25923	0.78

Antibacterial properties of essential oil

The antimicrobial activity of the essential oil included in this work was conducted by broth microdilution susceptibility method. The results summarized in Table 2, showed that the essential oil tested, presented an antibacterial activity with different degree. In fact, the MIC values indicate that the *M. longifolia* essential oil have a broad activity especially for *S. aureus* and *M. luteus*. The noted values are respectively, 0.78 and 0.19 mg/l. The antimicrobial activity can be attributed to the presence of high concentrations of menthol (32.51%), menthone (20.71%) and pulegone (17.76%).

Based on these results, it is possible to conclude that the essential oil has stronger and broader spectrum of antimicrobial activity as compared to the methanol extract. There is evidence in literature that gram+ bacteria are more sensitive to plant oil and extracts than gram- bacteria (Cosentino et al., 1999). However, the results in this study showed that essential oil of *M. longifolia* ssp. *Longifolia* did not have selective antimicrobial activities on the basis of the cell wall differences of bacterial microorganisms. This result may be explained by the high content of cispiperitone epoxide (18.4%), pulegone (15.5%) and piperitenone oxide (14.7%) in the essential oil of *M. longifolia* ssp. *longifolia* analyzed in the present study. Antibacterial and antifungal activities of these substances have been reported in previous studies (Karousou et al., 2007; Gulluce et al., 2007). This is the first study to provide data that essential oil of *M. longifolia* ssp. *longifolia* plants evaluated against a wide range of microorganisms possess potential antibacterial, antifungal and anticandidal activities that are comparable to standard drugs.

Morphological alteration of studied strains

Alterations in cells morphology and topography due to *M. longifolia* essential oil were examined by AFM (Figure 2). The control *S. typhimurium* and *E. coli* cells have a normal rod shape with a smooth surface (Figure 2A and B)

