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Research Article

In vitro Antibacterial Activity of Essentials Oils from *Mentha pulegium*, *Citrus aurantium* and *Cymbopogon citratus* on Virulent Strains of *Aggregatibacter actinomycetemcomitans*

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ABSTRACT

Giving the incidence of periodontitis and the increased resistance of oral bacteria to antibiotics, there is a need of alternative products, such as natural agents. The main objective of the present study was to investigate the *in vitro* antibacterial activity of selected Moroccan essential oils of *Mentha pulegium (*Pennyroyal*)*, *Cymbopogon citratus* (Lemongrass) and *Citrus aurantium* (Sour orange) against two virulent serotype b strains of *Aggregatibacter actinomycetemcomitans (Aa)*; JP2 and non-JP2 clones. This periopathogen, including the highly virulent JP2 clone, is known strongly associated with aggressive periodontitis. The essential oils were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). Their antimicrobial activities were determined by agar well diffusion and broth microdilution methods against a clinical Moroccan isolate of *Aa* sampled from periodontitis patients (JP2 clone) and a reference non-JP2 strain of *Aa*. All the tested essential oils induced significant inhibition zones for both strains and showed bactericidal effect. Statistically, no significant difference was found between two strains of *Aa*. The MICs and MBCs were ranged from 0,07 % to 0,3 % (v/v). *Cymbopogon citratus* was the most effective (MIC and MBC of 0,07%) against both two strains. Thus, the selected Moroccan essential oils exhibited a strong antibacterial activity on the highly pathogenic JP2 clone of *Aggregatibacter actinomycetemcomitans* as well as on non-JP2 strain suggesting the usefulness of these products as potential antimicrobial agents in periodontal diseases associated to this microorganism.

Keywords: *oral bacteria, Aggregatibacter actinomycetemcomitans, essential oils, antimicrobial activity, Minimum inhibitory Concentration.*

INTRODUCTION

Aggregatibacter actinomycetemcomitans, a gram-negative facultative anaerobic bacterium and a well-known periodontal pathogen, has been frequently associated with aggressive periodontitis, a disease that leads to a severe and rapid periodontal breakdown and tooth loss in relatively young subjects. Many recent studies have suggested that this pathogen may be involved in several complications of systemic diseases. Previous worldwide studies showed that various serotypes (a, b, c, d, e, f) of *A.actinomycetemcomitans* are associated with different forms of periodontitis, extra-oral infections, or periodontium health [1,2]. Actually, serotype b is frequently associated with aggressive periodontitis [1]. In Morocco, *A.actinomycetemcomitans* serotype b and particularly clone JP2, a highly virulent strain, characterized by a deletion of 530-bp in the promoter region of leukotoxin gene operon resulting in enhance production of the toxin [3], has been strongly associated with aggressive periodontitis [4-6]. Also, subjects harboring the serotype b JP2 clone have been shown to be at higher risk of developing aggressive periodontitis than those colonized by 'non-JP2 clones' strains [1]. Consequently, the eradication of this bacterium is essential for periodontium health and also subject well-being. This elimination is based upon mechanical treatment of periodontal pockets associated to antibiotics. However, there is sufficient evidence that antibiotic resistance has increased in the periodontal flora over the last decades [7], and searching for a new adjunctive treatment as an alternative or a complementary antimicrobial agent is essential. The use of natural products like essential oils, as antibacterial agents, is increasing in oral hygiene and dentistry. In Morocco, a large producer country of medicinal plants, many trials have been carried out on the safety and antimicrobial activity of Moroccan plant extracts and essential oils [8-13]. However, few data on the effects of Moroccan essential oils or their components on

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Table 1: Moroccan essential oils tested in this study and their medicinal properties in traditional use [14]

