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Original Article

SYNERGISTIC ANTIBACTERIAL EFFECT OF MYRTUS COMMUNIS AND THYMUS VULGARIS ESSENTIAL OILS FRACTIONAL INHIBITORY CONCENTRATION INDEX

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ABSTRACT

Objective: The present study attempts to investigate *in vitro* antibacterial activity of *M. communis* and *T. vulgaris* essential oils alone and in combination against two bacterial strains

Methods: The antibacterial activity of *M. communis* and *T. vulgaris* essential oils was assessed against *Staphylococcus aureus* and *Escherichia coli* by using the broth microdilution method then the fractional inhibitory concentration (FIC) index was used to define the interactions between both essential oils.

Results: Our results show that *T. vulgaris* was more effective compared to *M. communis* when both essential oils are tested alone against bacterial strains studied. Moreover, all applications of combination between M. communis and *T. vulgaris* essential oils displayed a partial synergistic antibacterial effect. Only, one combination against *S. aureus* and two combinations against *E. coli* were proved highly synergistic effect, increasing the antibacterial activity.

Conclusion: The antibacterial activity of both essential oils was enhanced by the combination (1/8 MIC Myrtle +1/4 MIC thyme) and two combinations (1/4 MIC Myrtle + 1/8 MIC thyme and 1/8 MIC myrtle + ¼ MIC thyme) which proved a highly synergistic effect against *S. aureus* ATCC 25922 and *E. coli* O128B12 respectively. These findings suggest 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 to enhancing its antibacterial properties.

Keywords: *M. communis, T. vulgaris*, Essential oils, Antibacterial activity, Combination, *staphylococcus aureus, Escherichia coli.*

INTRODUCTION

The lives of millions of people have saved by antibiotics favor; these later have contributed the major gains in life expectancy during the last century. However, the emergence of multi-drug resistant pathogens [1] has become a major public health concern. Indeed, the situation is particularly concerning in hospitals and food due to the colonization of the implant surface and infections of prosthetic devices [2]. Also the infectious diseases caused by resistant microorganisms are associated with prolonged hospitalizations, increased cost, and greater risk for morbidity and mortality.

Moreover, the resistance of many bacteria to antimicrobial agents, detergents, and disinfectant agents was a global problem major defy in different areas, such as food [3,4], the paper industry [5], arts [6] and environments [7–10]. Therefore, the need to develop alternative antimicrobial agents and antibiotics for combating the problem of microorganism resistance. Whence, recently the growing interest has focused on naturally occurring molecules, in particular plant oils and cruds extract which have been used for a wide variety of purposes for many years. Essential oils and some their components are known by their antimicrobial properties [11–13]. They are also used as fragrances and flavoring agents in foods and beverages [14], and for antioxidant activity [15,16]. In contrast, their combined antibacterial effects have not been reported extensively. Thus, we purpose in this study to investigate the effect of single and combined antibacterial effects of *Thymus vulgaris* and *Myrtus communis* essential oils against *Staphylococcus aureus* ATCC 25922 and *Escherichia coli* O128B12.

MATERIAL AND METHODS

Plant Material

Thymus vulgaris L. *(Labiateae*) and *Myrtus communis* L. (*Myrtaceae*) were freshly harvested and collected on October 2012 in the garden of the National Institute of Medicinal and Aromatic Plants. The plants were identified and deposited in the herbarium of the (NIMAP) Taounate-Morocco.

Essential Oils Extraction

Extraction of essential oils from the aerial part (leaves and stems) of plants was performed by a hydro-distillation method using Clevenger-type apparatus. The essential oils obtained were kept in dark at 4°C until further process.

Test Organisms

The essential oils of *M. communis* L. and *T. vulgaris* were tested for their synergistic antibacterial activity against two reference bacterial strains: *Staphylococcus aureus* ATCC 25922 (Gram-positive) and *Escherichia coli* O128B12 (Gram-negative). The strains were cultured on nutrient agar and incubated at 37 °C for 24 h and were then maintained in their appropriate agar medium at 4 °C throughout the study and used as stock cultures.

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

The MICs were determined using the broth microdilution assay as previously described [17] 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 (Oxoid, UK) supplemented with bacteriological agar (0.15% w/v) were distributed from the second to the 12th well of a 96-well polypropylene Microtiter plate. Essential oil dilution was prepared in Mueller Hinton Broth supplemented with bacteriological agar (0.15% w/v), 100 µl of these suspensions were added to the first test well of each Microtiter 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 was between 4 and 0.0039% (v/v) for myrtle and between 1 and 0.00097%. (v/v) for thyme. Plates were incubated at 37°C for 20 h. After incubation, 5 µl of resazurin were added to each well to assess bacterial growth as indicated by [17]. 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 minimum 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 plates. Experiments were also conducted in triplicate.

