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Full Length Research Paper

Chemical composition and antibacterial activity of essential oils from six Moroccan plants

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The essential oils (EOs) of six plants (*Artemisa herba alba*, *Rosmarinus officinalis*, *Ocimum basilicum*, *Lavandula officinalis*, *Mentha viridis* and *Mentha piperita*) widely distributed in Morocco were isolated and their chemical composition was investigated by gas chromatography-mass spectrometry (GC/MS). These EOs were tested *in vitro* against four pathogenic bacterial strains (*Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Streptococcus D*) and we determined the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of each EO. The oils of various plants showed high activity against all tested bacteria (MIC≤10 µL/ml), of which *K. pneumoniae* was the most sensitive strain (MIC≤5 µL/ml). In addition, the oil from *M. viridis* L. which contained high pulegone concentration (45%) exhibited a very interesting antibacterial activity against all the bacterial strains (MIC 2.5 µL/ml) and (MBC 2.5 µL/ml). The UV-visible study on the release of material absorbing at 260 nm showed significant leakage of cytoplasmic contents, indicating damage to the bacterial cell membrane integrity. Thus, these results indicate that the EOs represent a potential source of natural antibacterial substances that may be used against pathogenic systems.

Key words: *Artemisia herba alba*, *Rosmarinus officinalis*, *Ocimum basilicum*, *Lavandula officinalis*, *Mentha viridis*, *Mentha piperita*, essential oil composition, pulegone, antibacterial activity.

INTRODUCTION

Infectious diseases are the world's leading cause of premature death, killing almost 50 000 people every day (Ahmad and Beg, 2001). In the recent years, development of microbial resistance to antibiotics is of a global concern, imposing the need for a permanent search and development of new drugs (WHO, 2003; Levy, 1984; Silver and Bostian, 1993). Many efforts have been made to discover new antimicrobial compounds from various kinds of sources such as microorganisms and plants. Therefore, pharmaceutical companies have been motivated to develop new antimicrobial drugs in recent years, especially due to the constant emergence of resistant micro-organisms to conventional anti-microbials all over the world (Piddock and Wise, 1989; Mulligen et al., 1993). The recently emerged resistant *Escherichia coli* in Europe is one of the frightening examples (Turner, 2011). The drug-resistant bacteria and fungal pathogens have further complicated the treatment of infectious diseases in immunocompromised, AIDS and cancer patients (Rinaldi, 1991; Diamond, 1991).

Contrary to the synthetic drugs, antimicrobials of plant origin are not associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases (Iwu et al., 1999). Aromatic and medicinal plants had acquired particular attention in the field of

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Table 1. Region and period of each plant collection studied.

Plant species	Region of collection	Period of collection
Artemisia herba alba	Errachidia : Southeast Morocco	May 2009
Ocimum basilicum	Agadir : Southwest Morocco	May 2009
Mentha viridis	Marrakech : Southwest Morocco	May 2009
Rosmarinus officinalis	Rich : High Moroccan Atlas	June 2009
Lavandula officinalis	Azrou : Middle Moroccan Atlas	June 2009
Mentha piperita	Rabat : West Morocco	June 2009

intensive research on the natural antimicrobial compounds. They constitute a constant source of active reagents against pathogen germs (Mahady, 2005). Among these products, essential oils (EOs) produced by aromatic plants as secondary metabolites, have gained a net interest by many investigators (Ismaiel and Pierson, 1990; Bauer and Garbe, 2001; Oumzil et al., 2002; Zenasni et al., 2008). EOs are volatile natural complex compounds characterized by strong odour (Bakkali et al., 2008), and represent very complex natural mixtures which may contain more than sixty individual components at quite different concentrations (Senatore, 1996). Major components can constitute up to 85% of the EOs, whereas other components are present only as trace (Bauer et al., 2001). It has been recognized that some EOs have antimicrobial, antifungal, anticancer and antioxidants properties (Sara, 2004; Hong et al., 2004; Bozin et al., 2006; Alzoreky and Nakhra, 2003; Baser et al., 2002; Sylvestre et al., 2006).

