Determination of the minimum inhibitory concentrations of the antibacterial oregano and thyme essential oils in the presence of polysorbate 80

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Introduction

Plant-derived essential oils (EOs) have attracted the interest of the scientific community in recent years regarding their safety, as natural ingredients, as well as their application in food products (Alizadeh-Behbahani and Tabatabaei-Yazdi 2015; Fournomiti et al., 2015). Essential oils have shown remarkable antimicrobial potential against spoilage and pathogenic microorganisms. However, different antimicrobial activities of similar EOs may be observed when different methods are used (Bajalan et al., 2016). This study aims to evaluate and demonstrate the influence of polysorbate 80 (PS80) on the antimicrobial activity and minimum inhibitory concentrations (MICs) of oregano and thyme EOs against forty microorganisms.

Methods

1. Disk Diffusion Assay (DDA)

Scale 0.5 McFarland of forty pathogens

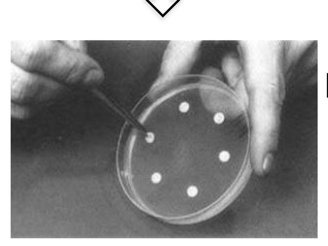
Bacillus cereus	*Acinetobacter baumannii* R
Bacillus subtilis	*Acinetobacter baumannii* S-1
Bacillus stearothermophilus	*Acinetobacter baumannii* S-2
Listeria monocytogenes SCOTT A	*Acinetobacter calcoaceticus* R
Listeria innocua 2030c	*Acinetobacter calcoaceticus* S
Staphylococcus aureus 18N (MRSA)	*Klebsiella pneumoniae*
Staphylococcus aureus 2037 M1 (MSSA)	*Proteus mirabilis*
Clostridium sporogenes 1.31	*Proteus vulgaris*
Clostridium sporogenes 1.34	*Pseudomonas aeruginosa*
Clostridium sporogenes 1.61	*Salmonella* Braenderup
Clostridium perfringens 1.16	*Salmonella* Enteritidis
Clostridium perfringens 1.19	*Salmonella* Enteritidis 417536
Enterococcus faecalis ATCC 29212	*Salmonella* Enteritidis 545047
Staphylococcus aureus ATCC 29213	*Salmonella* Typhimurium
Enterococcus faecalis DSMZ 12956	*Yersinia enterocolitica*
Enterococcus faecium DSMZ 13590	*Escherichia coli* ATCC 25922
Enterococcus flavescens DSMZ 7370	*Yersinia enterocolitica* NCTC 10406
Enterococcus casseliflavus DSMZ 20680	*Candida albicans*
Enterococcus gallinarum DSMZ 20628	*Saccharomyces cerevisiae*
Listeria monocytogenes L7946	
Listeria monocytogenes L7947	

Spread plate

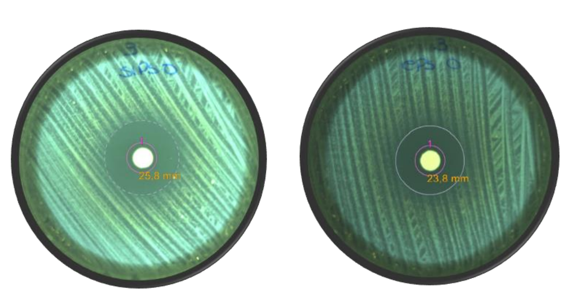
- Emulsified EOs were prepared with and without PS80 food grade solution
- Pre-heating at 50 ºC was realized in a 50 mL Falcon tube containing only Mueller-Hinton broth (MHb) and in another other one with MHb and PS80 (10% v/v) during 10 minutes at 500 rpm;
- Filter paper discs (6 mm) were impregnated in EOs solutions for 15 minutes;
- The discs remained at room temperature until complete evaporation for 30 minutes.

Essential oils tested:
Oregano (*Origanum vulgare* Leaf)
Thyme (*Thymus vulgare* Leaf)

Discs loaded were placed onto the surface of the inoculated agar.



