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RESEARCH ARTICLE

Bioinformatical and *in vitro* approaches to essential oil-induced matrix metalloproteinase inhibition

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Abstract

Context: Essential oils carry diverse antimicrobial and anti-enzymatic properties.

Objective: Matrix metalloproteinase (MMP) inhibition characteristics of *Salvia fruticosa* Miller (Labiatae), *Myrtus communis* Linnaeus (Myrtaceae), *Juniperus communis* Linnaeus (Cupressaceae), and *Lavandula stoechas* Linnaeus (Labiatae) essential oils were evaluated.

Materials and methods: Chemical compositions of the essential oils were analyzed by gas chromatography–mass spectrometry (GC–MS). Bioinformatical database analysis was performed by STRING 9.0 and STITCH 2.0 databases, and ViaComplex software. Antibacterial activity of essential oils against periodontopathogens was tested by the disc diffusion assay and the agar dilution method. Cellular proliferation and cytotoxicity were determined by commercial kits. MMP-2 and MMP-9 activities were measured by zymography.

Results: Bioinformatical database analyses, under a score of 0.4 (medium) and a prior correction of 0.0, gave rise to a model of protein (MMPs and tissue inhibitors of metalloproteinases) vs. chemical (essential oil components) interaction network; where MMPs and essential oil components interconnected through interaction with hydroxyl radicals, molecular oxygen, and hydrogen peroxide. Components from *L. stoechas* potentially displayed a higher grade of interaction with MMP-2 and -9. Although antibacterial and growth inhibitory effects of essential oils on the tested periodontopathogens were limited, all of them inhibited MMP-2 *in vitro* at concentrations of 1 and 5 µL/mL. Moreover, same concentrations of *M. communis* and *L. stoechas* also inhibited MMP-9. MMP-inhibiting concentrations of essential oils were not cytotoxic against keratinocytes.

Discussion and conclusion: We propose essential oils of being useful therapeutic agents as MMP inhibitors through a mechanism possibly based on their antioxidant potential.

Keywords: *Salvia fruticosa* M., *Myrtus communis* L., *Juniperus communis* L., *Lavandula stoechas* L., MMP-2, MMP-9, systems biology, periodontitis

Introduction

Bacteria-induced tissue degradation in periodontitis is driven by an inflammatory state, which activates tissue-degrading enzymes, mainly matrix metalloproteinases (MMPs), by the host (Sorsa et al., 2006). MMPs are necessary for tissue repair and cell migration; however, their over-expression or hyperactivity in periodontal tissues leads to degradation of tooth-supporting tissues (Mäkelä

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et al., 1999). Inhibition of excessively produced MMPs is the major goal in host-modulation therapy (Golub et al., 1995). Although the inhibition of MMPs is thought to decrease tissue degradation, studies on MMP knock-out animal models indicated that MMP-deficient animals are more prone to periodontal degradation in comparison with non-deficient controls (Kuula et al., 2009). Instead of the total inhibition of MMP secretion and activity, bringing their levels down to similar ones as typically existing in healthy tissues could be the aim in periodontal therapy.

Essential oils of several plants have been used in ethnomedicine because of their antifungal, antibacterial, or anti-inflammatory activities (Kalemba & Kunicka, 2003). Traditionally prepared essential oils are common home remedies against cold, cough, fever, gastrointestinal irritation, and inflamed wounds (Yesilada et al., 1995; Honda et al., 1996; Uzun et al., 2004). Essential oils are antibacterial or antifungal against several microorganisms (Filipowicz et al., 2003; Pitarokili et al., 2003; Dadalioglu & Evrendilek, 2004), including periodontitis-associated bacteria (Gursoy et al., 2009), but their effects on the enzyme activity of the host have not extensively been studied. There is only one study available in the periodontal literature showing that an essential oil may inhibit neutrophilic MMP-9 (Abraham et al., 2005), but still, the underlying mechanism for such an effect remains a question mark.

Bioinformatics represents a solid additional approach to traditional biological research. Strong efforts within the scientific community are made to develop novel computational tools to elaborate scaling hypothesis from large amounts of experimental knowledge and to test large data sets that would remain virtually impossible by other means. Computer modeling is able to perform efficient simulations from available experimental data, generating new hypotheses which could be later tested by other approaches *in vivo* or *in vitro*. Potential advantages of computer modeling over the wet laboratory work include relatively lower cost and faster execution, where different species can be monitored at the same time under a number of diverse conditions. For instance, some bioinformatic programs help the researcher to understand the function, regulation, and post-translational modifications of a gene of interest. Our group and others have successfully developed and applied computational tools in different areas of scientific research, such as redox biology, cancer biology and therapeutics, and evolutionary biology (Castro et al., 2006, 2007; Bonatto, 2007; Barea & Bonatto, 2009; Rybarczyk-Filho et al., 2011; Dalmolin et al., 2011; Papp et al., 2011). We have utilized bioinformatical methods in order to evaluate the current experimental landscape of interaction between essential oil components, MMPs and tissue inhibitors of metalloproteinases (TIMPs), as well as the potential compounds or proteins crosslinking with them (if there were any), by using protein–protein, chemical-chemical, and protein–chemical database and experimental data. This can be represented in a novel protein–chemical network where the characteristics of potential interactions will additionally be analyzed.

In the present study, we first aimed to evaluate the potential *in silico* co-interaction between selected essential oils and MMPs through bioinformatical approaches and, afterwards, to analyze the anti-gelatinolytic, cytotoxic, proliferative, and antibacterial (activity against common periodontopathogens) effects of essential oils *in vitro*.

Material and methods

Preparation of essential oils

Selection of essential oils was done according to their use in folk medicine and diversity in their chemical composition (Gursoy et al., 2009). Leaves of *Myrtus communis* Linnaeus (Myrtaceae) (myrtle), leaves and flowers of *Salvia fruticosa* Miller (Labiatae) (sage), and *Lavandula stoechas* Linnaeus (Labiatae) (lavender), and fruits of *Juniperus communis* Linnaeus (Cupressaceae) (juniper) were collected in the west-Mediterranean region of Turkey in May–July 2006. Samples were air-dried in shade for 2–4 days. Essential oils, obtained with the traditional water and steam distillation technique, were kept in dark at 4°C.