Common name	Species	Family plant	Medicinal properties
Pennyroyal	Mentha pulegium L.	Lamiaceae	expectorant, antispasmodic, stomachic, refreshing
Lemongrass	Cymbopogon citratus	Poaceae	Insectifuge, antiseptic
Sour orange	*Citrus aurantium L.*	Rutaceae	Anti infective, inotrope

oral bacteria, particularly on periodontal pathogens have been published. Thus, based on previous studies on antimicrobial effect of essential oils on various extra oral bacteria and fungus, we evaluate, *in vitro*, the antibacterial activity of 3 Moroccan essential oils named Mentha pulegium L., Cymbopogon citratus,and Citrus aurantium L. on virulent strains of *A.actinomycetemcomitans* including the highly leukotoxic JP2 clone and non-JP2.

MATERIALS AND METHODS

Bacterial strains and growth conditions: 2 virulent strains of *Aggregatibacter actinomycetemcomitans* were used for this study. The first one was a Moroccan clinical isolate (serotype b, JP2 clone), obtained from Kyushu Dental College, Japan. This strain was originally isolated from sampling of subgingival plaque in patients with aggressive periodontitis, at the Department of Periodontology, at the Center of Counseling and dental treatment in Rabat, Morocco. The second strain of *Aggregatibacter actinomycetemcomitans* was a serotype b non-JP2 clone, referenced as: CIP 101032, obtained from Pasteur Institute, Paris. The bacteria were maintained by cultivation on chocolate Agar with Vitox (Oxoid Deutschland Gmbh, Postfach, Wesel) or Brain Heart Infusion (BHI) agar, under an atmosphere of 5% CO_2 at 37°C for 48h.

Essential oils*:* Three essential oils derived respectively from *Mentha pulegium L. (*Pennyroyal*), Cymbopogon citratus* (Lemongrass) and *Citrus aurantium* L. (Sour orange), were tested in this assay. These oils were kindly provided by the National Institute of Medicinal and Aromatic Plants, Taounate, Morocco. They were extracted from plants, which were grown in the experimental garden of the institute. The Botanical identification was done by F A. and the authenticated voucher specimens were deposited in the Herbarium of photochemistry laboratory of the National Institute of Medicinal and Aromatic Plants - University of Sidi Mohamed Ben Abdellah, Fez, Morocco. Codes were assigned to different samples: *Mentha pulegium* L. (code: FA/RP/INPMA/104), *Cymbopogon citratus* (code: FA/RP/INPMA/105), *Citrus aurantium L.* (code: FA/RP/INPMA/106. The choice of essential oils was based on their documented antimicrobial effects and/or on anecdotal use in the population (Table 1) [14].

Essential oils extraction: A portion (100 g) of the aerial parts of the plants was hydrodistilled, for at least three hours, using a Clevenger-type apparatus. To eliminate any traces of water, the extracted oil was treated with anhydrous sodium sulfate (Na_2SO_4), filtered and then stored in the dark at 4°C. The yield calculation was done using the following formula: $Yield = \frac{Volume}{Mass} x\ 100$.

It was expressed in ml /100g of dry matter.

Chromatographic analysis and characterization of oils: Essential oils were analyzed by gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC/MS). Gas chromatography analyses were performed on a Hewlett-Packard (HP 6890) gas chromatograph (FID), equipped with a HP-5 capillary column (5 % phenyl methyl silicone). The characteristic of this column was: 30 m of length, 0.25 mm of diameter and 0.25 µm of film thickness. The temperature was programmed from 50°C (initial waiting 5min) to 200°C at 4°C/min. Gas chromatography conditions were as follows: N_2 as carrier gas (1.8 ml/min); split mode (Flow: 72.1 ml/min, ratio: 1/50) was used; temperatures of injector and detector were 275°C and 250°C respectively. Diluted samples (1/20 in Hexane) of 1 µl were injected manually. The machine was run by a computer system type ″HP ChemStation″. Gas chromatography coupled with mass spectrometry (GC/MS): The chemical composition of essential oils were analyzed using a gas chromatograph (TRACE GC Ultra) fitted to a mass spectrometer (Polaris Q-Ion Trap MS). Operating in electron-impact EI (70 eV) mode. VB-5 (Methylpolysiloxane 5% phenyl) and a column (30m × 0.25mm × 0.25µm thickness) were used (National Centre of Scientific and Technical Research – (NCSTR), Rabat, Morocco). The chromatographic conditions were as follows: Injector and detector temperatures at 220 and 300°C respectively; carrier gas, helium at flow rate of 1.4 ml/min; temperature program ramp from 40 to 300°C with gradient of 4°C/min (holding the initial and final temperature for 4min). The relative amount of individual components of the total oil was expressed as a percentage peak area relative to total peak area. Library search was carried out using the combination of NIST MS Search and literature. Oils constituents were also identified by their retention indices relatives to n-alkanes (C8-C24).