Morocco has an enormous unexplored potential of medicinal plants that are used in traditional medicine, some of which are misused because of lack of scientific data. The heterogeneous ecologic conditions have favoured the proliferation of more than 42, 000 plant species (Tahraoui et al., 2007; Hmamouchi, 1999). Our previous studies have shown that essential oil from some plants such as *Mentha suaveolens* and *Nepeta* spp. produced remarkable antibacterial property against several pathogenic bacteria (Oumzil et al., 2002; Zenasni et al., 2008). In this context, we were interested in analyzing in the present work the chemical composition of EOs obtained from six endemic plants of Morocco (namely: *Artemisia herba alba*, *Ocimum basilicum*, *Mentha viridis, Rosmarinus officinalis*, *Lavandula officinalis* and *Mentha piperita)* and testing their antibacterial activity against four pathogenic bacterial strains including *Enterococcus faecalis*, *E. coli*, *Klebsiella pneumoniae* and *Streptococcus D* by disc diffusion method and dilution assay. We also attempted to elucidate the mechanism of action by testing the EOs' capacity to disrupt the bacterial membrane structure in order to develop new type of disease control alternatives. The selection of medicinal plants is based on their traditional uses in Morocco (Hmamouchi, 1999; El-Hilaly et al., 2003; Bellakhdar et al., 1991).

MATERIALS AND METHODS

Whole plants were collected from different Moroccan regions where they are usually collected by herbalists for traditional use. Table 1 indicates the region and the period of each plant collection.

Extraction of essential oils

Fresh aerial parts of whole plants were desiccated at ambient temperature and 100 g of plant material were then subjected to steam distillation for 3 h. The extract recovered was subjected to successive ethyl acetate extractions (3 × 100 ml). After extraction, Na_2SO_4 (1 g Na_2SO_4 for 5 ml of EO) was added to the sample mixture to remove water. The sample mixture was then filtered on a filter membrane. The obtained EO was stored in sterile dark glass bottles in a freezer until use.

Analytical techniques

Gas chromatography-mass spectrometry (GC/MS) analysis of the EO was performed on a TRACE GC ULTRA equipped with non-polar VB5 (5% phenyl, 95% methylpolysiloxane) capillary column (30 m × 0.25 mm × 0.25 µM film thickness), directly coupled to a mass spectrometer (Polaris Q). The electron ionization energy was set at 70 eV. The temperature of injector and detector was set at 220 and 300°C, respectively. The oven temperature was programmed from 40 to 180°C at 4°C/min, then for 180 to 300°C at 20°C/min. The components of the oil were identified by comparison of their mass spectra with those in the Willey NIST 7^{th} Edition Library of mass spectral data. The composition of the oil sample was calculated from GC-MS peak areas and given by percentages. The Kovats retention indices (KI) were calculated by using n-alkanes C5 – C30 and the experimental values were compared with those reported in the literature (Adams, 2007).

Preparation of bacterial strains

The tested microorganisms included the following bacteria: *E. faecalis*, *E. coli*, *K. pneumoniae* and *Streptococcus D*. All pathogenic microorganisms isolated from patients were stored at the culture collection of the Microbiology Department (Microthec Unity) at the Institut National d'Hygiène (Rabat, Morocco). They were maintained in brain heart infusion (BHI) at -80°C. Prior to the experiment, cultures were prepared by subculturing 1 ml of each culture stock in 9 ml of BHI broth in order to obtain culture inoculates in an exponential growth phase of approximately 10^6 CFU/ml.

Disc diffusion method

The agar disc diffusion (ADD) method was employed for the

determination of antimicrobial activities of the tested EO as described previously (Oumzil et al., 2002). Briefly, the test was performed in sterile Petri plates containing BHI agar. Sterile filter paper discs (6 mm in diameter) were impregnated with 6 µL of oil and were placed on the Petri plates previously inoculated with a sterile microbial suspension (one microorganism per Petri dish). All Petri plates were sealed with sterile laboratory films to avoid eventual evaporation of the test samples, and then incubated at 37°C for 24 h. The diameters of inhibition zones were measured in millimetres.

Determination of MIC and MBC

We tested six serial concentrations of each EO (40, 20, 10, 5, 2.5, 1.25 and 0.625 µL/ml) diluted in BHI broth with 0.15% agar and strongly mixed for 2 min using a vortex. The MIC was assessed according to the procedure established by Canillac and Mourey (1995) and Oumzil et al. (2002). Briefly, 5 ml of culture medium was inoculated with 0.1 ml of a bacterium precultured in BHI at 37°C. The final concentration of bacteria was of 10^6 CFU/ml. The MIC is the lowest concentration of BHI (0.15% agar) for which no growth was detected after 24 h at 37°C (Canillac and Mourey, 1995). While for the determination of MBC, 0.1 ml of the cell suspensions from the tubes showing no growth were subcultured on nutrient agar plates and the Petri plates were incubated for 24 h at 37°C. The MBC was the highest dilution (lowest concentration) of the EO at which no growth occurred on the plates (Smith-Palmer et al., 1998).