Chemical composition analysis

Chemical compositions of the essential oils were analyzed by using gas chromatography–mass spectrometry (GC–MS) as previously described (Gursoy et al., 2009). Briefly, the GC–MS analyses were carried out using a Shimadzu QP 5050 (Kyoto, Japan) GC–MS system operating in the EI mode at 70 eV, equipped with an Cp-Wax 52 CB column (50 m × 0.32 mm; film thickness 1.2 μm). The initial temperature of the column being 60°C and was raised to 220°C at a 2°C/min rate. The carrier gas was helium, flow rate was 10 psi. The identification of the chemical constituents was based on data obtained from authentic samples and/or the Wiley, Nist, and Tutor libraries spectra.

Keratinocyte cultures

HaCaT human skin keratinocytes (generously provided by Hubert Fusenig, German Cancer Center, Heidelberg, Germany) were maintained as frozen stocks and cultured in a 5% CO_2 atmosphere at 37°C in Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum, 1% L-glutamine, and 1% penicillin G.

Bacterial cultures

Bacterial species and strains used in this study came from the culture collection of anaerobic bacteria of the National Institute for Health and Welfare (THL) and were: *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* NTCC 9710 and AHN 24195; *Porphyromonas gingivalis* ATCC 33277, AHN 24155, and AHN 24135; *Parvimonas micra* (formerly *Peptostreptococcus micros)* ATCC 33270, AHC 15107, and AHC 15154; *Fusobacterium nucleatum* ATCC 25586 and AHN 9508; *Prevotella intermedia* ATCC 25611 and AHN 8290; *Prevotella nigrescens* ATCC 33563 and AHN 8293.

The strains were revived from frozen (–70°C) stocks and subcultured for purity. Bacteria were grown on brucella blood agar enriched with hemin, and incubated at 37°C in an anaerobic chamber for 5 days.

In silico analysis

Development of an essential oil components-MMP interaction network model

By using a database resource search tool (STRING 9.0) for the retrieval of interacting genes (Szklarczyk et al., 2011), protein–protein interaction database and experimental data on MMPs and TIMPs were screened under a score of 0.4 (medium) and a prior correction of 0.0. Thereafter, by using a search tool for chemicals vs. chemicals and chemicals vs. proteins interactions (STITCH 2.0) (Kuhn et al., 2008, 2010), interactions between essential oil components, as well as essential oil components vs. MMPs and TIMPs were investigated under a score of 0.4 (medium) and a prior correction of 0.0 (Table 1). The results obtained from both *in silico* screenings were cross-linked in order to generate a network model representing the interaction between essential oil components, MMPs, and TIMPs (Model for Essential Oil components and MMP Interactions, the EOMI network).

Connectivity and clustering coefficient within the network model

Connectivity and clustering coefficient studies within the network were performed by using the ViaComplex software (Castro et al., 2009), which is an open-source application that builds landscape maps of gene expression networks.

Connectivity

Connectivity (k_i) of one node *i* is defined as the number of neighbors directly connected to a node.

Clustering coefficient

Clustering coefficient represents the fraction of possible associations between neighbors of a single node tending to cluster together. If one node is connected to a pair of nodes, then these three nodes may build a "triple". The number of triples represents the number of possible associations between the neighbors of one node. Clustering coefficient (C_i) is then calculated as:

$$C_i = \frac{2n_i}{k_i(k_i - 1)}$$

In this equation, n_i represents the number of links connecting the neighbors of node i (k_i) one to each other.

Antimicrobial activity

The agar dilution method was used to determine the minimum inhibitory concentration (MIC) of the essential oils on periodontal bacteria according to the Clinical Laboratory Standards Institute guidelines (CLSI, 2007). Brucella blood agars were prepared with serial two-fold dilutions of essential oils, ranging from 0.125 to 128 μL/mL. Bacterial inocula were prepared by adjusting their density to 0.5 McFarland standards with phosphate buffered saline (PBS) and inoculated to each agar plate with a multipoint inoculating device. The plates were incubated at 37°C in an anaerobic chamber for 5 days.

Antimicrobial activities of essential oils against the tested periodontal pathogens were analyzed with a disc diffusion assay with slight modifications (Murray et al., 1995). One 5-day-old colony from the first passage of each bacterial strain was inoculated on brucella blood agar. The 6 mm diameter discs were impregnated with 15 μL of each essential oil at concentrations of 1, 5, 10, 25, and 50 μL/mL in dimethyl sulfoxide (DMSO) and placed on to the inoculated agar plates. Antibacterial activities were interpreted as follows: –, no antimicrobial activity and an inhibition zone <1 mm; +, a modest antimicrobial activity and an inhibition zone corresponding to 2–4 mm; ++, a clear antimicrobial activity and an inhibition zone corresponding to 5–10 mm; +++, a strong antimicrobial activity and an inhibition zone >10 mm (Ojala et al., 2000). A penicillin G

Table 1. Table listing both compound and ensemble protein identifiers (CID and Ensemble Protein IDs) for the *in silico* approach.

Essential oil components	CID	MMPs/TIMPs	Ensemble Protein ID	Other compounds	CID
Menthol	CID000001254	MMP-1	ENSP00000322788	Hydrogen peroxide	CID000000784
Pulegone	CID000006988	MMP-2	ENSP00000219070	Hydroxyl radicals	CID000000961
Limonene	CID000022311	MMP-3	ENSP00000299855	Molecular oxygen	CID000000977
Menthone	CID000006986	MMP-7	ENSP00000260227		
p-Cymene	CID000007463	MMP-8	ENSP00000236826		
Carveol	CID000007438	MMP-9	ENSP00000361405		
α-Terpineol	CID000017100	MMP-10	ENSP00000279441		
Geranyl acetate	CID000007780	MMP-14	ENSP00000308208		
Dihydrocarvone	CID000022227	MMP-17	ENSP00000353767		
1,8-Cineole	CID000002758	MMP-20	ENSP00000260228		
Geraniol	CID000004458	MMP-25	ENSP00000337816		
Linalool	CID000006549	TIMP-1	ENSP00000218388		
Citral	CID000008843	TIMP-2	ENSP00000262768		
Camphor	CID000002537	TIMP-3	ENSP00000266085		
Thymol	CID000006989	TIMP-4	ENSP00000287814		

(10 U) disc was used as a positive control and pure DMSO as a negative control. The agar plates were incubated at 37°C in an anaerobic chamber for 5 days. After incubation, the inhibition zones were measured and the effect was calculated. All tests were done in triplicates.