In vitro antimicrobial assay

Agar well-diffusion method: The antibacterial activity of the selected Moroccan essential oils was evaluated by agar well-diffusion method. Initially, inoculate of micro-microorganisms were prepared by growing organisms on slant cultures for 24h, and suspending colonies in a sterile solution of 0.85% NaCl. Suspensions were adjusted to the turbidity of a 0.5 McFarland standards (approx. 1 X 10^8 CFU/mL). At first, the liquid bacterial culture was spread onto plates as described by Dorman & Deans 2000 [15]. After 15 min, the crude essential oil was poured in wells (6 mm diameter) made in the center of each agar plate. Doxycycline (disc: 30 µg) was used as positive control. All tests were performed in triplicate. The plates were incubated at 37°C in atmosphere of 5% CO_2 for 48h.

Table 2: Chemical composition of tested essential oils

RI	Constituents	*Mentha pulegium*	*Cymbopogon citratus*	*Citrus aurantium*
930	Tricyclene	-	0.04	-
936	□-Thujene	-	0.09	0.06
939	□-Pinene	0.52	2.22	0.82
952	Cyclohexanone-3-methyl	0.26	-	-
953	Camphene	-	-	0.05
980	□-Pinene	0.39	0.12	8.72
991	Myrcene	0.16	0.82	1.88
994	Octanol-3	1.86	-	-
1001	□-2-Carene	0.07	-	-
1011	□-2-Carene	-	-	2.32
1026	p-cymene	-	-	1.05
1031	Limonene	1.88	3.25	4.54
1072	*p*-Mentha-3,8-diene	1.44	-	-
1074	Linalool oxide	-	-	1.25
1088	□-Terpinolene	-	-	0.42
1098	Linalool	-	0.92	33.92
1153	Citronellal	-	32.77	0.07
1146	Isopulegol	-	0.81	-
1154	Menthone	0.19	-	-
1165	Borneol	-	0.12	0.07
1168	Pinocarvone	1.27	-	0.09
1171	Terpinen-4-ol	-	0.43	0.23
1173	Menthol	0.72	-	-
1192	□-Terpineol	-	-	3.54
1194	Dihydrocarvone	4.64	-	-
1228	Citronellol	-	12.67	-
1228	Nerol	-	-	0.37
1238	R(+)-pulégone	71.48	-	-
1240	Neral	-	0.44	0.05
1242	Carvone	5.66	-	-
1252	Peperitone	1.13	-	1.06
1255	Geraniol□	-	27.13	0.08
1270	Geranial	-	0.38	0.06
1295	Bornyl acetate	-	-	1.22
1298	Carvacrol	-	0.13	-
1354	Citronellyl acetate	-	5.08	-
1356	Eugenol	-	1.59	-
1383	Geranyl acetate	-	1.87	-
1391	□-Elemene	-	1.30	-
1418	□-Caryophyllene	0.33	-	1.21
1480	Germacrene D	-	1.86	-
1513	□-cadinene	-	0.32	-
1524	□-cadinene	-	1.83	-
1549	Elemol	-	1,92	-
1564	(E)-Nerolidol	-	-	5.24
1630	□-eudesmol	0.28	-	-
1649	□-eudesmol	0.49	0.34	-
1653	□-Cadinol	-	0.08	-
1722	(E,E)-Farnesol	-	-	3.08
1732	(E,E)-Farnesal	-	-	2.35
	Total	92.77	99.07	73.75
	Yield oil (%)	2.34	1.8	0.04

