Supplementary data

The novel quantitative assay for measuring the antibiofilm activity of volatile compounds (AntiBioVol)

Brożyna Malwina[1], Żywicka Anna[2], Fijałkowski Karol[2], Damian Gorczyca[3], Oleksy-Wawrzyniak Monika[1], Dydak Karolina[1], Migdał Paweł[4,5], Dudek Bartlomiej[6,7], Bartoszewicz Marzenna[1], Junka Adam[1,7*]

1 Department of Pharmaceutical Microbiology and Parasitology, Faculty of Pharmacy Wrocław Medical University, 50-556 Wrocław, Poland; adam.junka@umed.wroc.pl; malwina.brozyna@umed.wroc.pl;monika.oleksy@umed.wroc.pl karolina.dydak@umed.wroc.pl; marzenna.bartoszewicz@umed.wroc.pl

2 Department of Microbiology and Biotechnology, Faculty of Biotechnology and Animal Husbandry, West Pomeranian University of Technology, 70-311 Szczecin, Poland; anna.zywicka@zut.edu.pl; karol.fijalkowski@zut.edu.pl

3 Faculty of Medicine, Lazarski University, 02-662 Warszawa, Poland; damian.gorczyca@lazarski.pl

4 Bioimaging Laboratory, Łukasiewicz Research Network – PORT Polish Center for Technology Development, 54-066 Wrocław, Poland; pawel.migdal@port.org.pl

5 Department of Environment, Hygiene and Animal Welfare, Faculty of Biology and Animal Science, Wrocław University of Environmental and Life Sciences, 51-630 Wrocław, Poland; pawel.migdal@upwr.edu.pl

6 Laboratory of Microbiology, Institute of Genetics and Microbiology, University of Wrocław, 51-148 Wrocław, Poland; bartlomiej.dudek@uwr.edu.pl

7 Laboratory of Microbiology, Łukasiewicz Research Network – PORT Polish Center for Technology Development, 54-066 Wrocław, Poland adam.junka@port.org.pl, bartlomiej.dudek@port.org.pl

To whom correspondence should be addressed: Adam Junka, Medical University of Wrocław, Faculty of Pharmacy with Medical Analytics Division, Department of Pharmaceutical Microbiology and Parasitology, Borowska 211, 50-556 Wrocław, Poland. feliks.junka@gmail.com, tel no: +48 71 784 06 75