Incubation for 18-24h at 37 ºC (Yeasts were incubated 48 h at 25 ºC and *Clostridium* were incubated 48 h at 37 ºC in anaerobiosis)

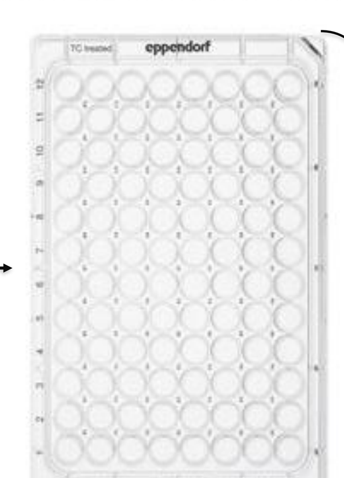


Essential oil without PS80   Essential oil with PS80

Only EOs that produced inhibition halos > 10 mm were selected for determination of Minimum Inhibition Concentrations

2. Minimum Inhibition Concentration (MIC)

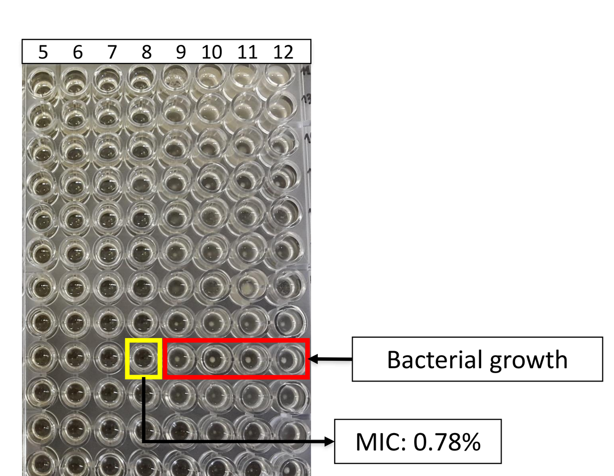
Dilution 1:2 in Mueller-Hinton of each EO + Dilution of each selected pathogen



Final concentration in each well:

EOs:
2- 50.00%   9- 0.39%
3- 25.00%   10- 0.20%
4- 12.500%   11- 0.10%
5- 6.25%   12- 0.05%
6- 3.13%
7- 1.56%
8- 0.78%

Pathogens: 10^6 UFC/ml



Incubation during 18-24 h at 37 °C (Yeasts were incubated 48 h at 25 °C and *Clostridium* were incubated 48 h at 37 ºC in anaerobiosis).

3. Statistical analysis

The comparison of the antimicrobial activity of EOs against each microorganism was carried out by one-way analysis of variance (ANOVA). The Tukey-Kramer test was used to determine the significant differences ($p < 0.05$) among group means.

Results

Table 1. Zones of growth Inhibition (mm; mean ± standard deviation) showing antimicrobial activity (including the disk diameter of 6.0 mm) and Minimum Inhibitory Concentration (MIC) (results are expressed in % of EO) of oregano and thyme EOs with and without polysorbate against different microorganisms