RI: Retention Index /values are in percentage

Determination of Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC): The minimum inhibitory concentrations of the respective essential oils against *A. actinomycetemcomitans* were determined according to a modification of the method described by Shapiro et *al*. 1994 [16]. The test was determined in sterile 96-well microplates with a final volume in each microplate well of 200 µl. Brain Heart

Infusion (BHI) broth was used for the 2 strains of *A. actinomycetemcomitans*. The turbidity of each liquid culture for use in the assays was adjusted to0.5 McFarland Units (approx. 1×10^8 CFU/mL) using sterile BHI. Each tested essential oil was dissolved in Tween 80/water (1/9), to enhance their solubility. Then, two fold serial dilutions of each original sample of essential oil were prepared in sterile culture medium to produce the concentration range of (1,25%- 0,019%). Aliquots (100 µl) of each dilution were dispensed into each well of 96-well cell culture plates with 100 µl of liquid culture. Amoxicillin (10 mg/mL) was used as positive control, Tween 80/water (1/9) as negative control. The plates were covered with plastic lids and incubated at 37°C for 48h under 5% CO_2. The determination of MIC values was done in triplicate and tests were duplicated, to obtain 18 values for each strain. After incubation period, 40 µL of a 2 mg/mL Triphenyl Tetrazolium Chlorid (TTC) indicator solution (indicator of microorganism growth) was added to every well and the plate was incubated at 37°C for about 2 hours [17]. The TTC indicator solution changes from clear to purple in the presence of bacterial activity. Whereas it remains clear when microbial growth was inhibited. MIC was defined as the lowest concentration of essential oil that showed no visible bacterial growth after incubation time (no color change (clear) of TTC). To determine the Minimum Bactericidal Concentration (MBC), 10 μL aliquots of cultures were taken from wells showing no visible turbidity, inoculated onto chocolate agar plates and incubated for 48h at 37°C under 5% CO_2 [18]. The MBC was considered as the lowest concentration of essential oil that killed 99.9% of microorganisms in culture on the agar plate after incubation period. This experiment (determination of MBC values) was performed in triplicate. The MBC/MIC ratio was also calculated to exhibit the nature of antibacterial effect of essential oils. When the ratio was lower than 4, the essential oil was considered as a bactericidal essential oil and when the ratio was higher than 4, it was considered as a bacteriostatic essential oil [19].

Statistical analyses***:*** Inhibition zone diameter, a continuous variable with a normal distribution, was presented as mean ± standard deviation. For statistical differences between the four groups (*Mentha pulegium, Cymbopogon citratus, Citrus aurantium*, Doxycycline), the One Way Analysis Of Variance (ANOVA) with Bonferroni correction was performed. The difference between JP2 and non-JP2 strains of *Aa* was tested by the Mann-Whitney test. *P* value < 0.05 was considered as statistically significant. Statistical analyses were carried out using SPSS for Windows (SPSS, Inc., Chicago, IL, USA).

RESULTS

Chemical composition**:** The chemical analyses showed that the major constituents of essential oils tested were as follow: β-pinene (8,73%), Linalool (33, 92%) and (E)-Nerolidol (5,24%) for *Citrus aurantium*, Geraniol (27,13%), Citronellol (12,67%), Citronellal (32,77%) and citronellyl acetate (5,08%) for *Cymbopogon citratus*, R(+)pulégone (71.48%) and carvone (5,66%) for *Mentha pulegium* (Table 2).