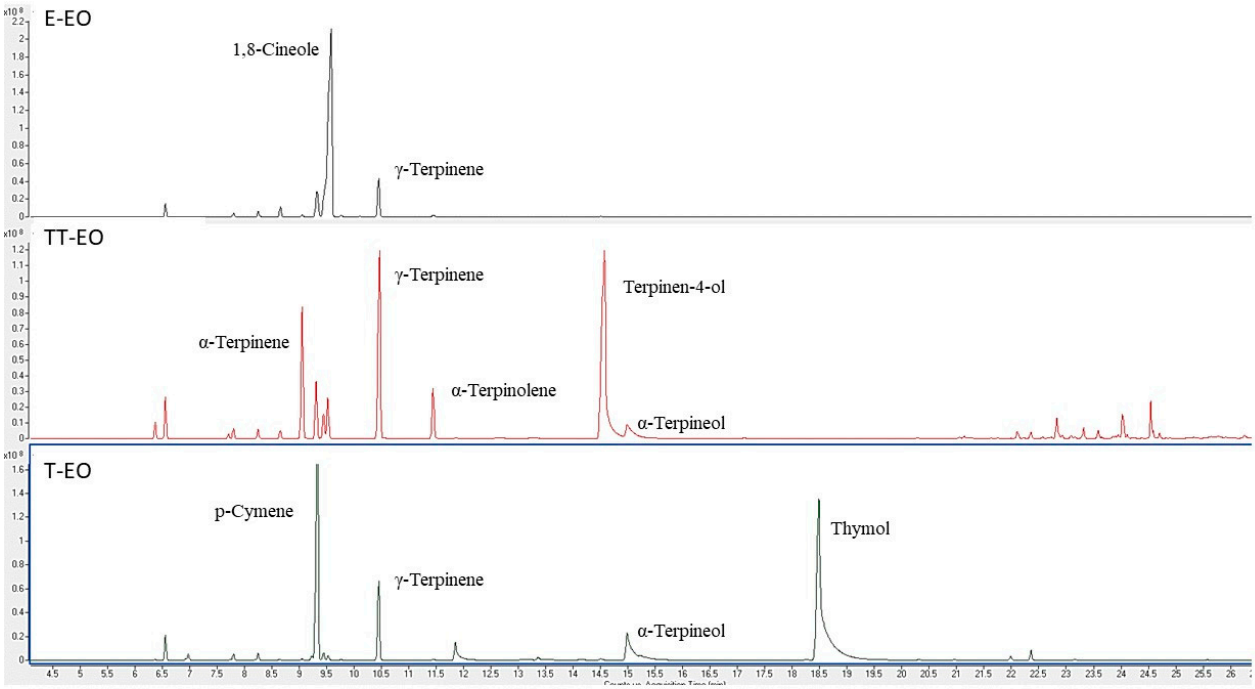


Fig.S1. GC-MS chromatograms of main EO ingredients. **E-EO** – eucalyptus oil; **TT-EO** – tea tree oil; **T-EO** – thyme oil. Results are presented as percentage content based on the peak area normalization.

RI	RT	Compound	E-EO	TT-EO	T-EO
929	6.37	α-Thujene		1.11 ± 0.02	
937	6.56	α-Pinene	2.30 ± 0.04	2.85 ± 0.06	2.20 ± 0.09
949	6.98	Camphene			0.73 ± 0.04
975	7.80	Sabinene		0.75 ± 0.02	0.64 ± 0.03
979	8.25	β-Pinene	1.09 ± 0.02	0.69 ± 0.01	0.81 ± 0.05
1005	8.65	α-Phellandrene	2.02 ± 0.02	0.61 ± 0.01	
1017	9.06	α-Terpinene		11.07 ± 0.17	
1025	9.31	p-Cymene	6.89 ± 0.07	4.69 ± 0.07	26.91 ± 0.99
1028	9.45	Limonene		2.08 ± 0.05	0.77 ± 0.04
1031	9.52	1,8-Cineole	79.10 ± 0.61	3.34 ± 0.06	
1060	10.47	γ-Terpinene	8.16 ± 0.07	19.07 ± 0.27	8.60 ± 0.03
1088	11.44	α-Terpinolene		4.34 ± 0.06	
1096	11.85	Linalool			3.45 ± 0.15
1141	13.36	Camphor			0.66 ± 0.06
1177	14.58	Terpinen-4-ol		33.27 ± 0.79	
1189	14.99	α-Terpineol		3.26 ± 0.13	7.84 ± 0.30
1289	18.49	Thymol			44.00 ± 0.46
1419	22.36	β-Caryophyllene		0.53 ± 0.01	1.00 ± 0.05
1440	22.83	Aromadendrene		1.83 ± 0.03	
1460	23.32	Alloaromadendrene		0.82 ± 0.02	
1496	24.03	Viridiflorene		2.35 ± 0.04	
1518	24.55	β-Cadinene		2.78 ± 0.03	

Tab.S1. Composition of main ingredients of tested EOs. **E-EO** – eucalyptus essential oil; **TT-EO** – tea tree essential oil; **T-EO** – thyme essential oil; **RI** - retention index; **RT** – retention time