Cell cytotoxicity analysis and cellular proliferation

HaCaT cells were grown in 96-well plates. Cells were washed twice with PBS, and fresh medium was then placed to each well. Each essential oil was added into the culture medium at 1 and 5 μL/mL concentrations. After incubating for 24 h, the medium in the wells was collected and the cytotoxic effect of each essential oil was measured with a colorimetric cytotoxicity detection kit (Roche, Mannheim, Germany), measuring the lactate dehydrogenase (LDH) activity in the culture media. Cellular proliferation was analyzed by using the Cell Titer 96 assay (Promega, USA). All tests were done in triplicates.

Analysis of MMP-2 and MMP-9 activities

HaCaT cells were grown in 96-well plates as described above. Essential oils were added into the culture medium at 1 and 5 μL/mL concentrations, together with 10 ng/mL of phorbol 12-myristate 13-acetate (PMA) (Sigma), a well-known MMP-9 inducer (Shin et al., 2007). After incubation for 24 h, the medium in the wells was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, by using 8% gels containing 1 mg of gelatin (Sigma)/mL. After electrophoresis, the gels were washed four times in 0.05 M Tris–HCl (pH 7.5), 0.02% NaN_3 buffer, 25% Tween 80. The second wash was supplemented with 10 mM $ZnCl_2$ and 100 mM $CaCl_2$. The incubation buffer consisted of 0.05 M Tris–HCl (pH 7.5), 0.02% NaN_3 buffer, 10 mM $ZnCl_2$, and 100 mM $CaCl_2$. After incubation for 24 h at 37°C, the gels were fixed and stained with 0.1% Coomassie blue R-250 in 30% methanol and 10% acetic acid. Finally, the gels were analyzed by the Bio-Rad Model GS-700 Imaging Densitometer using Scion Image 4.0.2 for Windows (Scion Corporation, USA). All tests were done in triplicates.

Statistical analysis

Differences in the epithelial cell toxicity, proliferation, and PMA-induced MMP-2 and -9 activity inhibition were analyzed by using the Student *t*-test ($p < 0.05$).

Results

The major constituents of essential oils were as follows: 1,8-cineole/eucalyptol (49.5%), camphor (13.3%), β-pinene (7.2%), α-pinene (5.8%), and camphene (5.1%)

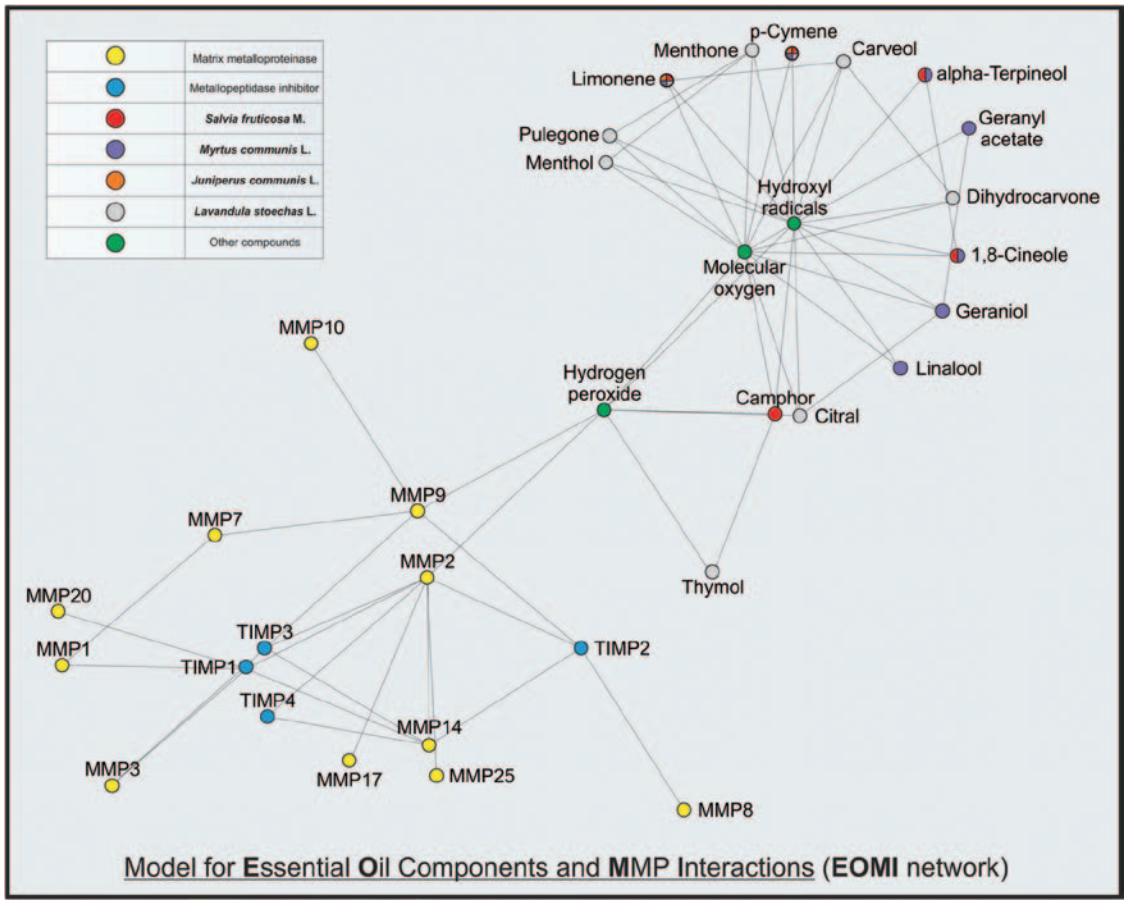

Figure 1. EOMI network. An essential oil components-MMP interaction network model was developed by utilizing the STRING 9.0 and STITCH 2.0 database resource search tools, under a score of 0.4 (medium) and a prior correction of 0.0.

for *S. fruticosa*, α-pinene (79.5%), β-pinene (4.0%), and 1,8-cineole/eucalyptol (3.9%) for *J. communis*, camphor (49.1%), fenchone (27.7%), and 1,8 cineol/eucalyptol (13.9%) for *L. stoechas*, and 1,8-cineole/eucalyptol (37.0%), α-pinene (30.2%), linalool (9.7%), terpineol (7.5%), and limonene (4.8%) for *M. communis*.

