INTRODUCTION

The oral species Aggregatibacter actinomycetemcomitans (Aa) is a periodontal pathogen, known as strongly associated with aggressive periodontitis, severe periodontal diseases which lead to rapid destruction of supporting tissues of teeth in young adults [1-3]. Genome analysis revealed that this species comprises discrete clonal lineages represented by different serotypes (a, b, c, d, e, f) associated with periodontitis or health, which may help to explain their differences in virulence and in association with disease [4-6]. Serotype b strain with a 530-bp deletion in the promoter region of the leukotoxin operon is designated as JP2 clone. This particular strain was found to be strongly associated with aggressive periodontitis in patients of African descent such as Moroccan adolescents [7]. The main characteristic of the virulent clone (JP2) is the expression of high levels of leukotoxin [8]. Consequently, the eradication of this bacterium is essential, based upon mechanical treatment of periodontal pockets associated to antibiotics. However, nowadays, there is sufficient evidence that antibiotic resistance has increased in the periodontal flora. Thus, the use of natural products such as essential oils, to overcome antibacterial resistances [9] and side effects of synthetic drugs, could be a good alternative as a new adjunctive treatment against this periopathogen. Moreover, these "essential oils," have become a necessity. The present study was conducted to evaluate the in vitro antibacterial activities of three selected essential oils from Moroccan aromatic medicinal plants (Origanum compactum, Thymus vulgaris and Cymbopogon martinii) against clinical Moroccan isolate of Aa JP2 strain.

METHODS

Antibacterial activity of essential oils was investigated using agar well diffusion method, then measured using broth microdilution method.

RESULTS: All the selected essential oils exhibited significant antibacterial activity on the highly pathogenic JP2 strain of Aa. Essential oil of Origanum compactum was found to be the most effective with a minimum inhibitory concentration (MIC) value of 0.03% (v/v) and a minimum bactericidal concentration value (MBC) of 0.07%.

CONCLUSION: The present findings indicate the possibility of exploiting these essential oils as potential antimicrobial agents in treatment of aggressive periodontitis associated to this pathogen.

KEYWORDS: Oral bacteria, Aggregatibacter actinomycetemcomitans, Essential oils, Antimicrobial activity, Minimum inhibitory Concentration.
In vitro cultures for 24h, and suspending colonies in a sterile solution of McFarland standards (approx. 1 × 10⁸ CFU/ml). At first, the liquid essential oil was dissolved in Tween 80/water (1/9). I noculate of The antibacterial activity of the selected Moroccan essential oils was expressed as a percentage peak area relative to total peak area. Library search was carried out using the combination of NIST MS 4 min. The relative amount of individual components of the total oil was determined according to a modification of the method described by Dorman and Deans 2000 [24]. After 15 min, the 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. Negative control consisted of 10% Tween 80, which was used to dissolve the bacterial culture was spread onto plates as described by Dorman and Deans 2000 [24]. After incubation for use in the assays was adjusted to 0.5 McFarland units (approx. 1 × 10⁶ CFU/ml) using sterile BHI. 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 48 h under 5% CO₂.

The determination of MIC values was done in triplicate and tests were duplicated. After incubation period, 40 µl of a 2 mg/ml Triphenyl tetrazolium chloride (TTC) indicator solution (indicator of microorganism growth) was added to every well and the plate was incubated at 37 °C for about 2 h [26]. 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 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% CO₂ [27]. 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 [28].

**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 (Origanum compactum, Thymus vulgaris, Cymbopogon martini, Doxycycline), the One Way Analysis Of Variance (ANOVA) with Bonferroni correction was performed. P value<0.05 was considered as statistically significant. MIC (%) (v/v), MBC (%) and the MBC/MIC ratio were expressed as mean ± standard deviation (SD). For statistical differences between the three tested essential oils concerning the inhibition zone diameter, the One Way Analysis Of Variance (ANOVA) with Bonferroni correction was used. 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: γ-terpinene (25.11%), Carvacrol (22.29%), Thymol (19.21 %), p-cymene (18.68 %) for Origanum compactum, Thymol (42.01%), p-Cymene (14.34 %), γ-Terpinene (12.04%), Carvacrol (5.07%) for Thymus vulgaris, Geraniol 84,12%, Geranyl acetate (6,67%) for Cymbopogon martini (table 2).