Antibacterial activity

Agar well-diffusion assay**:** After incubation time, all the essential oils tested resulted in consistent inhibition zones against the two strains of *A. actinomycetemcomitans* (Table 3). The average inhibition zone diameter caused by Doxycyline on JP2 and non JP2 strains of *A. actinomycetemcomitans* (22,67 ± 1,15 and 19,33 ± 0,57 respectively) was significantly smaller than that produced by the tested essential oils (*Mentha pulegium: 39* ± 1,00 and 34,67± 0,57, *Cymbopogon citratus: 42,33* ± 2,51 and 41,33 ± 1,15, *Citrus aurantium*: 40 ± 0,00 and 34,33 ± 1,15 respectively). Statistically, for all inhibition diameter values obtained, no significant difference was found between the two strains of *A. actinomycetemcomitans (P* value = 0.08).

MIC and MBC values determination: The serial diffusion assay in 96-well microplates revealed MICs values ranged from 0,07 to 0,3% (v/v) (Table 4). *Cymbopogon citrates* showed the most significant antibacterial effect on *Aa* with a MIC value of 0,07%. Concerning MBC, the values were equal or close to the MIC, indicating a good bactericidal activity of tested essential oils against *Aa*. For MIC and MBC values, no differences were observed between the 2 strains of *Aa*, the clinical isolate JP2 clone and the non JP2 reference strain.

DISCUSSION

Three essential oils were selected for this study based on their folk use for treatment of different diseases. Indeed, *Mentha pulegium* is one of the *Mentha* species, from plant family of Lamiaceae, well known for their therapeutic use in traditional medicine as expectorant, antispasmodic, stomachic, refreshing … [14, 20-23] and also for their antiseptic effect in the treatment of sinusitis, bronchitis and tuberculosis [24,25]. *Cymbopogon citratus*, from plant family of Poaceae, is mainly known for its insecticidal and antiseptic properties in Morocco [14]. Its antimicrobial properties were demonstrated by several studies, but its effect in oral infectious diseases has been less reported. *Citrus aurantium*, from plant family of *Rutaceae*, is less known in Moroccan folk medicine, but data in the literature related its various medicinal properties such as anti-infective mainly [26,27]. Our results showed that the three tested essential oils exhibited a strong antimicrobial activity against two virulent periopathogen strains of *A. actinomycetemcomitans;* JP2 and non-JP2. The antimicrobial properties of these oils are well documented against various bacteria, but less studied on oral pathogens particularly on the highly pathogenic strain of *A.actinomycetemcomitans* (serotype b JP2 clone) which is implicated in significant tissue damages of periodontium. As well known, the antimicrobial activity of an essential oil is related to its major components. All the oils tested were characterized by the dominance of various antibacterial compounds. The obtained results showed that *Cymbopogon citratus* was the most active oil against *Aa*. The chemical analyses of this oil revealed as main constituents Geraniol, Citronellol and Citronellal (Table

Table 3: Mean diameter of inhibition zones (mm) obtained by the agar diffusion method

Strains	Inhibition zone diameter (mm)†				
	Mentha pulegium	*Cymbopogon citratus*	*Citrus aurantium*	Doxycycline	P
A. actinomycetemcomitans (clinical JP2 strain)	39±1,00	42,33 ± 2,51	40 ± 0,00	22,67 ± 1,15*	<0,001
A.actinomycetemcomitans (non-JP2 strain)	34,67± 0,57	41,33 ± 1,15	34,33 ± 1,15	19,33 ± 0,57*	<0,001

Values are given as mean ± SD of triplicate experiment, †: diameter of inhibition zones including diameter of well 6 mm.
**: P<0,001: Doxycycline Vs Mentha pulegium, Cymbopogon citratus and Citrus aurantium*