	DDA with PS80		DDA without PS80		MIC with PS80		MIC without PS80	
	Oregano	Thyme	Oregano	Thyme	Oregano	Thyme	Oregano	Thyme
Bacillus cereus	37.7±0.5	28.4±2.8	39.7±3.7	36.1±2.2	12.50	3.13	0.05	0.20
B. stearothermophilus	34.2±0.5	26.6±1.5	35.1±2.9	41.0±4.9	12.50	6.25	0.39	0.39
B. subtilis	22.9±0.4	18.6±0.5	24.4±2.3	23.2±1.1	12.50	6.25	0.05	0.10
Clostridium sporogenes 1.31	20.9±0.1	25.7±0.7	39.7±1.1	13.5±0.9	6.25	6.25	0.05	0.20
Clostridium sporogenes 1.34	17.3±2.6	19.0±0.8	37.4±0.0	18.8±1.7	6.25	12.50	0.05	0.20
Clostridium sporogenes 1.61	25.8±0.1	28.1±0.4	11.1±0.4	14.1±1.8	3.13	6.25	0.05	0.20
Clostridium perfringens 1.16	20.7±3.9	20.9±2.1	12.1±1.0	22.0±1.0	3.13	3.13	0.05	0.10
Clostridium perfringens 1.19	18.9±1.6	20.4±1.1	37.4±0.0	16.1±2.1	3.13	6.25	0.05	0.10
E. faecalis ATCC 29212	21.7±5.2	10.8±0.2	29.6±3.5	28.9±1.1	12.50	12.50	0.05	0.20
E. faecalis DSMZ 12956	23.1±2.1	10.1±0.5	36.6±2.3	16.8±2.0	12.50	6.25	0.05	0.20
E. faecium DSMZ 13590	36.3±0.5	16.7±3.2	34.5±2.3	28.3±4.4	6.25	6.25	0.05	0.20
E. flavescens DSMZ 7370	40.2±3.6	37.4±1.1	37.2±0.8	30.1±0.4	6.25	6.25	0.10	0.05
E. gallinarum DSMZ 20628	30.4±0.0	26.3±0.4	29.6±2.4	28.7±3.2	6.25	6.25	0.05	0.20
E. casseliflavus DSMZ 20680	27.9±1.1	17.3±2.1	28.0±2.5	26.8±1.1	6.25	6.25	0.10	0.10
L. monocytogenes 7946	31.1±0.2	31.1±0.8	38.4±2.5	36.0±1.4	0.39	3.13	0.05	0.05
L. monocytogenes 7947	31.5±3.3	32.1±2.4	35.9±2.4	33.7±5.9	0.39	0.78	0.05	0.05
L. monocytogenes SCOTT A	14.0±2.4	11.4±1.7	14.8±0.8	13.0±2.8	3.13	6.25	0.05	0.10
Listeria innocua 2030c	34.4±0.8	29.4±2.5	39.5±3.3	32.1±1.7	3.13	6.25	0.05	0.10
St. aureus ATCC	30.8±1.1	28.4±3.6	28.8±1.3	29.9±2.5	3.13	6.25	0.05	0.05
St. aureus 18N (MRSA)	35.1±3.2	30.5±0.4	30.0±1.6	34.7±3.0	6.25	6.25	0.05	0.10
St. aureus 2037 M1 (MSSA)	30.2±0.5	29.3±2.6	31.0±0.1	30.0±1.6	0.78	3.13	0.05	0.10
A. baumannii	36.5±0.1	30.1±3.5	37.4±4.0	32.6±3.7	1.56	1.56	0.05	0.05
A. baumannii	36.8±0.0	28.1±2.7	37.7±3.5	38.3±3.3	1.56	1.56	0.05	0.05
A. baumannii	35.8±0.8	30.1±0.6	33.5±0.0	32.4±0.0	0.78	1.56	0.05	0.05
A. calcoaceticus	29.8±1.8	26.0±1.3	29.4±1.5	30.4±1.2	0.78	1.56	0.05	0.05
A. calcoaceticus	32.5±1.8	30.1±0.8	33.0±1.8	31.4±1.4	1.56	1.56	0.05	0.05
Escherichia coli ATCC	33.0±1.9	24.7±0.5	33.9±1.8	31.9±2.7	3.13	3.13	0.10	0.20
Klebsiella pneumoniae	16.6±2.8	16.8±3.3	24.7±2.1	20.4±3.5	6.25	3.13	0.10	0.20
Proteus mirabilis	32.4±2.7	28.6±0.4	30.1±2.8	36.7±1.8	1.56	3.13	0.05	0.05
Proteus vulgaris	23.6±1.0	16.0±1.8	24.9±2.1	22.6±2.8	3.13	12.50	0.10	0.20
Pseudomonas aeruginosa	22.2±0.7	17.8±1.6	21.1±2.1	18.5±0.2	6.25	12.50	0.05	0.05
S. Braenderup	23.3±1.8	16.8±0.4	25.1±1.6	22.1±2.5	3.13	12.50	0.10	0.10
S. Enteritidis	23.4±0.0	18.8±2.1	23.3±0.5	20.9±1.5	3.13	12.50	0.10	0.10
S. Enteritidis 417536	17.0±0.7	16.1±1.3	19.6±1.7	20.5±2.5	1.56	3.13	0.05	0.05
S. Enteritidis 545047	16.3±3.0	18.4±3.4	19.8±0.3	20.0±0.2	3.13	6.25	0.05	0.05
S. Tiphymurium	30.7±1.3	24.6±0.4	33.3±3.0	29.9±1.8	1.56	3.13	0.10	0.10
Yersinia enterocolitica	36.1±3.2	31.2±1.3	40.4±4.0	47.8±3.3	1.56	3.13	0.05	0.10
Yersinia enterocolitica NCTC10406	22.1±1.1	18.6±1.5	23.3±2.5	21.5±1.0	1.56	3.13	0.05	0.10
Candida albicans	44.5±0.4	35.7±0.4	55.3±2.6	50.7±2.8	3.13	0.78	0.05	0.10
Saccharomyces cerevisiae	44.5±4.5	26.7±4.4	50.0±1.8	37.5±0.1	0.78	1.56	0.05	0.05

- For the Disk Diffusion Assay, comparing both techniques, it was observed that in most microorganisms no differences were seen in the size of the halo;
- However, some differences were observed for MIC determination: MICs of each EO were different in the presence and absence of PS80, for all microorganisms.

Conclusions

The PS80 was used to facilitate the homogenization of EOs, but this may turn the antimicrobial compounds in EOs less available, with no direct contact with the target organisms limiting the microbial reduction capacity. This study reinforces the need for using defined standard methods for the *in vitro* determination of MICs.

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