Determination of Fractional Inhibitory Concentration (FIC)

The effects of interactions between *M. communis* and *T. vulgaris* essential oils against bacterial strains were evaluated using the checkerboard technique [18]. The concentrations of both essential oils were prepared in Mueller Hinton Broth supplemented with bacteriological 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. In other word from 8 × MIC to 1/128 × MIC concentration. 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 –A well was considered as growth control.

Inoculum size 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 hours. 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 ∑ FICI values are interpreted as follows: ≤ 0.5= synergistic; 0.5-0.75 = partial synergy; 0.76-1.0 = additive; > 1.0-4.0 = indifferent (non-interactive); > 4.0 = antagonistic.

RESULTS

Minimal Inhibitory and Bactericidal Concentrations

The results of two essential oils which were evaluated for their antibacterial activity against *S. aureus* ATCC 25922 and *E. coli* O128B12 are shown in table 1. As can be noted in this finding, all essential oils tested were shown an important antibacterial effect. Indeed, the MIC values of *M. communis* essential oil ranged from 1 to 0.5 % (v/v) and for *T. vulgaris* essential oil are 0.03125 % (v/v) against strains studied. Hence, *T. vulgaris* essential oil exhibit a higher antibacterial effect with MIC values 16 fold and 32-fold least compared to *M. communis* essential oil against *E. coli* and *S. aureus* respectively. Also, it noted that *S. aureus* (Gram- positive) was more resistant to the *M. communis* essential oil with MIC value of 1% (v/v) than *E. coli* (Gram-negative). However, both strains were inhibited by the same MIC value of *T. vulgaris* essential oil (0.03125 %).

Table 1: Determination of MIC values of *M. communis and T. vulgaris* essential oils against *Staphylococcus aureus* ATCC 25922 and *Escherichia coli* O128B12

Concentration % (v/v)	*Staphylococcus* aureus ATCC 25922		*Escherichia coli* O128B12	
	T. vulgaris	*M. communis*	*T. vulgaris*	*M. communis*
4	*	*	*	-
2	*	-	*	-
1	*	-	*	-
0.5	*	+	*	-
0.25	*	+	*	+
0.125	-	+	-	+
0.0625	-	+	-	+
0.03125	-	+	-	+
0.01562	+	+	+	+
0.00781	+	+	+	+
0.0039	+	+	+	+
0.00195	+	+	+	+

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

Regarding the MBC values of both essential oils tested (Table 2). We found that MBC values could well be similar to their MIC values against *E. coli* and 2 fold higher toward *S. aureus*. In fact, the MBC values of *T. vulgaris* and *M. communis* essential oils were 0.0625 %; 2 % and 0.03125 %; 0.5 % (v/v) against *S. aureus* and *E. coli* respectively. It can therefore be interpreted that they act by a bactericidal action.

Fractional inhibitory concentrations and FIC index

The results for interaction effect between essential oils of *M. communis* and *T. vulgaris* toward bacterial strains studied are presented in table 3.

FIC index values for the combined application of *M. communis* and *T. vulgaris* essential oils ranged from 0.375 to 0.625. Also, as can be noted in this table, the combination (1/8 MIC +1/4 MIC of *M. communis* and *T. vulgaris* essential oils respectively) was displayed a synergistic effect against *S. aureus* with a FIC index of 0.375. Moreover, three combinations were exhibited an inhibitory effect on the growth of *S. aureus* with a FIC index values of 0.562 and 0.625 which were > 0.5, indicating a partial synergistic interaction.

Regarding to the effect of combined application of essential oils studied against *E. coli*, the results demonstrated that two combinations (1/4 MIC Myrtle + 1/8 MIC thyme and 1/8 MIC myrtle + ¼ MIC thyme) were given a synergistic antibacterial effect toward E. coli with a FIC index value of 0.375. While, the combination (1/64 MIC myrtle + ½ MIC thyme) was shown a partial synergistic effect with a FIC index value of 0.516. In summary, all combined applications done between *M. communis* and *T. vulgaris* essential oils showed an antibacterial activity with synergistic and partial synergistic outcomes against two bacterial models (Gram-positive and Gram-negative) and any antagonistic action was founded.