In silico analysis by using STRING 9.0 and STITCH 2.0 gave rise to the EOMI network for the interaction between the essential oil components, MMPs, and TIMPs (Figure 1). The EOMI network shows the direct interconnection between essential oil components and MMP-2 and -9 through previous interactions with $^{\bullet}OH$, O_2, and H_2O_2. Analysis of the main characteristics of the network by the ViaComplex software revealed that both $^{\bullet}OH$ and O_2 displayed the highest connectivity (Figure 2a and 2b) to a highly clustered group of essential oil components (Figure 2c and 2d).

The MIC values for the essential oils ranged from 0.25 to 8 μL/mL (Figure 3). None of the essential oils showed clear antimicrobial activity on the tested periodontopathogens (Table 2). There was a modest antibacterial activity at 50 μL/mL concentration of all essential oils against *P. micra* ATCC 33270 and AHC 15107, and at 50 μL/mL concentration of *J. communis* essential oil against *P. intermedia* ATCC 25611 and *P. nigrescens* AHN 8293. Penicillin G (the positive control) was strongly effective against all examined bacterial strains, while DMSO (the negative control) did not have any antibacterial effect.

When MMP-9 (a 92 KDa band) and MMP-2 (a 72 KDa band) activities were measured by a gelatin-zymography technique, the concentrations of 1 and 5 μL/mL of essential oils from *M. communis* and *L. stoechas* significantly inhibited the MMP-9 activity, while all essential oils tested significantly reduced the MMP-2 activity when compared with the positive control (PMA) (Figure 4).

The treatment of HaCaT cells for 24 h with 1 and 5 μL/mL of essential oils from *S. fruticosa*, *M. communis*, *J. communis*, and *L. stoechas* did not change the cellular proliferation (Figure 5a) and none of the essential oils showed any cytotoxic effect on MMP-inhibiting concentrations (Figure 5b). The concentrations of essential oils higher than 5 μL/mL were not analyzed by zymography, since those concentrations triggered the cellular detachment of keratinocytes.

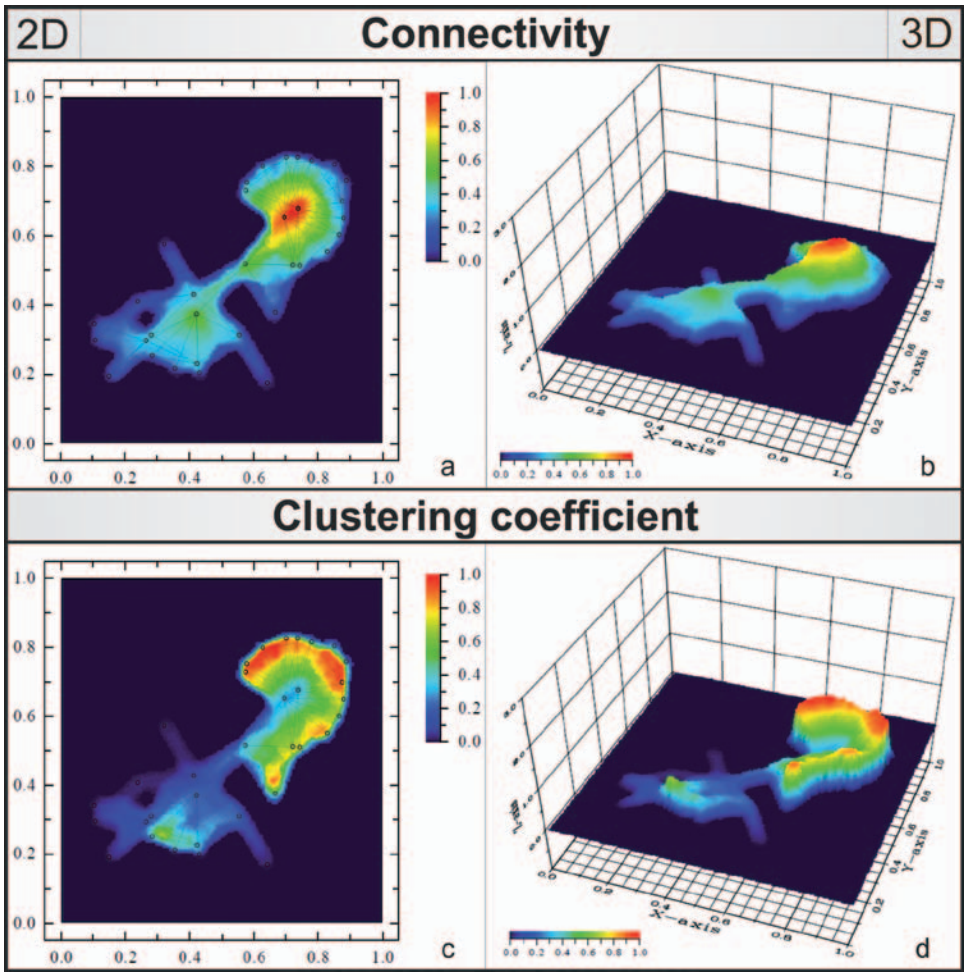

Figure 2. Software analysis (ViaComplex) of the properties within the EOMI network. Figure shows 2D and 3D representations of connectivity (a, b) and clustering coefficient (c, d) of each component in the network model.

