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Combined effect of essential oils against bacteria associated with deterioration of historical wood

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Abstract
Microbial deterioration of wood is becoming a very serious economic problem, due to emergent resistance of microorganisms to the conventional antimicrobial agents. In this study, the antibacterial effect of the combined applications of *Myrtus communis* and *Thymus vulgaris* essential oils against tow decaying wood bacteria was studied. The minimal inhibitory concentrations and minimal bactericidal concentrations were determined using the broth microdilution and subculture on plates assays. Furthermore, the fractional inhibitory concentration was evaluated by the checkerboard technique. The results showed that *M. communis* and *T. vulgaris* essential oils displayed minimal inhibitory concentration values of 1 and 0.03125% respectively. The fractional inhibitory concentration index values of combined applications of both essential oils have given 0.562 and 0.625 against strains studied, suggesting a partial synergic interaction. The combination (1/8 minimal inhibitory concentration of myrtle + 1/4 minimal inhibitory concentration of thyme) has demonstrated a strong synergistic effect towards *Bacillus subtilis*, by decreasing the minimal inhibitory concentration values 8-fold for myrtle and 4-fold for thyme essential oils when tested alone. These findings reinforce the suggestion that the mixture of these essential oils at suitably low concentrations could be a promising alternative to replace synthetic antimicrobial agents and lead to new research about natural products in order to find new ecofriendly preservative of all wood objects.

1. Introduction
Wood has always been the material widely used in our society because of its availability, aesthetic qualities and its ease of implementation. Especially, cedar wood was widely employed in various constructions and decorations with the appearance of majestic buildings such as mausoleums, magnificent palaces and riads [1]. However, despite these advantages, the cedar wood has a major drawback due to its susceptibility to different abiotic and biotic agents of deterioration including fungi, insects and bacteria that causing invaluable losses and creating significant economic impact [2,3]. Moreover, decay of wood represents a serious economic problem in the paper industry [4], arts [5] and environments [6]. Therefore, it is necessary to have recourse to its preservation in order to protect it and provide its sustainable use.
The global concerns related to the environment, high costs and the microbial resistance to synthetic antimicrobial agents, detergents and disinfectant have become a major defy. Thus, there is a major challenge to look for alternatives treatment measures and to develop solutions with competitive cost and strong environmental profile [7,8]. Hence, in the past ten years, the growing interest has focused on naturally occurring molecules, in particular essential oils and other plant extracts as a potential source of wood protection agents to prevent its biodeterioration [9–12].
The sufficient information available regarding microbial and enzymatic degradation of wood and wood products have already been reported [13–15]. In addition, the antifungal and antibacterial activities of essential oils and their majors components against pathogenic microorganisms of different fields have been demonstrated in several works [16–20]. In contrast, no work has been published previously on its combined antimicrobial effect against bacteria which are part of the wood decomposers microbial diversity. Thereby, this investigation was done to evaluate the effect of single and combined antibacterial effects of *Thymus vulgaris* and *Myrtus communis* essential oils against bacteria isolated from decayed wood of the old Medina of Fez city (Morocco).

2. Experimental

2.1. Plant material and essential oils preparation

The plants used in this study were *Thymus vulgaris* L. (*Labiateae*) and *Myrtus communis* L. (*Myrtaceae*). They were harvested from the garden of the National Institute of Medicinal and Aromatic Plants, Taounate (Morocco). The essential oils extraction from fresh aerial part (leaves of *M. communis*; leaves and steams of *T. vulgaris*) of these plants was performed by hydro-distillation method, for 2 h, using Clevenger-type apparatus [21]. The essential oils recovered were stored in darkness at 4°C until the use.