Bactericidal activity

E. coli were grown overnight at 37°C in 100 ml BHI broth. A series of increasing concentrations of each EO were prepared in the culture broth medium and 500 µL of viable bacteria were inoculated into each tube, shaken and incubated at 37°C for 24 h. The density of the each culture (designed as bacterial growth) was measured at a wavelength of 600 nm after each time point. To detect genetic material release, 1 ml sample of each tube were centrifuged at 1200 g for 5 min to remove all trace of bacteria. The supernatant was re-suspended in PBS and used to measure UV 260 nm absorption by Camspec UV/visible spectrophotometer at each time point. We used untreated bacteria as negative control and bacteria treated with penicillin-streptomycin (100 U/ml - 100 µg/ml) as positive control.

RESULTS

Chemical composition of the essential oils

The results obtained by GC-MS analysis of the EOs are presented in Table 2. *A. herba alba* contained eucalyptol (27.29%), camphor (23.42%) and chrysanthenone (21.76%) as the major compounds. The oils from *R. officinalis* are also characterized by a high percentage of eucalyptol (56.85%). Moreover, the main volatile components of the *O. basilicum* EO are linalol (53.98%) and methyl trans-cinnamate (15.33%). For *L. officinalis* EO, we found that linalyl acetate (44. 96%) and linalol (44.64%) were the main components. The chemical composition of EO of the two *Mentha* species (*M. viridis* and *M. piperita*) is qualitatively similar, although there are some differences in the concentrations of individual components; linalol (52%) and linalyl acetate (25.9%) are the predominant compounds in the oil of *M. piperita*, but the essential oil of *M. viridis* contained the largest amount of pulegone (45%).

Antibacterial activity of the essential oils

The results of the disk diffusion test indicate that each EO showed different degree of growth inhibition (Figure 1). The maximum inhibition was recorded against *E. coli* with the EO of *O. basilicum* (20 mm) Figure 1A. *Streptococcus D* and *Enterococcus faecalis* were susceptible to *L. officinalis* EO with inhibition zone 12 mm and 17 mm respectively (Figure 1B and C). *R. officinalis* EO and *L. officinalis* exhibited significant activity against *K. pneumoniae* with similar inhibition zone of about 16 mm (Figure 1D). In addition, the antibacterial activity indicated that oils from *A. herba alba*, *R. officinalis*, *O. basilicum*, *L. officinalis* and *M. piperita* presented comparable activity against all strains of tested bacteria (MIC≤10 µL/ml) (Table 3).

Furthermore, the EO of *M. viridis* was found to be more active at lower dilution against the chosen pathogenic bacterial strains (MIC = 2.5 µL/ml and MBC = 2.5 µL/ml; Table 3). In addition, *K. pneumoniae* was more sensitive to the oils studied (MIC≤5 µL/ml). In the case of *O. basilicum*, *E. coli* was the most sensitive (diameter of inhibition = 20 mm) (Figure 1A) and MIC was of about 5 µL/ml of oil dilution (Table 2).

Bactericidal activity and cell lysis

The mechanism of action of EOs from our selected plants was studied by examining their bactericidal and cell lytic activity against *E. coli*, and comparing it with penicillin-streptomycin cocktails. The antibacterial activity of the EOs was determined using EO concentration corresponding to 2xCMI. We first tested antibacterial effect of all EOs against *E. coli*. EO treated bacteria showed growth arrest after 24 h. *E. coli* showed high sensitivity to all EO, with 75% of growth inhibition in the presence of EO from *M. viridis* and *M. piperita* as compared to untreated bacteria (T^-) (Figure 2A). Bacteriostatic agents limit the growth of bacteria by interfering with protein production, DNA replication or other aspects of bacterial cellular metabolism. This is in contrast to bactericides which kill bacteria (Pankey and Sabath, 2004).