**Antibacterial activity**

**Agar well-diffusion assay**

After incubation time, all the tested essential oils and positive control (Doxycycline) resulted in consistent inhibition zones against Aa JP2 strain (table 3). No inhibition zone was observed for the negative control (10% Tween 80). The difference was statistically significant (P value<0.001) between the negative control and all the tested agents (Origanum compactum, Thymus vulgaris, Cymbopogon martini and Doxycycline).

**MIC and MBC values determination**

The serial diffusion assay in 96-well microplates revealed MICs values ranged from 0.03 to 0.07% (v/v) (table 4). Origanum compactum showed the most significant antibacterial effect on Aa with a MIC value of 0.03%. Concerning MBC, the values were ranged from 0.07 to 0.15% (v/v).

For MBC/MIC ratio, all the values found were lower than 4, considering thus tested essential oils as bacterial agents.

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

| Common name | Species                | Family plant | Medicinal properties                             |
|-------------|------------------------|--------------|--------------------------------------------------|
| Oregano     | Origanum compactum     | Lamiaceae    | Intestinal antiseptic, diuretic, antacid, stomachic, antispasmodic[20] |
| Thyme       | Thymus vulgaris        | Lamiaceae    | Pulmonary and intestinal antiseptic, expectorant, diuretic, stomachic, anthelmintic, antispasmodic[20] |
| Palmarosa   | Cymbopogon martini     | Poaceae      | Insect repellent [21] anthelmintic [22] antifungal [23] |

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Thymus vulgaris Vs Cymbopogon martinii (P= 0.008) ° °: P<0.05: Origanum compactum Vs Cymbopogon martinii (P= 0.008).

\[ n=6; \text{Values are given as mean ±SD. MIC and MBC values are expressed in percentage (%). Data were analyzed by one-way ANOVA. °: P<0.05:} \]

| RI | Constituents                  | Origanum compactum | Thymus vulgaris | Cymbopogon martini |
|----|-------------------------------|--------------------|-----------------|---------------------|
| 931| α-Thujene                     | 2.10              | 0.22            | 0.05                |
| 939| α-Pinene                      | 0.54              | 1.87            | 0.14                |
| 948| Camphene                      | 0.17              | -               | 0.07                |
| 973| Sabine                       | 0.23              | -               | 0.09                |
| 980| β-Pinene                      | 0.16              | 0.79            | 0.06                |
| 991| Myrcene                      | 2.21              | 2.18            | 0.34                |
| 995| δ-2-carene                   | 0.09              | -               | -                   |
| 1005| α-Phellandrene               | 0.26              | 0.51            | -                   |
| 1011| β-2-Carne                    | 0.08              | -               | 0.08                |
| 1008| m-cymene                     | 0.14              | -               | -                   |
| 1088| Terpinolene                  | 0.07              | 0.78            | -                   |
| 1098| Linalool                     | 1.24              | 4.41            | 2.42                |
| 1143| Camphor                      | 0.05              | -               | -                   |
| 1165| Borneol                      | 0.20              | -               | -                   |
| 1177| Terpinen-4-ol                | 0.34              | 1.08            | -                   |
| 1184| p-cymen-8-ol                 | 0.09              | -               | -                   |
| 1189| α-terpinol                   | 0.99              | -               | -                   |
| 1228| Nerol                        | -                 | 0.13            | -                   |
| 1235| Thymol methyl ether          | -                 | 2.14            | -                   |
| 1240| Nerol                        | -                 | 0.21            | -                   |
| 1255| Geraniol                     | -                 | -               | 84.12               |
| 1270| Geraniol                     | -                 | -               | 2.16                |
| 1290| Thymol                       | 19.21             | 42.01           | -                   |
| 1298| Carvacrol                    | 22.29             | 5.07            | -                   |
| 1352| Terpinyl acetate             | -                 | 0.41            | -                   |
| 1356| Eugenol                      | -                 | 0.41            | -                   |
| 1383| Geranyl acetate              | -                 | -               | 6.67                |
| 1391| β-Elemene                    | -                 | 0.15            | -                   |
| 1401| Methyl Eugenol               | -                 | 0.41            | -                   |
| 1418| β-Caryophyllene              | 1.03              | 0.82            | -                   |
| 1430| β-copaene                    | -                 | 0.16            | -                   |
| 1454| α-Humulene                   | -                 | 0.26            | -                   |
| 1480| Germacrene D                 | 0.34              | -               | -                   |
| 1509| β-Bisabolene                 | -                 | 0.37            | -                   |
| 1513| γ-cadinene                  | 0.05              | -               | -                   |
| 1581| Caryophyllene oxide          | 0.06              | 0.32            | 0.64                |

RI: Retention Index /values are expressed in percentage.