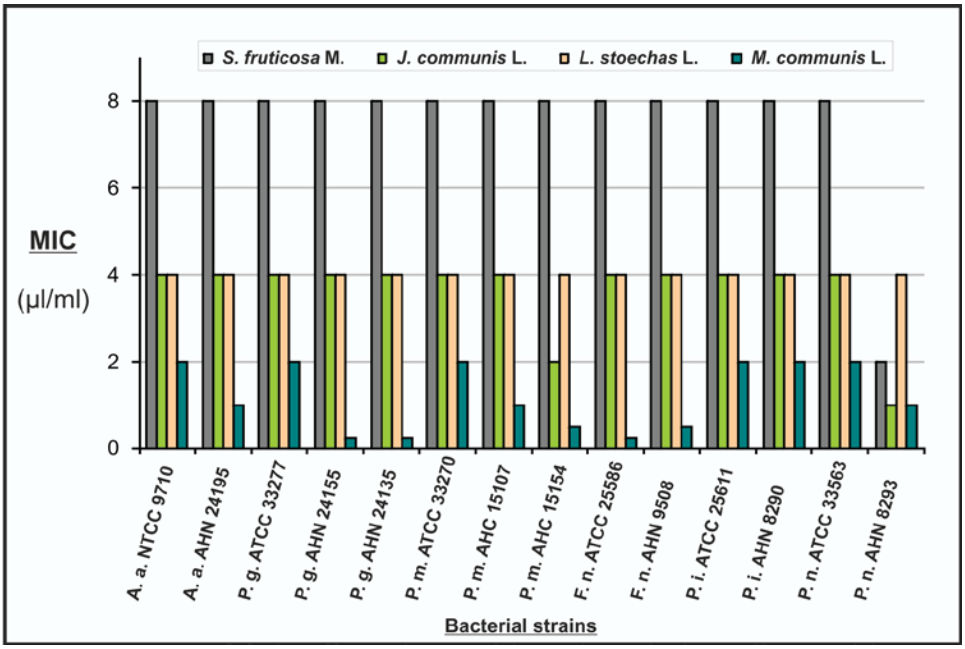

Figure 3. MIC of the tested essential oils against periodontal bacteria (A.a.: *Aggregatibacter actinomycetemcomitans*; F.n.: *Fusobacterium nucleatum*; P.g.: *Porphyromonas gingivalis*; P.m.: *Parvimonas micra*; P.i.: *Prevotella intermedia*; P.n.: *Prevotella nigrescens*).

Table 2. Antibacterial effects of essential oils against laboratory (NTCC and ATCC) and clinical (AHN and AHC) strains of six periodontal pathogens.

	Essential oils (µL/mL)																			
	Salvia fruticosa M.					*Myrtus communis* L.					*Juniperus communis* L.					*Lavandula stoechas* L.				
Bacterial species and strains	1	5	10	25	50	1	5	10	25	50	1	5	10	25	50	1	5	10	25	50
Aggregatibacter actinomycetemcomitans																				
NTCC 9710	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AHN 24195	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Porphyromonas gingivalis																				
ATCC 33277	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AHN 24155	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AHN 24135	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Parvimonas micra																				
ATCC 33270	–	–	–	+	+	–	+	+	+	+	–	–	–	–	+	–	–	–	+	+
AHC 15107	–	–	–	–	+	–	–	–	+	+	–	–	–	–	–	–	–	–	+	+
AHC 15154	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–
Fusobacterium nucleatum																				
ATCC 25586	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AHN 9508	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Prevotella intermedia																				
ATCC 25611	–	–	–	–	–	–	–	–	+	+	–	–	–	–	+	–	–	–	–	–
AHN 8290	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
Prevotella nigrescens																				
ATCC 33563	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AHN 8293	–	–	–	–	–	–	–	–	+	+	–	–	–	–	+	–	–	–	–	–

–, no antibacterial effect; +, a modest antimicrobial activity.

Discussion

The present study provides significant highlights about the inhibitory effect of essential oils over MMP-2 and -9 activities, based on both *in silico* and *in vitro* data. Bioinformatical tools may represent a "bridge" interconnecting different areas of research, such as biochemistry, pharmacology, molecular biology, medicine, etc. This is essential for developing a new way to do science: a modern, multidisciplinary, and, therefore, integrative approach that allows the researcher to work with large amounts of data.

Periodontitis is an infectious disease, characterized by breakdown of the tooth-supporting tissues and triggered by inflammatory response mechanisms of the host. The