2.2. Essential oil analysis

For gas chromatographic (GC) analysis, the essential oils samples were diluted in methanol (1/20 v/v). The analysis was performed on GC Hewlett-Packard type (HP 6890 series) coupled with a mass spectrometer (HP 5973 series) equipped with the HP-5MS capillary column (30 m x 0.25 mm, film thickness is 0.25 µm). The carrier gas was helium (1.2 ml/min). The column temperature was programmed from 45 to 240°C at 2°C/min. The fragmentation was done by impact electronics in a field of 70 eV. A sample volume of 1 µl was injected in a split mode (leakage ratio: 1/20) at a temperature of 250°C.

The identification of the components was made by the comparison of their mass spectra with those of the library (NIST 98) and by comparison of their retention indices (IR) with those of bibliography [22]. The retention indices were determined in relation to homologous series of n-alkanes (C_9-C_{23}) under the same operating conditions.

2.3. Bacterial strain isolation and molecular identification

The bacterial strains used throughout this work were isolated from degraded historical wood samples in the old Medina of Fez city, Morocco.

For molecular identification, the genomic DNA was extracted using thermal shock. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the primers, fD1 (5’AGAGTTTGATCCTGG-CTCAG3’) and Rs16 (5’TACGGCTACCTTGTTACGAC TT3’) [23]. DNA sequencing was performed using ABI 3130 (Applied Biosystems) according to the manufacturer's instructions. Comparative sequence analysis was performed by comparing sequences with those available in the online databases provided by the National Centre for Biotechnology Information (NCBI) using the Gen bank BLASTN tools.

2.4. Determination of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The MICs were determined using the broth microdilution assay as previously described [24], with slight modifications. Agar at 0.15% (w⁄v) was used as emulsifier and resazurin was used as bacterial growth indicator. Firstly, 50 µl of Mueller Hinton Broth (MHB) (Oxoid, UK) supplemented with bacteriological agar (0.15% w/v) were distributed from the second to the 12th well of a 96-well polypropylene microtitre plate. Essential oil dilutions were prepared in MHB supplemented with agar (0.15% w/v). 100 µl of these suspensions were added to the first test well of each microtitre line, and then 50 µl of scalar dilution were transferred from the second to the 11th well. The 12th well was considered as growth control. Then, 50 µl of a bacterial suspension were added to each well at a final concentration of approximately 10^6 CFU/ml. The final concentration of the essential oil ranged between 4 and 0.0039% (v⁄v) for myrtle and between 1 and 0.00097% (v⁄v) for thyme. After incubation at 37°C for 20 h, 5 µl of resazurin were added into each well to assess bacterial [24]. After further incubation at 37°C for 2 h, the MIC was determined as the lowest essential oil concentration that prevented a change in resazurin color. Bacterial growth is detected by reduction of blue dye resazurin to pink resorufin. Experiments were conducted in triplicate.

The minimal bactericidal concentration (MBC) corresponded to the lowest concentration of the essential oil yielding negative subcultures after incubation at 37°C for 24 h. It is determined by spotting 2 µl from negative wells on LB (Luria-Bertani) agar plates. Experiments were also conducted in triplicate.

2.5. Determination of Fractional Inhibitory Concentration (FIC)

The effects of interactions between *M. communis* and *T. vulgaris* essential oils against tow *Bacillus* strains isolated from decayed cedar wood were evaluated using the checkerboard technique [25]. The concentrations of both essential oils were prepared in MHB supplemented with agar (0.15% w/v). Along the x-axis across the checkerboard plate, 50 µl of each myrtle essential oil concentration was added into each well from the first to the 11th well. As for the y-axis, 50 µl of each thyme essential oil concentration was added into each well from 4 × MIC to 1/16 × MIC. The 12th well was considered as growth control.