Furthermore, leakage of cytoplasmic contents is an indicator of damage to the bacterial cytoplasmic membrane. Therefore, bacterial cell membrane integrity was examined by quantification of the released of material absorbing at 260 nm (DNA and RNA) after adding EOs at the indicated concentrations by spectrophotometry in the supernatant fluid. In the same time, the viable bacteria

Table 2. Chemical composition (%) of six essential oils from *Artemisia herba alba* (AHA), *Rosmarinus officinalis* (RO), *Ocimum basilicum* (OB), *Lavandula officinalis* (LO), *Mentha viridis* (MV), *Mentha piperita* (MP).

Compounds	Percentage (%) of compounds							
	AHA	RO	OB	LO	MV	MP	KI*	KI**
α-pinene	3.40	15.34	-	-	-	-	919	939
Camphene	3.87	4.85	-	-	-	-	950	953
β-pinene	-	6.52	-	-	-	-	985	980
Myrcene	-	-	-	-	1.5	2.9	1000	991
Eucalyptol	27.29	56.85	9.33	2.08	2.5	1.5	1027	1033
Ocimene	-	-	1.70	-	-	0.2	1038	1040
Linalyl acetate	-	-	-	44.96	0.5	25.9	1072	1061
Linalool	-	-	54	44.64	-	52	1089	1098
Thujone	3.83	-	-	-	-	-	1099	1102
Alcohol fenchylique	1.76	-	-	-	-	-	1108	1112
Chrysanthenone	21.76	-	-	-	-	-	1133	1123
Camphor	23.42	12.99	1.99	2.66	-	-	1141	1143
Menthone	-	-	-	-	33	0.5	1160	1154
Borneol	4.02	-	2.85	2.51	-	-	1164	1165
Limonene	-	-	-	-	5.5	0.2	1200	1189
α-Terpineol	-	-	-	-	-	4.5	1229	1237
Pulegone	-	-	-	-	45	1.2	1235	1237
Carvone	-	-	-	-	0.5	0.4	1249	1242
Geraniol	-	-	-	-	4.5	1.2	1255	1255
Chrysanthenyl acetate	3.14	-	-	-	-	-	1277	1262
Methyl trans-cinnamate	-	-	15.3	-	-	-	1295	1301
Eugenol	2.30	-	-	-	-	-	1358	1356
Eugenol methyl ether	-	-	1.89	-	-	-	1399	1401
Trans- caryophyllene	2.66	3.45	-	-	-	-	1404	1404
Cis-Caryophyllene	-	-	3.43	-	7	0.9	1420	1418
Humulene	-	-	2.63	-	-	-	1438	1440
Caryophyllene oxide	-	-	-	3.15	-	-	1571	1581
Farnesol	-	-	4.56	-	-	-	1695	1697
Others	2.55	-	2.32	-	-	7.6	-	-

I: Kovats retention indices; KI* experimental values and KI**: literature values.

decrease significantly after 24 h when compared to control. The release of material absorbing at 260 nm after 24 h in the treated cultures with EOs showed significant leakage compared to untreated bacteria (T^-) (Figure 2B). When compared to *E. coli* treated with penicillin-streptomycin at the indicated concentration, EOs showed slightly lower capacity to damage bacterial membrane except for *M. piperita* which showed higher activity than the plant species (Figure 2B). This phenomenon was observed as soon as 1 h of incubation (data not shown), thus indicating membrane damage related to the addition of the EOs. For the first time, it was determined that Eos exhibit their activity by damaging the cell membrane and inducing leakage of the tested bacteria. The same results were confirmed at 48 h and five days of culture (data not shown). These results largely confirmed the strong antibacterial agents of the EOs studied.

DISCUSSION

The results obtained by GC-MS analysis shows that each plant species has a specific quantitative and qualitative composition. The reasons of this variability can be due to different geographical sources, the genotype and the climate; all of this variability influences the chemical composition and the relative concentration of each constituent (Masotti et al., 2003; Angioni et al., 2006; Cosentino et al., 1999). For example, in our study, the oils from *R. officinalis* are characterized by a high percentage of Eucalyptol, although EO of this plant growing in Algeria belongs to 1,8-cineole chemotype (Boutekedjiret et al., 1998). In a previous study, it was shown that EO from *Mentha suaveolens* subspecies present variable chemical composition that is different from those of *Mentha* species studied here (Oumzil et al.,