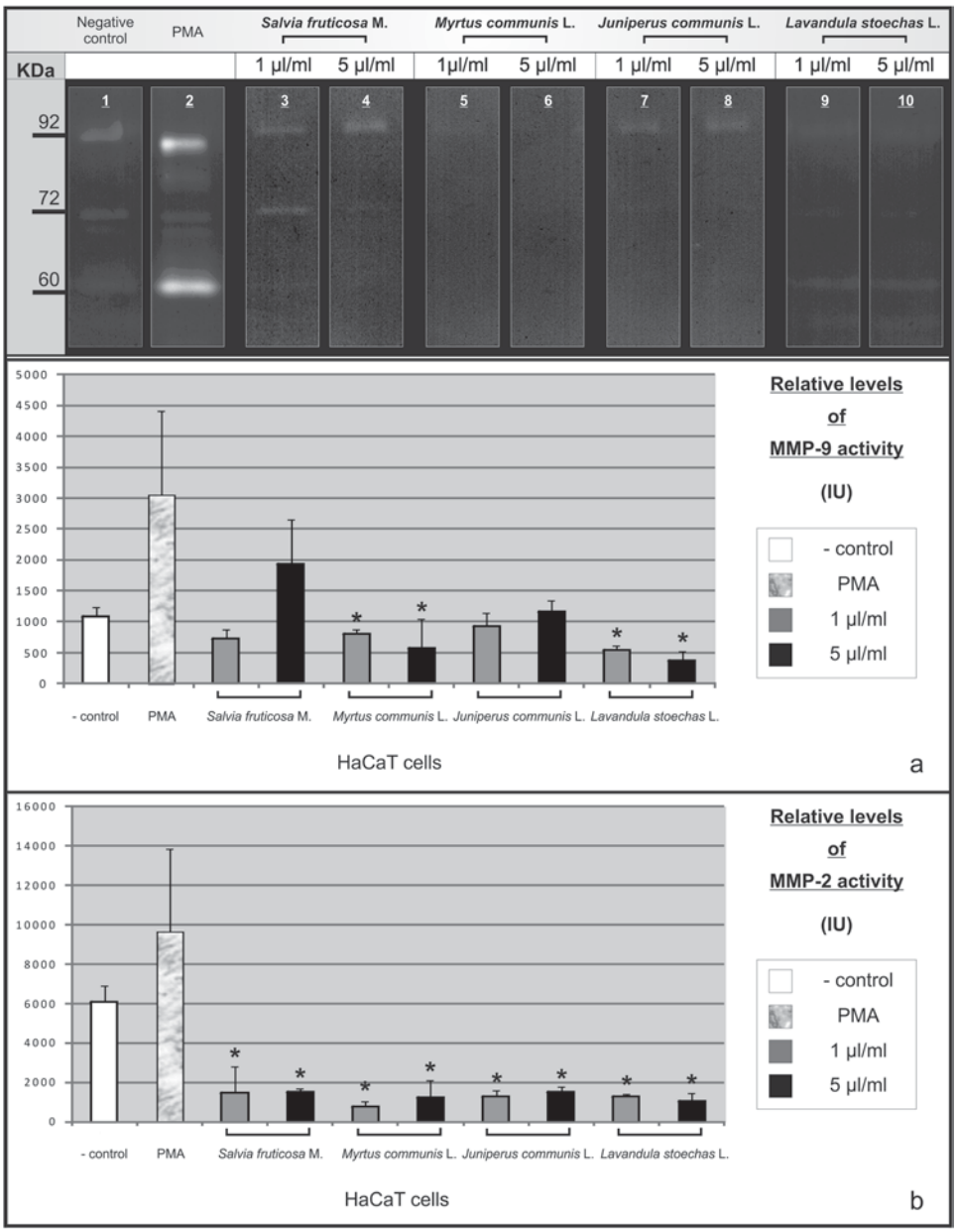

Figure 4. Effect of essential oils on MMP-9 and MMP-2 activities of keratinocytes. HaCaT human skin keratinocytes were treated with essential oils at 1 and 5 µL/mL concentrations in the culture medium, together with 10 ng/mL of PMA, a well-known MMP-9 inducer. Untreated HaCaT and PMA-treated cells represent the negative and positive control, respectively. Gelatinolytic activities seen on 92 and 72 kDa areas represent the MMP-9 (a) and MMP-2 (b) activities, respectively (*Statistical difference with the positive control; $p < 0.005$).

inflammatory response is initiated as an immediate reaction to bacterial challenge in the subgingival area. However, both extension and exacerbation of the inflammatory response leads to the destruction of periodontal tissues. When periodontitis is treated with anti-infective strategies, the treatment outcome is not always optimal and predictable in the healing process (Preshaw, 2008). Therefore, controlling an excessive host response and minimizing the tissue destruction can be a milestone for the treatment of periodontitis (Tonetti & Chapple 2011). Essential oils have been considered potential therapeutic agents in periodontal therapy, however, their beneficial effects as an adjunctive treatment option have been underestimated when limiting their use as antimicrobial agents solely (Feng et al., 2011). According to our findings in the present study, some essential oils could be used for the modulation of the host response, since they were able to inhibit an up-regulated gelatinolytic activity at non-antibacterial concentrations against periodontal pathogens. An essential oil-based management of excessive tissue destruction may lead researchers to develop new treatment modalities in the area of periodontology. In addition, if the beneficial use of essential oils in host-modulation therapy could be demonstrated *in vivo*, their applications would not only be limited to periodontal diseases; in fact, those may be applied to other inflammatory diseases, such as inflammatory lung diseases or gastrointestinal diseases.