The inoculum of approximately 2.10^6 CFU/mL was then added into all the wells. The 96-well plate was then sealed and incubated at 37°C for 20 h. After incubation, 10 µl of resazurin were added to each well to assess bacterial growth. After further incubation at 37°C for 2 h, the FIC index values were then calculated using the following formula:

$$\sum \text{FICI} = \text{FIC(A)} + \text{FIC(B)}$$

Where

$$\text{FIC (A)} = \frac{\text{MIC (A) in combination}}{\text{MIC (A) alone}}$$

And

$$\text{FIC (B)} = \frac{\text{MIC (B) in combination}}{\text{MIC (B) alone}}$$

The $\sum$ FICI values are interpreted as follows: $\leq$ 0.5= synergistic; 0.5-0.75 = partial synergy; 0.76-1.0 = additive; > 1.0-4.0 = indifferent (non-interactive); > 4.0 = antagonistic.

3. Results and Discussion

3.1. Chemical composition of essential oils

The gas chromatography-mass spectrometry (GC-MS) analysis (Table 1) shows that 29 and 47 compounds were identified in essential oils of *T. vulgaris* and *M. communis* representing 88.6 and 97.89%, respectively. The major constituents of thyme oil were thymol (40.0%), γ-terpinene (12.0%), p-cymene (12.0%), linalool (4.4%) and carvacrol (3.1%), beside other compounds with relatively low levels, including thymol methyl ether (2.1%), myrcene (2.1%), α-thujene (2.1%), α-terpinene (2.0%) and α-pinene (1.7%). Regarding the myrtle oil, the finding demonstrated that it was dominated by 1.8-cineol (27.65%), α-pinene (24.26%), limonene (14.32%) and myrtenyl acetate (13.05%).

Table 1: Chemical composition of *T. vulgaris* and *M. communis* essential oils.

Compounds*	RI	*T. vulgaris*	*M. communis*
		Percentage (%)**	Percentage (%)**
α –Thujene	931	02.10	00.12
α –Pinene	939	01.70	24.26
Camphene	953	-	00.04
Sabinene	976	-	00.23
β –Pinene	980	00.70	00.22
Octan-1-en-3-ol	988	00.10	-
Myrcene	991	02.10	00.21
α Phellandrene	1005	00.50	00.21
α –Terpinene	1018	02.00	00.06
p-Cymene	1026	12.00	-
Limonene	1031	01.00	14.32
1.8-Cineole	1033	00.80	27.65
(E) β –Ocimene	1040	-	00.12
γ –Terpinene	1062	12.00	01.98
α -Terpinolene	1088	00.80	00.09
Linalool	1098	04.40	02.21
Fenchol	1112	-	00.04
Allo-ocimene	1129	-	00.06
Terpin-1-ol	1134	-	00.04

Trans-Pinocarveol	1139	-	00.14
Verbenol	1140	-	00.21
Terpinene-4-ol	1177	00.10	00.25
α –Terpineol	1189	-	03.34
Myrtenol	1194	-	01.58
Cis-Carveol	1217	-	00.10
Neral	1228	-	00.12
Thymol methylether	1235	02.10	-
Geraniol	1255	-	00.08
Linalyl acetate	1257	-	00.49
Geranial	1270	-	00.12
Verbenyl acetate	1282	-	01.05
Thymol	1290	40.00	-
Bornyl acetate	1295	-	00.22
Carvacrol	1298	03.10	-
Myrtenyl acetate	1335	-	13.05
Carvyl acetate	1337	-	00.25
δ Elemene	1339	-	00.12
Terpinyl-4-acetate	1340	-	00.18
Terpinyl acetate	1352	00.40	00.57
α –Copaene	1376	-	00.29
Geranyl acetate	1383	-	01.79
β –Elemene	1391	00.10	-
Methyl eugenol	1401	00.40	00.75
β –Caryophyllene	1418	00.80	00.25
β –Copaene	1430	00.10	-
γ Patchoulene	1441		00.12
α-Humulene	1454	00.30	00.35
Germacrene D	1480	00.30	-
Citronnellyl Isobutyrate	1482	-	00.08
Viriflorene	1493	-	00.07
β –Bisabolene	1509	00.30	-
Geranyl Isobutyrate	1514	-	00.09
δ-Cadinene	1520	00.10	
Isobornyl-2-methyl Butyrate	1522	-	00.03
Citronnellyl-n-butyrate	1529	-	00.08
Geranyl-n-butyrate	1562	-	00.07
Isoeugenol acetate	1563	-	00.07
Caryophyllene oxide	1581	00.30	00.12
Total		88.60	97.89

RI: Retention index, *: Identification by GC-MS- **: Percentages of compounds provided by gas chromatogram.