2). All these compounds exhibited good antibacterial activity, as showed in previous studies [28-32]. Indeed, alcohols and aldehydes compounds are well known to possess an antimicrobial efficacy and a broad spectrum [15,33] more significant than other chemical constituents. *Cymbopogon citratus* contains also, another minor component; limonene, which was found to possess an efficient antibacterial property [34], and that may contribute to improve the observed antimicrobial activity. *Citrus aurantium* was mainly composed of Linalool (alcohol), such as reported in the literature [35]. This component is known to have antimicrobial activity against various microbes; oral and non-oral bacteria [34, 36-40]. In a recent study, it has been reported that Linalool exhibit a high antibacterial activity against *Aa* [34]. Other compounds of this oil, though in minor concentration, have previously been known to possess antimicrobial properties too. These include α-terpineol [32], Geraniol [29, 30] and Terpinen-4-ol [27]. α-terpineol and Terpinen-4-ol showed an antimicrobial effect on *Aa* in previous trials [34, 36, 37, 41]. It is also well known that diversity and the synergistic effects of major and minor constituents present in the essential oils should be taken into consideration to account for their biological activity [38]. *Mentha pulegium*, the less active oil in our study, has been shown be dominated by R(+)-pulegone (ketone). Referring to the literature, although this major constituent showed a good antibacterial activity [40], ketones are known to have weaker activity than alcohols and aldehydes as demonstrated in a previous work [42]. In this study, we used the agar well-diffusion assay to evaluate the inhibitory activity of the essential oils against studied strains of *Aa*. The inhibition zones diameters obtained were superior than 20 mm (Table 3) which demonstrated a high susceptibility of the tested bacterium to the selected essential oils as showed by Duraffourd *et al.* 2002 [43]. No statistical difference of inhibition by the tested essential oils was found between the two strains (*P* value= 0,08). The antibacterial effect of these agents seemed to be greater than that obtained by Doxycycline, the positive control, against both JP2 and non-JP2 strains of *Aa* (Table 3). Although, according to literature, a good susceptibility of *Aa* was demonstrated towards this antibiotic (MIC90 of 1 microgram/mL) [44]. In the second step of our experimentation, antibacterial activity of the selected oils was then studied by determining the MIC and MBC, by 96-well microplates assay. This broth microdilution method, widely used in different studies, is more accurate than others utilized for testing the antibacterial activity of essential oils [45]. However, to enhance the solubility of tested oils, as known poorly soluble in water, and to optimize the contact microorganism-oil, an emulsifier such as Tween 80 has been used in our assay, as recommended by many researchers in earlier reports [46, 47]. Then, a colorimetric MIC method, using TTC (Triphenyl Tetrazolium Chloride), have been adopted to define the endpoint of bacterial growth, as described in several studies [17, 48-52]. TTC (Triphenyl Tetrazolium Chloride), a redox indicator, changes the color in response to bacterial growth, which enhances the detection of growth [48]. However, this indicator might exhibit an inhibitory effect on bacterial growth if used at high concentration, which may lead to false interpretations of susceptibility and decreased reproducibility. In our report, an initial concentration of 2 mg/ml of TTC have been used, which have decreased in each well of microplate to a final amount of 0,03%. This concentration (<0.125%), applied in our broth microdilution method, doesn't have any toxic effect on bacterial growth as found by Rahman et *al.* 2004 [48]. Thus, MIC was defined as the lowest concentration of oil that inhibited visible growth, as indicated by the TTC staining. All tested essential oils showed bactericidal activity with promising results of MBC and MBC/MIC ratio which was lower than 4 (Table 4). MBC values were equal or almost to MICs. *Cymbopogon citratus* showed the highest inhibitory (MIC of 0,07%) and bactericidal activity (MBC of 0,07%) in comparison with *Citrus aurantium* and *Mentha pulegium (*MIC of 0,3% for both, MBC of 0,3% and 0,6% respectively). These findings concerning this oil (*Cymbopogon citratus)* seem to be mostly in accordance with those obtained in previous researches against other facultative anaerobic bacteria [53-55] such as *Escherichia coli* (MIC of 0,06%) [48], *Actinomyces naeslundii* and *Porphyromonas gingivalis* (MIC of 0.44 and 0.22 mg/ml respectively) [56]. The second effective essential oil, in this study, *Citrus aurantium*, also exhibited strong effectiveness against *Aa* (MIC and MBC of 0,3%). These findings exceed those found by Ben Housna et *al*. 2013 [57] and Hammer et *al.* 1999 [18] against a panel of Gram negative bacteria. This may be explained by the no standardized methods employed in different studies to assess antimicrobial activity of essential oils (Agar dilution method or broth dilution method by macro or microdilution, exposure of micro-organisms to plant oil, the solubility of oil or oil components, and the use and quantity of an emulsifier) [46]. Concerning *Mentha pulegium*, the bactericidal properties against strains of *Aa* have been found to be the weakest in comparison with *Citrus aurantium* and *Cymbopogon citratus;* this could be