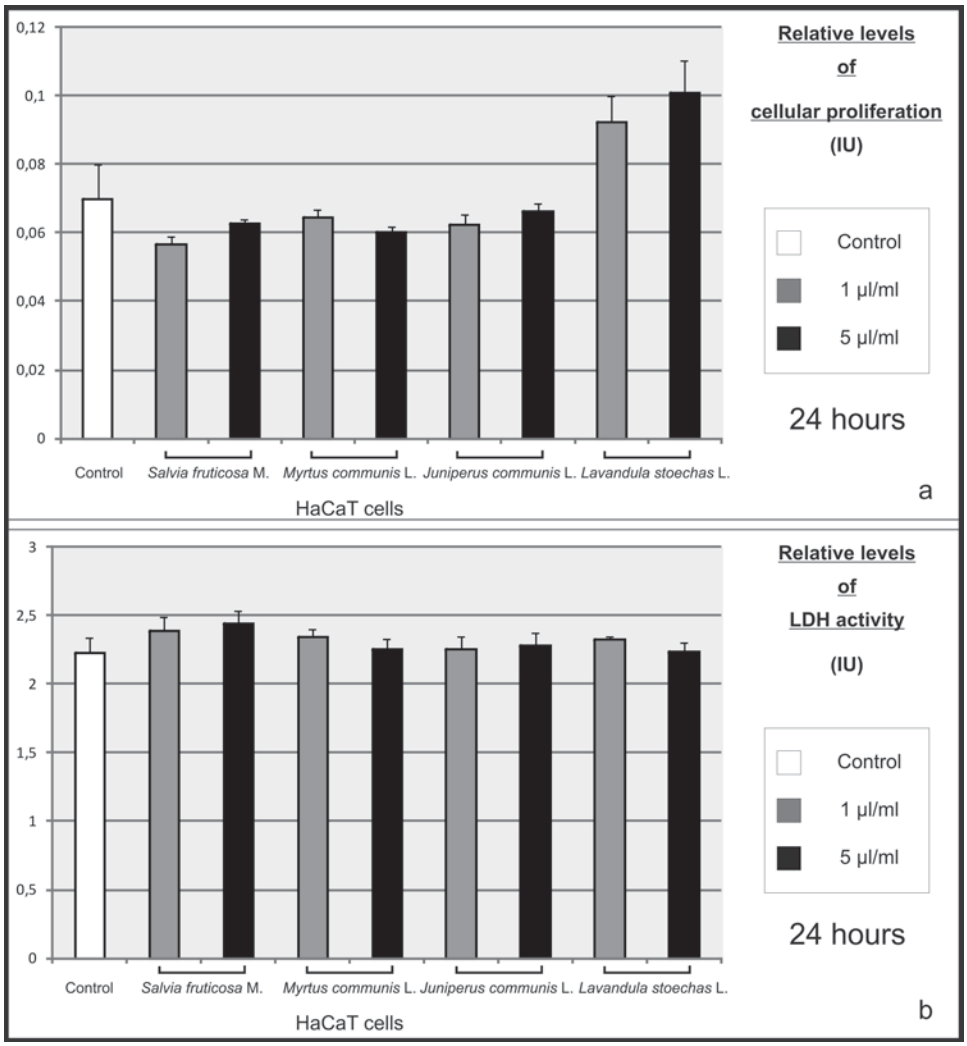

Figure 5. Effect of essential oils on proliferation and viability of HaCaT cells. HaCaT keratinocytes were treated with essential oils at concentrations of 1 and 5 µL/mL. After incubating for 24 h, no significant differences were found in the proliferation (a) and LDH activity (b) of these cells, when compared to control.

Our bioinformatical approach was based on the STRING (version 9.0) tool (Szklarczyk et al., 2011), which covers more than 1100 completely sequenced organisms, the STITCH (version 2.0) tool (Kuhn et al., 2008, 2010), which links proteins from 630 organisms to over 74000 different chemicals (including 2200 drugs), and the ViaComplex tool for studying the characteristics within the network (Castro et al., 2009). The characteristics of these tools let researchers to analyze a large amount of information coming from experiments and databases or even text mining. Hydroxyl radicals, molecular oxygen, and hydrogen peroxide were provided by the database for interconnecting the essential oils components, MMPs, and TIMPs *in silico*. In fact, one can already find in the literature various reports demonstrating the antioxidant potential of the essential oils used in our study (Emami et al., 2007; Papageorgiou et al., 2008; Aidi Wannes et al., 2010; Messaoud et al., 2011). According to our knowledge, however, this is the first time when a bioinformatical approach is being used in such a study context. Our *in silico* approach took into consideration databases and experimental data on MMPs and TIMPs exclusively under a score of 0.4 (medium) and a prior correction of 0.0. Text mining or text analytics was avoided. This means that our software analysis did not gather information from comments, hypotheses and/or discussions in the literature, for developing the EOMI network model.

In the present study, HaCaT non-tumorigenic human keratinocytes were selected as cell lines. Although this cell line has a non-oral origin, it is a widely used model in oral epithelial cell studies (Uitto et al., 1992; Boukamp et al., 1988; Gursoy et al., 2008a) and its MMP-2 and -9 secretion characteristics are well demonstrated (Gursoy et al., 2008b).

In periodontitis, specific pathogenic bacteria exert their virulent effect by inducing host cells to secrete destructive enzymes, such as MMPs (Ding et al., 1995) (Figure 6). Among them, MMP-9 (a 92 kDa gelatinase) and MMP-2 (a 72 kDa gelatinase) are enzymes that are secreted by keratinocytes, fibroblasts, neutrophils, macrophages, and osteoclasts (Sorsa et al., 2006). Increase in the MMP-9 production initiates the degradation of