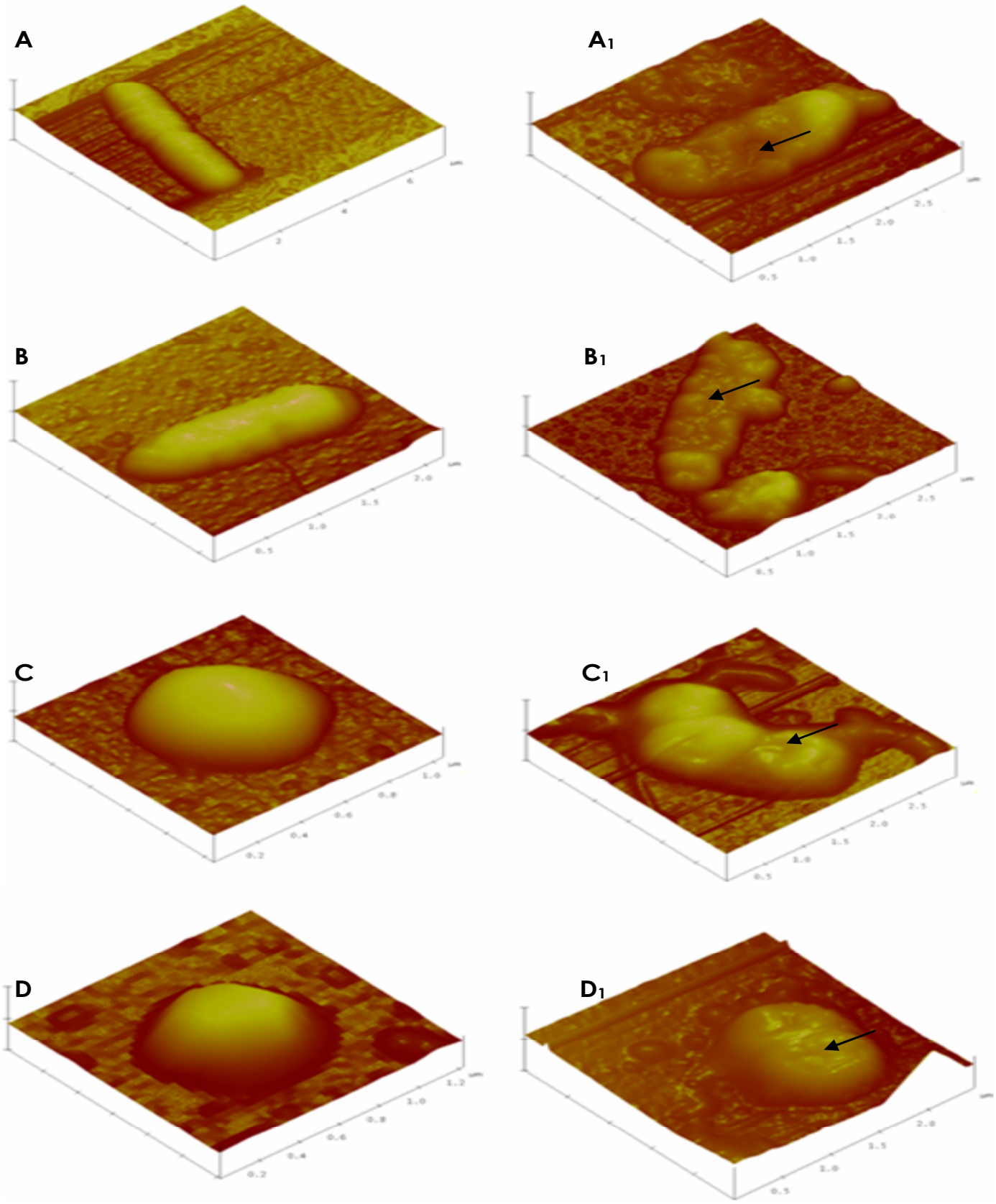

Figure 2. Atomic force microscopy of treated bacteria with *M. longifolia* essential oil. A, B, C and D: Control *S. typhimurium* LT2 DT104, *E. coli* ATCC 35218, *M. luteus* NCIMB 8166 and *S. aureus* ATCC 25923, respectively; A1, B1, C1 and D1 are cells obtained after *M. longifolia* essential oil treatments.

whereas, *M. luteus* and *S. aureus* cells have a normal coccoid shape (Figure 2C and D). After 24 h of bacteria treatment with *M. longifolia* essential oil, we have noted a big damage in *S. typhimurium* and *E. coli* (rod bacteria), whereas damage is less important in coccoid bacteria (*M. luteus* and *S. aureus*). Figure 2 represents damage in tested cells for *S. aureus* and *E. coli*; a hole can be seen on surface and cell wall. It has disappeared to reveal fine cytoplasm structure. It seems that coccoid bacteria are more resistant to *M. longifolia* essential oil than rod bacteria such as *Salmonella* strains. These results are in agreement with the report of Kalchayanand et al. (2004), who suggested the morphological changes of two gram-pathogens (*E. coli* O157:H7 and *S. typhimurium*) when they are exposed to hydrostatic pressure and bacteriocin mixture. Pressurization produced extensive changes in the cell wall. Similar results have been reported by Braga and Ricci (1998) in the case of treated *E. coli* after its exposure to cefodizime. These results indicate possibility that exopolysaccharide on outer membrane of cells might be untangled and released or the peptidoglycan or the cytoplasmic membrane perturbed (Slavik et al., 1995).

Generally, gram+ bacteria are considered more sensitive to essential oil than gram- bacteria (Lis-Balchin, 2003) because of their less complex membrane structure. Sikkema et al. (1994, 1995) showed that, as a result of their lipophilic character, cyclic monoterpenes will preferentially partition from an aqueous phase into membrane structures. This resulted in membrane expansion, increased membrane fluidity and inhibition of a membrane-embedded enzyme. Lambert et al. (2001) reported that the mode of action of essential oils is related to an impairment of a variety of enzyme systems, mainly involved in energy production and structural components synthesis. They also explain the mode of action through leakage of ions, ATP, nucleic acids and amino acids. Examination of *E. coli* cells using electron microscopy after exposure to tea tree oil revealed a loss of cellular electrondense material and coagulation of cytoplasmic constituents, although it was apparent that these effects were secondary events that occurred after cell death (Gustafson et al., 1998). Tea tree oil also stimulates leakage of cellular potassium ions and inhibits respiration in *E. coli* cell suspensions, providing evidence of a lethal action related to cytoplasmic membrane damage (Cox et al., 1998).