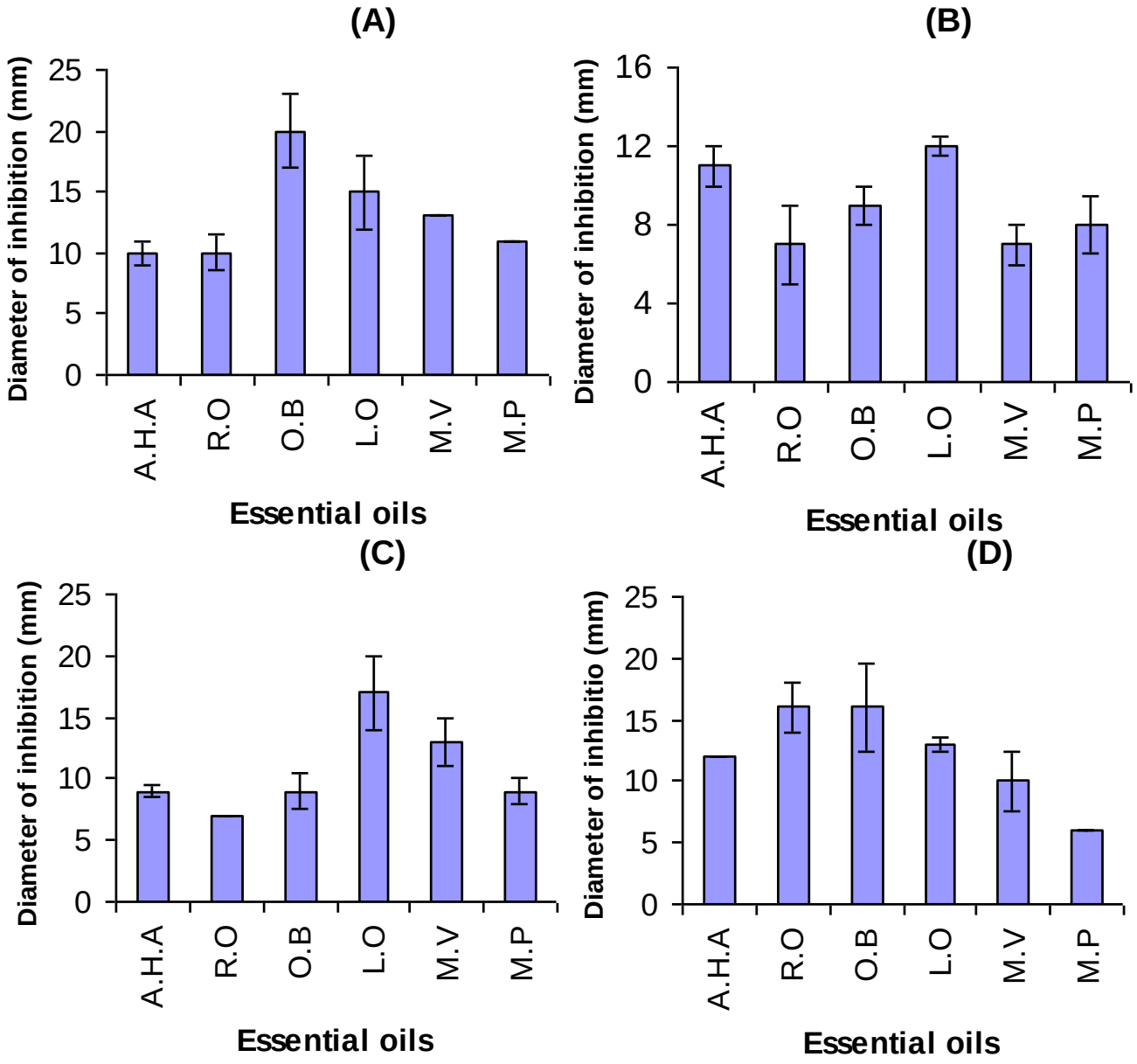

Figure 1. Antibacterial activity* of EO from *Artemisia herba alba* (A.H.A), *Rosmarinus officinalis* (R.O), *Ocimum basilicum* (O.B), *Lavandula officinalis* (L.O), *Mentha viridis* (M.V) *and Mentha piperita* (M.P) against *E. coli* (A), *Streptococcus D* (B), *E. faecalis* (C) and *K. pneumoniae* (C). *: Mean zone of inhibition (Ø mm) and standard deviation.