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

|                          | Origanum compactum | Thymus vulgaris | Cymbopogon martini | Doxycycline | Tween 80 (10%) | P     |
|--------------------------|--------------------|-----------------|--------------------|-------------|----------------|-------|
| Inhibition zone diameter (mm) | 17.67±2.08        | 18.67±1.15      | 22.67±1.52         | 22.67±1.15  | 6±0.00 **  | 0.001|

Values are given as mean±SD of triplicate experiment; †: diameter of inhibition zones including diameter of well 6 mm. °: diameter of well (6 mm). °: P<0.001: Tween 80 (10%) Vs Origanum compactum, Thymus vulgaris, Cymbopogon martini and Doxycycline.

**Table 4: Minimum Inhibitory Concentrations (MIC) (%) (v/v) and Minimum Bactericidal Concentrations (MBC) (%) (v/v) of essential oils against Aa JP2 strain**

|                          | Origanum compactum | Thymus vulgaris | Cymbopogon martini |
|--------------------------|--------------------|-----------------|--------------------|
| MIC (%)                  | 0.03±0.01          | 0.06±0.01       | 0.05±0.01          |
| MBC (%)                  | 0.07±0.01          | 0.15±0.01       | 0.07±0.01          |
| MBC/MIC                  | 2.4±0.97 **        | 2.4±0.69 **     | 1.26±0.41          |

n=6; Values are given as mean±SD. MIC and MBC values are expressed in percentage (%). Data were analyzed by one-way ANOVA. °: P<0.05: Thymus vulgaris Vs Cymbopogon martini (P= 0.008) **; P<0.05: Origanum compactum Vs Cymbopogon martini (P= 0.008).
DISCUSSION

In this study, the obtained results exhibited a potent antibacterial effect of the tested essential oils on a highly virulent periodontopathogen: JP2 clone of Aa. Actually, we tested selected Moroccan essential oils (Origanum compactum, Thymus vulgaris and Cymbopogon martini), which have never been studied on oral bacteria especially on the highly JP2 strain of Aa, a gram-negative facultative anaerobic bacterium and a well-known periodontopathogen, strongly involved in aggressive periodontitis [1-3]. The choice of essential oils was based on their documented medicinal properties particularly antimicrobial effects and/or on their therapeutic use in traditional medicine in Moroccans (table 1). Indeed, Origanum compactum and Thymus vulgaris, belonging to plant family of Lamiaceae, is widely used for their antimicrobial properties [29, 30]. And Cymbopogon martini, from plant family of poaceae, is mainly known for its insecticidal and antiseptic properties [21-23]. The in vitro antibacterial activity of these tested essential oils against Aa JP2 strain was qualitatively and quantitatively assessed by the presence of inhibition zone diameters and MIC and MBC values. As shown in table 3 and 4, all tested essential oils exhibited good antibacterial activity against the studied microorganism. The inhibition zones were in range of 17.67-22.67 mm, which demonstrated a good susceptibility of the tested bacterium to the selected essential oils. Indeed, as showed by Durrafour et al. [31], a tested microorganism is considered not sensitive for a diameter smaller than 8 mm, moderately sensitive for a 8-14 mm diameter, sensitive for a 14-20 mm diameter, and very sensitive for a diameter larger than 20 mm. The significant and important antibacterial activity of all these tested oils could be attributed to its major components. Indeed, the obtained results showed that Origanum compactum was dominated by phenols (thymol 19.21% et carvacrol 22.29%), which are known responsible of bactericidal activity of essential oils [32, 33]. Thymol is the major component present at high concentration (42.01%) in Thymus vulgaris, which would also explain the potent antimicrobial activity of this tested oil, as reported in an anterior study [34]. Actually, in a previous report, Thymol showed significant antibacterial effect on Aa [25]. Cymbopogon martini was characterized by the dominance of phenols, as Geraniol (94.12%). This chemical constituent could be at the origin of its marked antibacterial efficacy, because of their high antimicrobial activity demonstrated in previous studies [35, 36]. Indeed, according to the literature, alcohols and phenols are well known for their antimicrobial efficacy more than other chemical compounds (such as terpene hydrocarbons) [24, 37, 38].