Conclusion

The *M. longifolia* essential oil has important compounds (menthol, menthone, pulegone, etc) having interesting antimicrobial activities. Our results show this antimicrobial effect explained by cell wall bacteria damaged at MIC concentrations observed by AFM. This susceptibility is more accentuated in *S. typhimurium* and *E. coli* (rod bacteria), whereas, the damage is less important in coccoid bacteria (*S. aureus* and *M. luteus*). On the other hand, this essential oil can be used in natural preservatives in food against the well known causal agents of food borne diseases and food spoilage.

ACKNOWLEDGMENT

We are grateful to Moez Abroud from Centre de Recherche Technologique de Borj Cédria, (Tunisia) for the technical assistance in AFM technique.

REFERENCES

Abad MJ, Ansuategui M, Bermejo P (2007). Active antifungal substances from natural sources. Archivoc pp. 116-145

Adams RP (2001). Identification of essential oil components by gas chromatography-quadrupole mass spectroscopy. Allured Publishing Co., Illinois, USA.

Bauer K, Grabe D, Surburg H (2001). Common Fragrance and Flavor Materials: Preparation, Properties and Uses. Wiley-VHC. Weinheim p. 293.

Braga PC, Ricci D (1998). Atomic Force Microscopy: Application to Investigation of *Escherichia coli* Morphology before and after Exposure to Cefodizime. Antimicrob. Agents Chemother. 42: 18-22.

Burt S (2004). Essential oils: their antibacterial properties and potential applications in foods- a review. Int. J. Food Microbiol. 94: 223-253.

Colwell RR, Spira WM (1992). The ecology of *Vibrio cholerae*, *In* D. Barua, and WBI. Greenough (ed.), Cholera. Plenum, New York., N p. 107-127.

Cosentino S, Tuberoso CIG, Pisano B, Satta M, Mascia V, Arzedi E (1999). *In-vitro* antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils. Lett. Appl. Microbiol. 29: 130-135.

Cox SD, Gustafson JE, Mann CM, Markham JL, Liew YC, Hartland RP, Bell HC, Warmington JR, Wyllie SG (1998). Tea tree oil causes K+ leakage and inhibits respiration in *Escherichia coli*. Lett. Appl. Microbiol. 26: 355-358.

European Pharmacopoeia (1975). Maisonneuve SA, Saintee-Ruffine 3: 68.

Ghoulami S, Idrissi A, Il Fkih-Tetouani S (2000). Phytochemical study of *Mentha longifolia* of Morocco. Fitoterapia 72: 596-598.

Gill AO, Holley RA (2006). Disruption of *E. coli*, *Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant oil aromatics. Int. J. Food Microbiol. 108: 1-9.

Gulluce M, Sahin F, Sokmen M, Ozer H, Daferera D, Sokmen A, Polissiou M, Adiguzel A, Ozkan H (2007). Antimicrobial and antioxidant properties of the essential oils and methanol extract from *Mentha longifolia* L. ssp. *longifolia*. Food Chem. 103: 1449-1456.

Gustafson JE, Liew YC, Chew S, Markham JL, Bell HC, Wyllie SG, Warmington JR (1998). Effects of tea tree oil on *Escherichia coli*. Lett. Appl. Microbiol. 26: 194-198.