3.2. Isolated and identified bacteria

The results of the molecular identification of bacterial isolates from the deteriorated wood indicated that these latter were *Bacillus subtilis* and *Bacillus safensis* with access numbers of JN700079.1 and KT027733.1 respectively, and the genetic similarity of 100% with the existing NCBI sequences (Figure 1 and 2). This finding is consistent with the work of Suberkropp [26], which reported the presence of six different bacterial genera with a logarithmic growth during the early stages of wood degradation. In addition, another study has demonstrated the presence of five different bacterial strains belonging to the genera of *Bacillus*, *Pseudomonas*, *Klebsiella*, *Acinotobacter* and *Oceanobacillus* isolated from decayed wood [27].

The bacterial degradation of wood was reported by many authors. They have shown that these microorganisms are a source of concern problem for historical building, archaeological remains and wooden objects. Indeed, it have a real impact on the durability of wood, its color and its physical chemical characteristic by producing enzymes (cellulase and ligninase) which are responsible of wood constituents degradation [2,13,28,29].

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Query  1     TGCTGATCCGCGATTACTAGCGATTCMGCTTCACGCAGTCGAGTTGCAGACTGCGATCCG  60
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Query  301   CAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGACGTCCTATCTCTAGGATTGTCA  360
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Sbjct  993   CAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGACGTCCTATCTCTAGGATTGTCA  934

Query  361   GAGGATGTCAMSACCTGGTAAGGTTCTTCGCGTTGCTTC  399
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Sbjct  933   GAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTC  895
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Figure 1: Nucleotide sequence of *Bacillus subtilis*

3.3. Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The antibacterial activities of *M. communis* and *T. vulgaris* essential oils against *B. subtilis* and *B. safensis* isolated from decayed cedar wood are shown in Tables 2 and 3.

According to the results (Table 2), the MIC values of *M. communis* and *T. vulgaris* essential oil are ranged from 1 to 0.03125% (v/v) against the tested bacterial strains. The MIC values of *M. communis* essential oil were 16 and 32-fold higher than those of *T. vulgaris*, indicating the stronger antibacterial effect of thyme essential oil. Indeed, it was capable of inhibiting the development of *B. safensis* and *B. subtilis* at MIC values of 0.0625 and 0.03125% (v/v) respectively. However, *M. communis* essential oil was not capable to inhibit the growth of these bacterial strains at concentration ranging from 0.5 to 0.03125% (v/v). These findings could be due to their varied chemical compositions and the antibacterial effectiveness of their major compounds.

Regarding the MBC values of both essential oils tested, found after spotting 2 µl from negative wells on LB plates, the results indicated that MBC values of the essential oils tested were almost similar to their MIC values against both bacterial strains studied and 2 fold higher towards *B. subtlis* in the case of thyme essential oil (Table 3). In fact, the MBC values of *T. vulgaris* and *M. communis* essential oils were 0.0625 and 1% (v/v) respectively. Thus, it can be concluded that both exhibited a bactericidal effect. The antimicrobial activity of *M. communis* and *T. vulgaris* essential oils were reported in several studies [30,31]. It could be attributed to their chemical composition, which are rich in hydrocarbon and oxygenated monoterpenes, and mainly to their major compounds including thymol, linalool, carvacrol, γ-terpinene, p-cymene, α-pinene and 1.8-cineole. Indeed, phenolic compounds are known for their highest efficiency and broadest spectrum of antimicrobial activity [19,32,33].