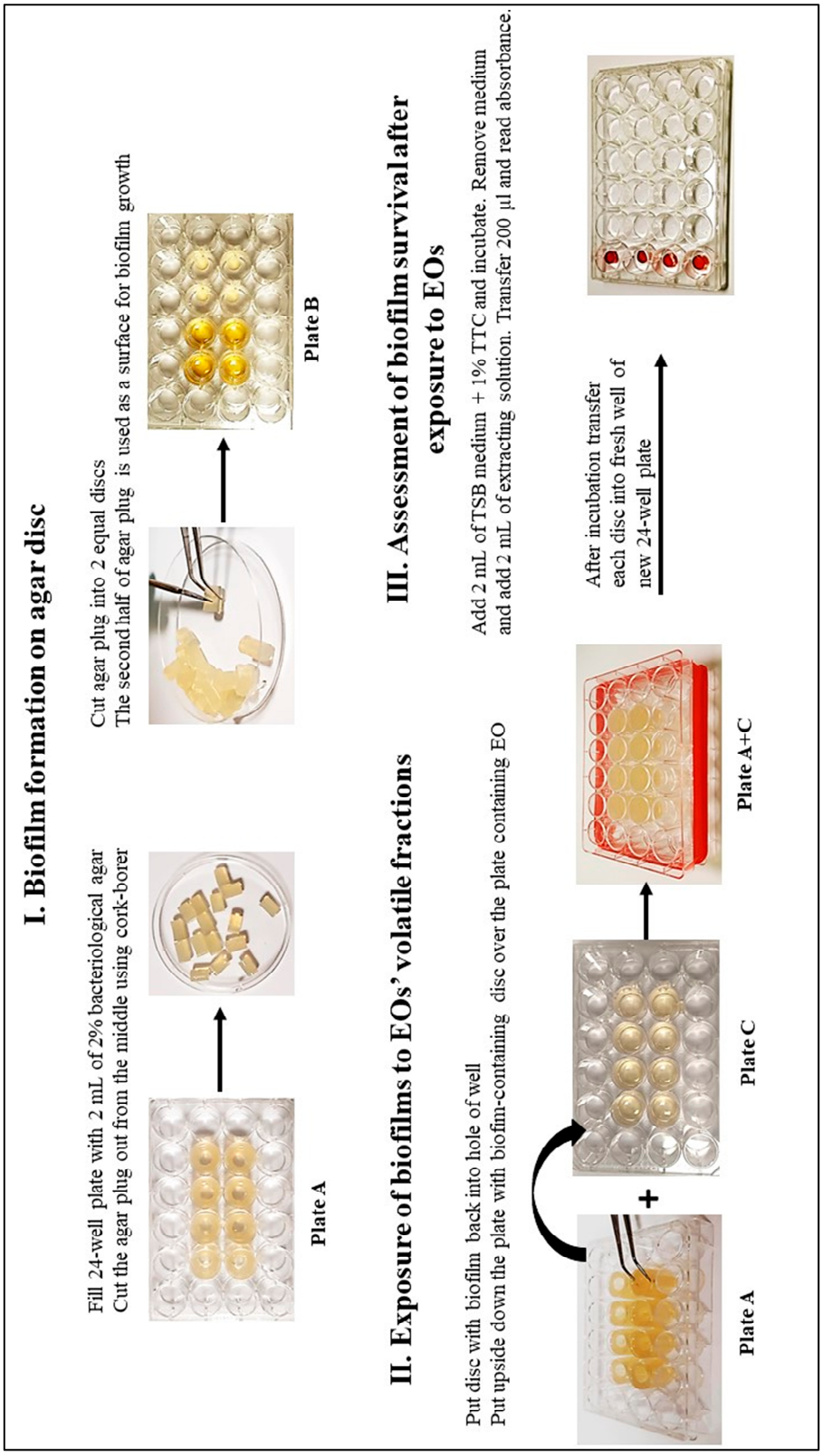
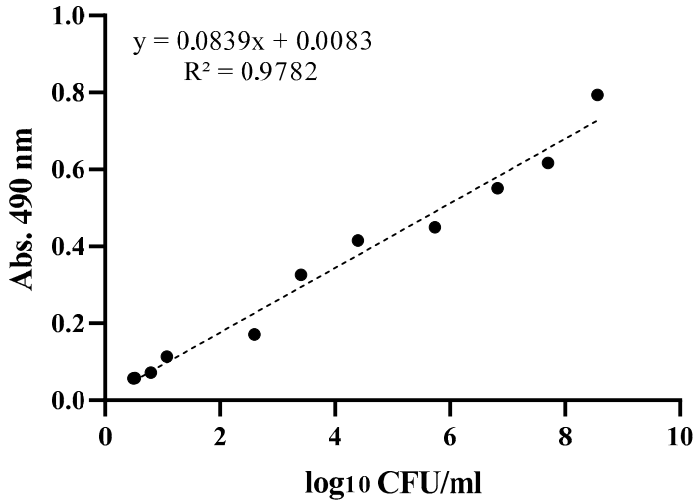


Fig. S2. Photographic presentation of AntiBioVol test performance.