Table 2: Determination of MBC values of *M. communis and T. vulgaris* essential oils against *Staphylococcus aureus* ATCC 25922 and *Escherichia coli* O128B12

Concentration % (v/v)	*Staphylococcus aureus* ATCC 25922		*Escherichia coli* O128B12	
	T. vulgaris	*M. communis*	*T. vulgaris*	*M. communis*
4	*	*	*	-
2	*	-	*	-
1	*	+	*	-
0.5	*	+	*	-
0.25	*	+	*	+
0.125	-	+	-	*
0.0625	-	+	-	*
0.03125	+	*	-	*
0.01562	+	*	+	*
0.00781	+	*	*	*
0.0039	*	*	*	*
0.00195	*	*	*	*

+: 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 FIC, FIC index and outcome of interactions of *M. communis* and *T. vulgaris* essential oils combination against *S. aureus* ATCC 25922 and *E. coli* O128B12

Bacterial strain	Essential oil	MIC % (v/v)		FIC	FICI	Outcome
		Alone	Combination			
S. aureus ATCC 25922	*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	0.007813	0.250		
	M. communis	1	0.0625	0.0625	0.562	Partial synergy
	T. vulgaris	0.03125	0.015625	0.5		
E. coli O128B12	*M. communis*	0.5	0.125	0.25	0.375	Synergy
	T. vulgaris	0.03125	0.003907	0.125		
	M. communis	0.5	0.0625	0.125	0.375	Synergy
	T. vulgaris	0.03125	0.007813	0.25		
	M. communis	0.5	0.007813	0.0156	0.515	Partial synergy
	T. vulgaris	0.03125	0.015625	0.5		

DISCUSSION

For several years, essential oils of *M. communis* and *T. vulgaris* are known for their antimicrobial activity which reported in several studies [19,20]. These antibacterial properties could be mainly attributed to its chemical composition, which are rich with monoterpenes hydrocarbon and oxygenated monoterpenes, and to their major compounds, including phenolic and alcoholic terpenes (thymol; linalool and carvacrol) and γ-terpinene p-cymene, α-pinene and 1.8-cineole (data not shown). In fact, the broader spectrum activity exhibited by phenolic compounds was investigated by many authors [13,21]. Furthermore, the antibacterial effect of combination application between *T. vulgaris* and other aromatic plants essential oils and extracts have been studied by many authors [22–24]. And the interactions of their major components [25,26]. Nevertheless, up to now, there are no literature data on the antibacterial effect of combined application between *M. communis* and *T. vulgaris* essential oils**.**

In this paper, the essential oils of these two plants domesticated and cultivated in the National Institute of Aromatic and Medicinal Plants (Morocco) have shown an important antibacterial activity against *S. aureus* ATCC 25922 and *E. coli* O128B12 which were founded more susceptible to *T. vulgaris* essential oil with a MIC value 32-fold less than that of *M. communis.* These results could be explained by the chemical composition of *T. vulgaris* essential oil, which is known by its high contents with thymol, γ-terpinene, p-cymene, linalool and carvacrol, these latter are known for their stronger antibacterial activity [27–29].

Regarding to the FIC index of interaction between these essential oils. The combination (1/8 MIC myrtle +1/4 MIC thyme) and two combinations (1/4 MIC Myrtle + 1/8 MIC thyme and 1/8 MIC myrtle + ¼ MIC thyme) were given an important synergistic antibacterial effect which is higher than application of either essential oil alone toward *S. aureus* and *E. coli* respectively. This can be outstanding to synergistic interaction between their main components. Our results are in agreement with several works which have reported the antibacterial activity of these bioactive molecules in different combinations [26,28].

The weak antibacterial activity of *M. communis* essential oil founded in this study could be due to its major components (1,8 Cineole, α-pinene, limonene, myrtyl acetate, α -terpineol and γ terpinene), which are hydrocarbons and oxygenated monoterpenes known for their relatively weak antibacterial effect [21]. Though, its combination with *T. vulgaris* essential oil (thymol, carvacrol) increases its antibacterial property against bacterial strains studied. This result corroborated with previously published data [25,30,31], which described the capacity of hydrocarbons to facilitate the penetration of carvacrol into the cell by interacting with the cell membrane. It also confirmed that carvacrol and 1,8 cineole were showed a synergistic interaction toward many bacteria strains. This increased antimicrobial activity caused by its combined application could be partially explained by their different structure and mechanisms of action [25].

In conclusion, the results presented in this study showed that all applications of combination between *M. communis* and *T. vulgaris* essential oils were displayed a partial synergistic effect toward bacterial studied. However, only the combination (1/8 MIC Myrtle +1/4 MIC thyme) and two combinations (1/4 MIC Myrtle + 1/8 MIC thyme and 1/8 MIC myrtle + ¼ MIC thyme) were proved highly synergistic effect against *S. aureus* ATCC 25922 and *E. coli* O128B12 increasing the antibacterial activity. This finding reinforced 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 on natural products to exploit its antibacterial properties in medical and food areas and it can register as an ecological solution at lower cost.

CONFLICT OF INTEREST STATEMENT

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript

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