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Query  1     TGACGGAGCACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGG  59
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Query  360   TAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCC  419
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Sbjct  691   TAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCC  750

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Query  480   CGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACG  539
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Sbjct  811   CGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACG  870

Query  540   GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACC  599
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Query  600   AGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCCCTTCGGGGACAGAGTGAC  659
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Query  660   AGGTGGTGCATGGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTTAAGTCCCGCAA-  718
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Query  719   CGAGCGCAACCCTTTGATCTTTAGTTTGCCAGCATTTCAGTTTGGG  764
             ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1049  CGAGCGCAACCCTTTGATCTTAAGTT-GCCAGCATT-CAGTTTGGG  1092
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Figure 2: Nucleotide sequence of *Bacillus safensis*

The prominent role of a phenolic group and the system of delocalized electrons in the chemical structure of thymol and carvacrol for their strong antibacterial activity has already been reported [34]. The same statements can be made from this study, which proved that the thyme essential oil predominated by thymol, has shown stronger antibacterial effect against *B. safensis* and *B. subtilis* than that of myrtle with 16 and 32-fold lower MIC values. Unlike phenolic terpenes, the hydrocarbon ones showed ineffective antimicrobial activity when used as singular compounds [19]. The α-pinene, limonene, γ-terpinene and p-cymene have been reported previously to show very low or no antimicrobial activity against 25 genera of bacteria [35]. Therefore, the weak antibacterial property of *M. communis* essential oil shown here (in this work) could be due to its high content of α-pinene and limonene (Table.1). Moreover, anther work has demonstrated that 1.8 cineole (major component of myrtle essential oil studied) has shown moderate antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* compared to linalool, terpinen-4-ol and α-terpineol [36]. However, it was more effective than p-cymene, γ-terpinene, α- terpinene and terpinolene.

Table 2: Antimicrobial susceptibility, MIC values of *M. communis & T. vulgaris* essential oils for *B. subtilis* and *B. safensis*

Concentration % (v/v)	MIC			
	B. subtilis		***B. safensis***	
	M. communis	*T. vulgaris*	*M. communis*	*T. vulgaris*
4	-	*	-	*
2	-	*	-	*
1	-	-	-	-
0.5	+	-	+	-
0.25	+	-	+	-
0.125	+	-	+	-
0.0625	+	-	+	-
0.03125	+	-	+	+
0.01562	+	+	+	+
0.00781	+	+	+	+
0.0039	+	+	+	+
0.00195	*	+	*	+
0.00097	*	+	*	+

+: presence of growth; -: absence of growth; *: not done; positive control: bacterial suspensions and Mueller-Hinton Broth supplemented with agar (0.15% w/v).

Table 3: Determination of MBC values of *M. communis and T. vulgaris* essential oils against *B. subtilis* and *B. safensis.*

Concentration % (v/v)	MBC			
	B. subtilis		***B. safensis***	
	M. communis	*T. vulgaris*	*M. communis*	*T. vulgaris*
4	-	*	-	*
2	-	*	-	*
1	-	-	-	-
0.5	*	-	*	-
0.25	*	-	*	-
0.125	*	-	*	-
0.0625	*	-	*	-
0.03125	*	+	*	*
0.01562	*	*	*	*
0.00781	*	*	*	*
0.0039	*	*	*	*
0.00195	*	*	*	*
0.00097	*	+	*	*

+: presence of growth; -: absence of growth; *: not done; positive control: bacterial suspensions and Mueller-Hinton Broth supplemented with agar (0.15 % w/v).