Table 3. Minimal inhibitory concentrations (MIC) (µL/ml) and minimal bactericidal concentration (MBC) (µL/ml) of selected essential oils from *Artemisia herba alba*, *Rosmarinus officinalis*, *Ocimum basilicum*, *Lavandula officinalis*, *Mentha viridis* and *Mentha piperita* against four pathogenic bacteria.

Plant species	Test organism (MIC/MBC)			
	E. coli	***Streptococcus D***	***E. faecalis***	***K. pneumoniae***
Artemisia herba alba	10/10	10/10	10/10	2.5/2.5
Rosmarinus officinalis	10/10	10/10	10/10	5/5
Ocimum basilicum	5/5	10/10	5/5	5/5
Lavandula officinalis	10/10	10/10	10/10	5/5
Mentha viridis	2.5/2.5	2.5/2.5	2.5/2.5	2.5/2.5
Mentha piperita	10/10	2.5/5	2.5/2.5	2.5/2.5

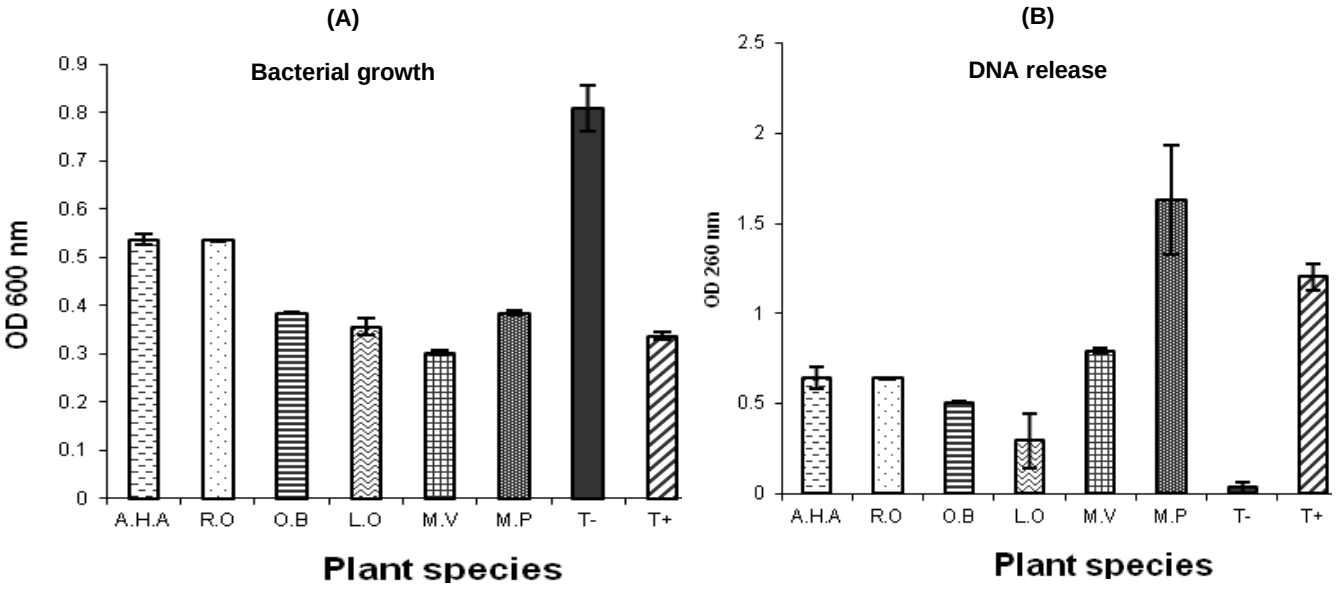

Figure 2. Bacterial growth (measured as absorbance at 600 nm) registered after 24 h of exposure to each essential oils on *E. coli* (A) and leakage of material cytoplasmic (measured as absorbance at 260 nm) (B). T-: Untreated bacteria, T+: positive control (penicillin-streptomycin).

2002). The EO of *M. viridis* was found to be more active at lower concentration against all the pathogenic bacterial strains we used. These results could be due to differences in chemical composition of the oils as we have reported previously (Oumzil et al., 2002; Zenasni et al., 2008).