Concerning the 96-well microplates assay (MIC and MBC values determination), Origanum compactum has been found to be the most active oil with the highest inhibitory (MIC of 0.03%) and bactericidal activity (MBC of 0.07%) in comparison with other tested oils (Thymus vulgaris and Cymbopogon martini). These results are in agreement with those obtained in previous works on other tested non-oral Gram-negative bacteria [39-42]. Essential oils of Thymus vulgaris and Cymbopogon martini exhibited MIC of 0.06 % and 0.05% respectively, reflecting also a strong antibacterial activity on Aa. These findings confirm those found in the literature on other extraroral bacteria [27, 41]. More recently, Kedzia et al. 2013 [43], tested Thymus vulgaris on Aa and reported a potent antimicrobial effect of this oil on Aa (MICs ≤ 62-500 µg/ml). Otherwise, the CMB/CMI ratio was calculated in this study, providing information on the nature of the antibacterial effect of tested essential oils [28]. All the studied essential oils have been found to be bactericidal on Aa JP2 strain. However, some statistical differences have been registered between both Origanum compactum and Thymus vulgaris with Cymbopogon martini (table 4). This may be probably related to chemical composition relative to each tested oils. Indeed, as we mentioned above, phenols, as major compound of Origanum compactum and Thymus vulgaris [but not found in Cymbopogon martini], are well known responsible of bactericidal activity of essential oils [32, 33].

In our study, it is worth noting that this virulent oral pathogen (Aa JP2 strain) was a clinical isolate, sampled from subgingival biofilm in periodontitis patients. Earlier studies have demonstrated significant inhibitory activity of similar tested essential oils (Origanum compactum, Thymus vulgaris and Cymbopogon martini) against other various clinical isolates of different origins (respiratory, intestinal...). Indeed, Cymbopogon martini proved to be potent in inhibition of pathogenic Gram negative bacteria, clinically isolated from vaginal infections, as reported by Schwietz et al. 2006 [44]. Similarly, Thymus vulgaris showed antibacterial activity against clinical isolates of the respiratory tract including facultative anaerobic Gram negative species (Haemophilus influenzae) [45]. Origanum compactum, in previous studies, also showed antibacterial efficacy on clinical isolates of Gram-negative bacteria of intestinal, respiratory and skin origins [19, 42]. Thus, it should be noted that although clinical isolates belong to bacterial biofilm, and may develop antimicrobial resistance such as acquired resistance to antibiotics which is often reported, no particular bacterial resistance or adaptation to these essential oils has been described in all these studied clinical isolates. This may be probably related to the mode of action of essential oils affecting several targets of bacterial structures at the same time [46]. Therefore, according to our obtained results, the highly leukotoxic clone (JP2) of Aa has been shown to be very sensitive to natural agents; three selected essential oils (Origanum compactum, Thymus vulgaris and Cymbopogon martini). However, the evaluation of the safety and toxicity of these products is still required. Otherwise, the tested microorganism (JP2 Aa) is a virulent periodontal pathogen growing within a biofilm in patients with periodontal infections. This biofilm is composed of various bacteria and bacteria species, which are also associated with aggressive periodontitis. Thus, further in vitro and in vivo studies are needed to evaluate the antimicrobial activity of these selected essential oils on the entire subgingival biofilm sampled from periodontitis patients, in order to consider the possibility of exploiting these new natural products as effective antibacterial agents in the treatment of aggressive periodontitis.

CONCLUSION

This in vitro study showed sensitivity of the highly virulent JP2 clone of Aa to the selected essential oils: Origanum compactum, Thymus vulgaris and Cymbopogon martini. Based on these findings, we could suggest the usefulness of these natural products as potential antimicrobial agents in periodontal diseases associated to this virulent microorganism.

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CONFLICT OF INTERESTS

All the author(s): Lakhdar Leila, Farah Abdelrah, Idriss Lahlo Amine, Sana Rida, Anbal Bouziane and Oumkeltoue Oumniedclare that there is no conflict of interest regarding the publication of this paper.

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