Table 4: Minimum Inhibitory Concentrations (MIC)(%) (v/v) and Minimum Bactericidal Concentrations (MBC) (%) (v/v) of essential oils against *A. actinomycetemcomitans*

Strains	*Mentha pulegium*			*Cymbopogon citratus*			*Citrus aurantium*		
	MIC (%)	*MBC (%)*	*MBC/ MIC*	*MIC (%)*	*MBC (%)*	*MBC/ MIC*	*MIC (%)*	*MBC (%)*	*MBC/MIC*
A.actinomycetemcomitans (clinical JP2 strain)	0,3	0,6	2	0,07	0,07	1	0,3	0,3	1
A.actinomycetemcomitans (non-JP2 strain)	0,3	0,6	2	0,07	0,07	1	0,3	0,3	1

probably related to its chemical composition (ketone) as indicated before. Although, the obtained values of MIC and MBC demonstrated a good antibacterial activity of this oil in accordance with previous findings [58,59]. Another report by Shapiro et *al* 1994 [16] using microdilution method, such as employed in our study, have revealed similar MIC values (0,3 %) on *Aa* by *Mentha piperita* (Lamiaceae), which is from *Mentha* species too. This supports our results concerning this oil. Our results exhibited no difference concerning MIC and MBC values between *Aa* clinical isolate JP2 and reference non-JP2 strain. Actually, until now, no particularly bacterial resistance or adaptation to essential oils in clinical isolates has been described, probably because of their mode of action affecting several targets of bacterial structures at the same time [60]. Thus, according to our obtained results, and since the JP2 and non-JP2 strains of *Aa* are both serotype b which is known to be virulent and associated with aggressive periodontitis, we could suggest that the tested oils may be useful as adjunctive or complementary treatment in this disease. However, the evaluation of the cytotoxicity of the tested essential oils is still needed. Otherwise, the microorganism tested in the present investigation is a virulent periodontal pathogen growing within a biofilm in patients with periodontal infections, which is composed of other various and complex periopathogens such as *Porphyromonas gingivalis, Fusobacterium nucleatum….* which are known strongly associated with the progression and severity of periodontal disease and the recurrence of periodontal treatment [61,62]. Therefore, in our research project, it is expected to perform further experimental studies by testing these selected Moroccan essential oils and others used and well known in Moroccan folk medicine against *Aa* in biofilm form and major virulent periodontal bacteria growing within the biofilm, in order to consider the usefulness of these natural products as potential antimicrobial agents in aggressive periodontitis. Consequently, clinical trials will be conducted to confirm these results.

In conclusion and within the limitation of this study, our results concerning the sensitivity of *Aa* to the selected essential oils; *Mentha pulegium*, *Citrus aurantium* and *Cymbopogon citratus*, are promising, providing a powerful basis for more *in vitro* and *in vivo* investigations, in order to assess the real efficiency of the studied oils as alternative or complementary antibacterial agents.

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CONFLICT OF INTEREST

All the author(s): Lakhdar Leila, Farah Abdellah, Bajjou Tahar, Rida Sana, Bouziane Amal and Ennibi Oumkeltoum declare that they have no competing interests.

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