3.4. Fractional Inhibitory Concentrations and FIC index

The results of the antibacterial combined effect between essential oils of *M. communis* and *T. vulgaris* are presented in Table 4. The FIC index values for the combined application of *M. communis* and *T. vulgaris* essential oils ranged from 0.375 to 0.625. Also, as it can be noted in this table, only the combination (1/8 MIC of myrtle + 1/4 MIC of thyme) inhibited the growth of *B. subtilis* with a FIC index of 0.375, which was < 0.5, indicating a synergistic interaction. Moreover, three combinations of *M. communis* and *T. vulgaris* essential oils (1/2 MIC+ 1/16 MIC), (1/2 MIC + 1/8 MIC) and (1/8 MIC + 1/2 MIC) have displayed a partial synergistic effect against this strain with a FIC index of 0.562 and 0.625 respectively. Regarding *B. safensis*, the results demonstrated that all fours combinations generated by the checkerboard assay have displayed FIC index values ranged from 0.5 to 0.562, indicating the partial synergistic effect of the essential oils studied.

Table 4: Determination of FIC, FIC index and outcome of interactions of *M. communis* and *T. vulgaris* essential oils combinations against *B. subtilis* and *B. safensis* strains.

Bacterial strain	**Essential oil**	**MIC % (v/v)**		**FIC % (v/v)**	**FICI**	**Outcome**
		Alone	Combination			
B. subtilis	*M. communis*	1	0.5	0.5	0.562	Partial synergy
	T. vulgaris	0.03125	0.001954	0.0625		
	M. communis	1	0.5	0.5	0.625	Partial synergy
	T. vulgaris	0.03125	0.003907	0.125		
	M. communis	1	0.125	0.125	0.375	Synergy
	T. vulgaris	0.03125	0.0078125	0.25		
	M. communis	1	0.125	0.0625	0.562	Partial synergy
	T. vulgaris	0.03125	0.015625	0.5		
B. safensis	*M. communis*	1	0.5	0.5	0.562	Partial synergy
	T. vulgaris	0.0625	0.003907	0.0625		
	M. communis	1	0.5	0.5	0.625	Partial synergy
	T. vulgaris	0.0625	0.0078125	0.125		
	M. communis	1	0.25	0.25	0.5	Partial synergy
	T. vulgaris	0.0625	0.015625	0.25		
	M. communis	1	0.125	0.125	0.625	Partial synergy
	T. vulgaris	0.0625	0.03125	0.5		

The antibacterial effect of combination between *T. vulgaris* and other aromatic plants essential oils have been studied by many authors [37,38]. Moreover, the effect of interactions between their major components has also been studied [18]. But until now, there is no literature on the antibacterial effect of combination between *M. communis* and *T. vulgaris* essential oils. Indeed, the results of FIC index of interaction between these essential oils demonstrated that combination (1/8 MIC of myrtle + 1/4 MIC of thyme) exhibited an important synergistic antibacterial effect, which was higher than the application of either essential oil alone. This can be due to synergistic interaction between their main components. In fact, several works have reported the antibacterial activity of these bioactive molecules in different combinations [39]. The combination of *M. communis* essential oil, which was found weakly active (alone), with *T. vulgaris* essential oil, increased the antibacterial property against the studied wood bacteria. This result corroborated with previously published studies [18,40], which described the capacity of hydrocarbons to interact with cell membrane, thus facilitating the penetration of carvacrol into the cell. It also confirmed that carvacrol and 1.8 cineole showed a synergistic interaction towards many bacteria strains, which could be partially explained by their different structures and mechanisms of action [18].

Conclusion

The present study deals with the assessment of the antibacterial effect of *T. vulgaris* and *M. communis* essential oils alone and in the binary combinations against tow bacteria isolated from decayed cedar wood. The results showed that most combinations have displayed a partial synergistic effect against bacterial studied greater than that obtained with the applications of each essential oil alone. Moreover, only the combination (1/8 MIC of myrtle + 1/4 MIC of thyme) has exhibited a highly synergistic effect decreasing the MIC values 8-fold and 4-fold for *M. communis* and *T. vulgaris* essential oils when tested alone. This finding suggests that the mixture of these essential oils at suitably low concentrations could be a promising environmental alternative to replace synthetic and chemical antimicrobial agents, and that further research should be undertaken on natural products for the benefit of better protection and preservation of archaeological monuments and all wood objects.

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