Indeed, it has been reported that pulegone play an important role in antibacterial activity (Andersen and Jensen, 1984; Oumzil et al., 2002). Our findings may suggest the potential use of *M. viridis* oils in treatment of infections caused by those pathogenic germs. Moreover, we found that *K. pneumoniae* was the most sensitive germ to EO from *M. viridis*. In the case of *O. basilicum*, *E. coli* was the most sensitive; this oil contains eucalyptol which was reported to impart microbial effects on *K. pneumoniae* (Fabio et al., 2007). Thus, one may take into consideration that the inherent activity of an oil can be expected from the chemical configuration of the components, the proportions in which they are present and to interactions between them (Dorman and Deans, 2000). Considering the large number of different group of chemical compounds present in EOs, it is most likely that their antibacterial activity is not attributable to one specific mechanism of action, but there are several targets in the cells (Skandamis and Nychas, 2001; Carson et al., 2002) and the mechanisms of action have not been yet studied (Lambert et al., 2001; Oumzil et al., 2002).

Our study showed that many essential oils possess important antibacterial activity against the four pathogenic bacteria species studied. Among the EO tested, *M. viridis* was the most active on all the bacteria tested with MIC≤2.5 µL/ml. In addition, *O. basilicum* was the most active on *E. coli*. These findings suggest an interesting antibacterial potential of *M. viridis* and *O. basilicum* EO and the possible development of new drugs based on the EO components, in treating infections caused by pathogen germs with high morbidity and mortality worldwide. The measurement of growth inhibition of bacterial by disc diffusion method and dilution assay is not sufficient. Additional studies are required on the mode of action in pathogenic bacteria as effects on bacterial cell membranes.

The disruption of the bacterial membrane structure has not yet been well characterized in term of the mode of action. Most antimicrobial agents may be categorized according to their principle mode of action. It is postulated that polymixin, hexachlorophene and chlorhehexidine exert their inhibitory effects by increasing bacterial membrane permeability, causing leakage of bacterial cell and they get partitioned into the lipid bilayer of the cell membrane, causing frequent fundamental changes in bacteria membrane and function, thus rendering it more permeable and provoke whole cell lysis (Hugo and Longworth, 2011; Joswick et al., 1971; Pankey and Sabath, 2004). Release of intracellular components is a good indicator of membrane integrity; small ions as potassium and phosphate tend to each out first, followed by large molecules such as DNA, RNA and other materials (Hugo and Longworth, 2011; Joswick et al., 1971). On the other hand, the mechanisms by which EOs can inhibit microorganisms involve different modes of action. The cytoplasmic cell membrane undoubtedly is the target of many antimicrobials agents. Although, the antibacterial properties of essential oils has been

reviewed in the past. The mechanism of action has not been studied in great detail (Lambert et al., 2001). In line with this, EOs by penetrating through the cell wall and cytoplasmic membrane disrupt and permeabilize them and provokes leakage of cytoplasmic constituents: metabolites and ions (Cowan, 1999; Thoroski et al., 1989). This activity has been also demonstrated for essential oils from oregano and thyme (Horne et al., 2001).

In addition chemical compounds from essential oils also act on cytoplasmic cell membrane (Knobloch et al., 1989). Carvacrol and thymol damaged cell membrane and increase its permeability (Lambert et al., 2001). When tested at concentration higher than their minimum inhibitory concentration, carvone and eugenol disintegrate the outer membrane (Thoroski et al., 1989; Oosterhaven et al., 1995). Leakage of cytoplasmic contents is an indicator of damage to the bacterial cytoplasmic membrane. The UV-visible study on the release of material absorbing at 260 nm showed significant leakage. This indicates membrane damage related to the addition of the essential oils studied here. To the best knowledge, no previous study was undertaken to provide comparative data on the mode action of EOs against pathogenic bacteria. For the first time, it was determined that *M. piperita* and *M. viridis* essential oils exhibit their activity by highly damaging the cell membrane of the tested bacteria in our experimental condition when compared to using antibiotics such as penicillin and streptomycin. Nevertheless, other studies should be aimed at determining the exact mechanism of action of each EO by comparison to the most potent antibiotics used in therapeutics and effects *in vivo*.

Many bacteria can cause fatal diseases; in fact despite the existence of potent antibiotic agents, resistant or multi-resistant strains are continuously emerging. In an effort to discover new lead compounds, many research groups screen natural components to detect secondary metabolites with relevant antibacterial activities. In conclusion, the essential oils produced in Morocco offer a promising way for research of the phytochemical active principle in therapeutic indications (Hmamouchi, 1999). However, studies should be conducted to determine the mechanism of action of each EO and compare with the most potent antibiotics used in therapeutics.

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