Hafedh H, Mejdi S, Hichem BJ, Zine M, Amina B (2008). Comparison of chemical composition and antimicrobial activities of *Mentha longifolia* L. ssp. *longifolia* essential oil from two Tunisian localities (Gabes and Sidi Bouzid). Ann. Microbiol. 58(3): 103-110.

Hafedh H, Najla T, Emira N, Mejdi S, Hanen F, Riadh K, Amina B (2009). Biological activities of the essential oils and methanol extract of two cultivated mint species (*Mentha longifolia and Mentha pulegium*) used in the Tunisian folkloric medicine. World J. Biotechnol. Microbiol. 25: 2227–2238.

Hausner M, Wuertz S (1999). High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. Appl. Environ. Microbiol. 65: 3710-3713.

Helander IM, Alakomi HL, Kyosti LK, Mattila-Sandholm T, Pol I, Smid EJ, Gorris GM, Von WA (1998). Characterization of the action of selected essential oil components on Gram-negative bacteria. J. Agric. Food Chem. 46: 3590-3595.

Juven BJ, Kanner J, Schved F (1994). Factors that interact with the antibacterial action of thyme essential oil and its active constituents. J. Appl. Bacteriol. 76: 626-631.

Karousou R, Balta M, Hanlidou E (2007). “Mints”, smells and traditional uses in Thessaloniki (Greece) and other Mediterranean countries. J. Ethnopharmacol. 109: 248-257.

Knobloch K, Pauli A, Iberl B (1988). Antibacterial activity and antifungal properties of essential oil components. J. Essent. Oils Res. 1: 119-128.

Lambert RJW, Skandamis PN, Coote P (2001.) A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J. Appl. Microbiol. 91: 453-462.

Lis-Balchin M (2003). Feed additives as alternatives to antibiotic growth promoters: botanicals. Proceedings of the 9th International Symposium on Digestive Physiology in Pigs, Banff AB, Canada. University of Alberta, publisher. 1: 333-352.

Mimica-Dukic N, Bozin B, Sokovic M, Mihajlovic B, Matavulj M (2003). Antimicrobial and antioxidant activities of three *Mentha* species essential oils. Planta. Med. 69: 413-419.

Morris JA, Khettry A, Seitz EW (1979). Antimicrobial activity of aroma chemicals and essential oils. J. Am. Chem. Soc. 56: 595-603

Poolman B, Driessen AJM, Konings WN (1987). Regulation of solute transport in Streptococci by external and internal pH values. Microbiol. Rev. 51: 498-508.

Rasooli I, Rezaei MB (2002). Bioactivity and chemical properties of essential oils from *Zataria multiflora* Boiss and *Mentha longifolia* (L.) Huds. J. Essent. Oil Res. 14: 141-146.

Sikkema J, De Bont JAM, Poolman B (1994). Interactions of cyclic hydrocarbons with biological membranes. J. Biol. Chem. 269: 8022-8028.

Sikkema J, De Bont JAM, Poolman B (1995). Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Rev. 59: 201-222.

Slavik MF, Kim WJ, Walker JT (1995). Reduction of *Salmonella* and *Campylobacter* on chicken carcasses by changing scalding temperature. J. Food Prot. 58: 689-691.

Trumpower BL, Gennis RB (1994). Energy transduction by cytochrome complexes in mitochondrial and bacterial respiration: the enzymology of coupling electron transfer reactions to transmembrane proton translocation. Ann. Rev. Biochem. 63: 675-716.

Ultee A, Bennink MHJ, Moezelaar R (2002). The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus. PhD thesis, ISBN 90-5808-219-9. Appl. Environ. Microbiol. 68(4): 1561-1568.

Ultee A, Kets EPW, Alberda M (2000). Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. Arch. Microbiol. 174(4): 233-238.

Ultee A, Smid EJ (2001). Influence of carvacrol on growth and toxin production by *Bacillus cereus*. Int. J. Food Microbiol. 64: 373-378.

Uribe S, Ramirez T, Pena A (1985). Effects of β-pinene on yeast membrane functions. J. Bacteriol. 161: 1195-1200.