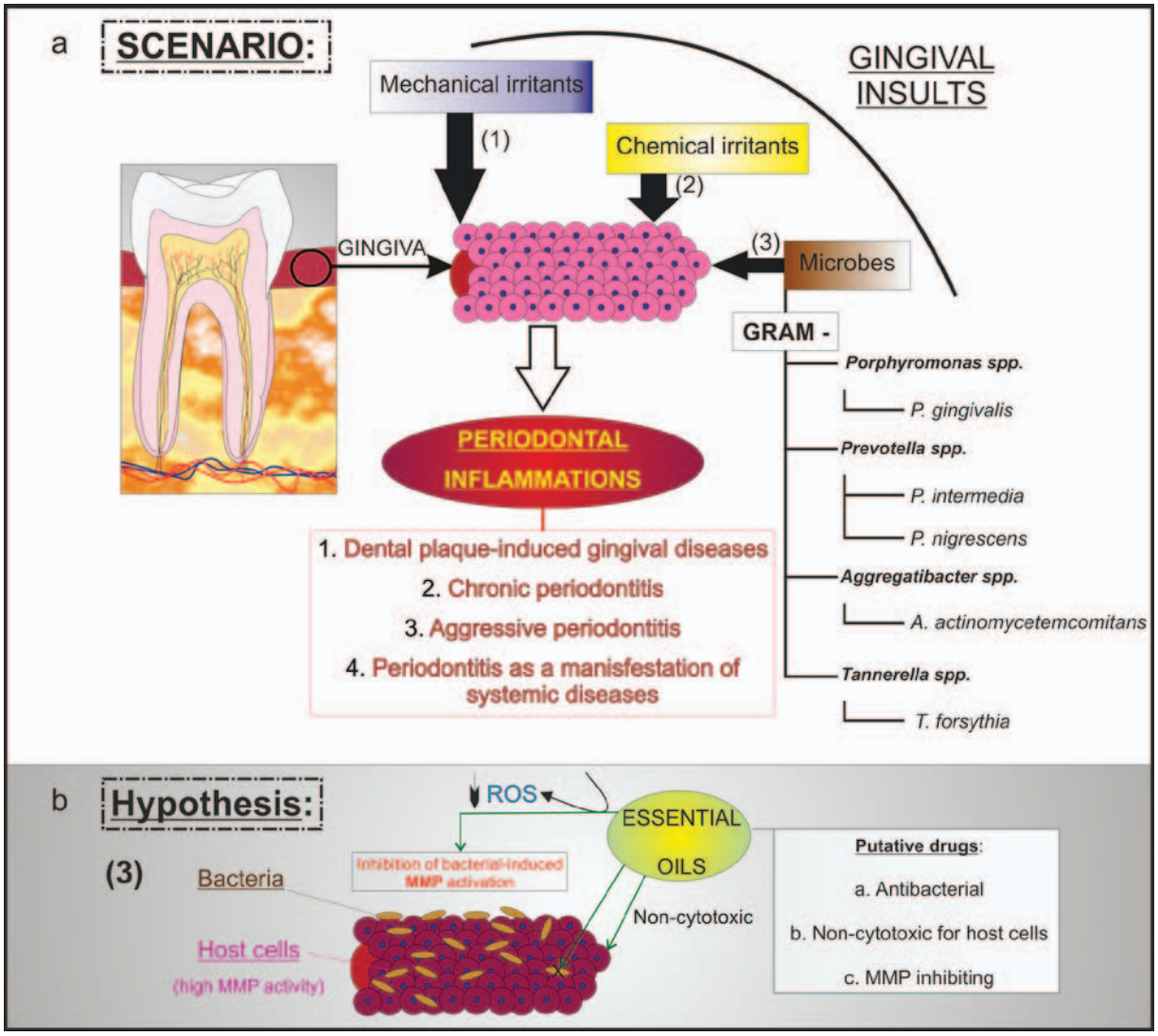

Figure 6. Hypothetical essential oil-induced inhibition in periodontitis. Mechanical and chemical irritants, as well as microbes can trigger periodontal inflammation (a). In such scenarios, specific bacteria exert their virulent effect by inducing host cells to secrete destructive enzymes, such as MMPs. In this work, we hypothesized that essential oil-induced MMP inhibition may occur thanks to the antioxidant properties of these compounds and ROS scavenging (b).

the basement membrane and connective tissue, events that can be clinically detected as loss of periodontal attachment (Mäkelä et al., 1994; Dong et al., 2009). Therefore, there is a search for developing new, efficient, and safe therapeutic strategies to bring the excessive MMP expression and activity to physiological levels. In order to achieve this goal, a deeper comprehension about the molecular mechanisms of MMP production by the host cells would be necessary. In the present study, the EOMI network showed direct interconnections between essential oil components and MMP-2 and MMP-9 through previous interactions with $^{\cdot}OH$, O_2, and H_2O_2. Further analyses revealed that both $^{\cdot}OH$ and O_2 displayed the highest connectivity to a highly clustered group of essential oil components. Further studies are warranted to explain these interactions in detail. If the mechanism of interaction between essential oils and MMPs, such as MMP-2 and -9, occurs through the interaction with $^{\cdot}OH$ and O_2, then one could predict a possible synergism between these components over MMPs.

Oxidative environments are necessary for the activation of these MMPs, since oxidants stimulate proteolytic cleavage, a crucial mechanism of zymogen activation (Morgunova et al., 1999; Nelson & Melendez, 2004). When cells consume oxygen, superoxide anion ($^{-}O_2$) is produced by electron leakage at mitochondrial complexes I and III (Finkel & Holbrook, 2000). $^{-}O_2$, H_2O_2, and $^{\cdot}OH$ (the latter can be generated by Fenton or Harber–Weis reactions from $^{-}O_2$ and H_2O_2) are commonly generated by reactive oxygen species (ROS) in the cells (Kamata & Hirata, 1999; Dalmolin et al., 2007). This is in line with the observation that periodontitis is associated with a cumulative increase in ROS and MMP productions (Asman et al., 1984; Shapira et al., 1991).

Interestingly, essential oils have been found to exert antioxidant or anti-inflammatory activities, which can

provide beneficial effects in protecting the tissues from exacerbated host response (Miguel et al., 2011). Plants have evolved antioxidant mechanisms, such as catalase and peroxidase systems, that can remove ROS from the cells (Noctor & Foyer, 1998). Plant monoterpenes can also scavenge O_3 in experimental conditions (Fares et al., 2008). Since MMPs can be activated through a ROS-dependent pathway (Meli et al., 2003), scavenging of ROS may inhibit the MMP activity. This could be the mechanism by which essential oils are able to inhibit MMPs (Figure 6b).

Antimicrobial effect against microorganisms is a common characteristic of several essential oils. In order to simulate the pathogenic microbial community present at infected subgingival sites, we selected six bacterial species that have been associated with periodontitis (Socransky & Haffajee, 2005). It is known that there are strain-dependent variations within a bacterial species, therefore, we also included more than one strain of each species, representing both clinical and laboratory strains, in the study. The four essential oils selected for the present study had either a weak antibacterial effect or no effect. Even though possible antibacterial effects could be found upon treatment with higher concentrations of essential oils, those concentrations could also be cytotoxic against host cells (Prashar et al., 2004). Microbes are able to generate defense mechanisms against natural antimicrobial compounds, for example, by degrading monoterpenes in anaerobic conditions (Harder & Probian, 1995). The degradation of active essential oil compounds by periodontopathogenic bacteria was not in the scope of the present study. However, in a clinical use, anti-enzymatic monoterpenes can be utilized with strong antibacterial phenolic components, such as carvacrol, for improving the success of periodontal therapy (Botelho et al., 2008).

In the present study, none of the essential oils displayed cytotoxic effects against keratinocytes. However, at concentrations higher than 5 μL/mL, all essential oils induced cellular detachment on our *in vitro* model. Detachment of cells after essential oil treatment has already been described (Takarada et al., 2004; Gursoy et al., 2009), but the underlying mechanism for such an effect is still unknown.

Conclusions

To the best of our knowledge, this is the first study integrating bioinformatics in the context of essential oils vs. MMP inhibition. Although the essential oils tested had no clear antibacterial activity against any of the examined periodontal pathogens, essential oils, even at non-antibacterial concentrations, act as potential agents for the inhibition of excessive MMP-2 and MMP-9 secretions.

In summary, based on our *in silico* and *in vitro* results, it can be postulated that some essential oils could be used as functional MMP inhibitors and, as such, as potential therapeutic agents in diseases where excessive MMP activity causes tissue destruction, such as periodontal diseases.

Declaration of interest

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