A



B

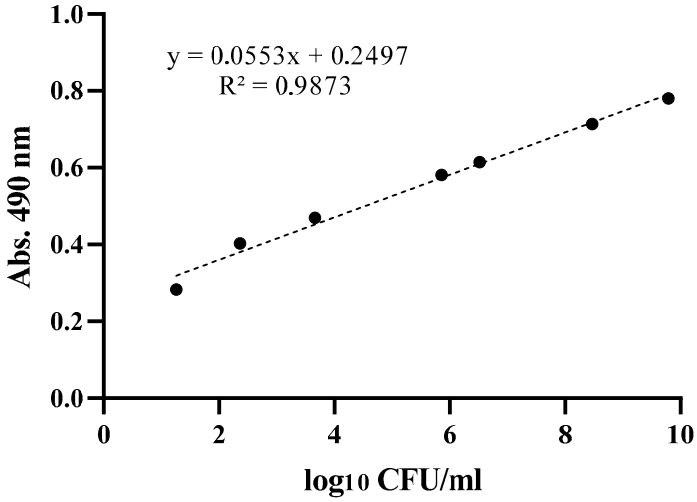


Fig. S3. Calibration curves for absorbance measurements at 490 nm (TTC assay) versus number of log10 colony forming units (CFU) / mL **[A]** *S. aureus*, **[B]** *P. aeruginosa*.

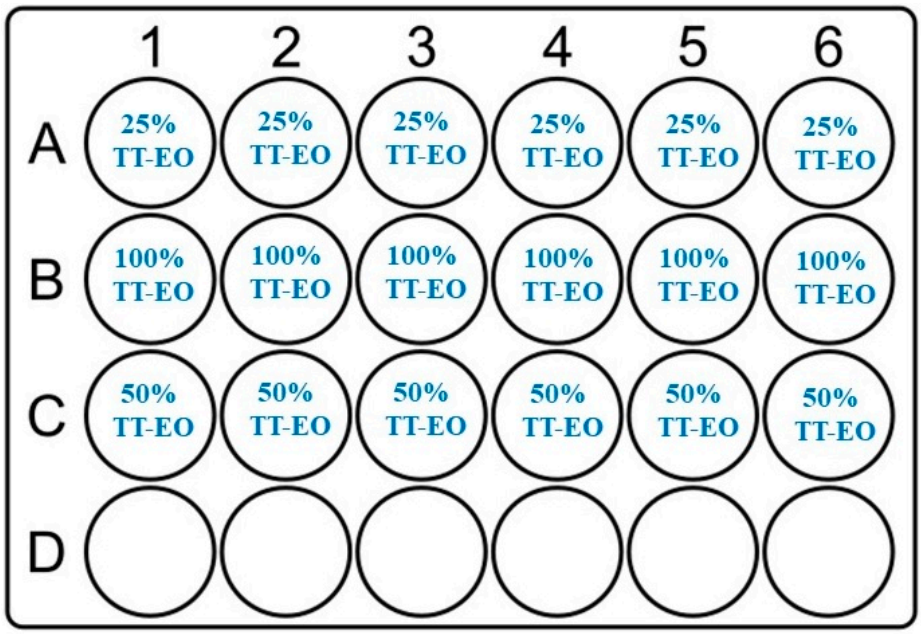


Fig. S4. Distribution of 3 TT-EO's concentrations in a single test plate. **TT-EO** – tea tree oil; 25%, 50%, 100%: applied concentrations [v/v] of TT-EO.

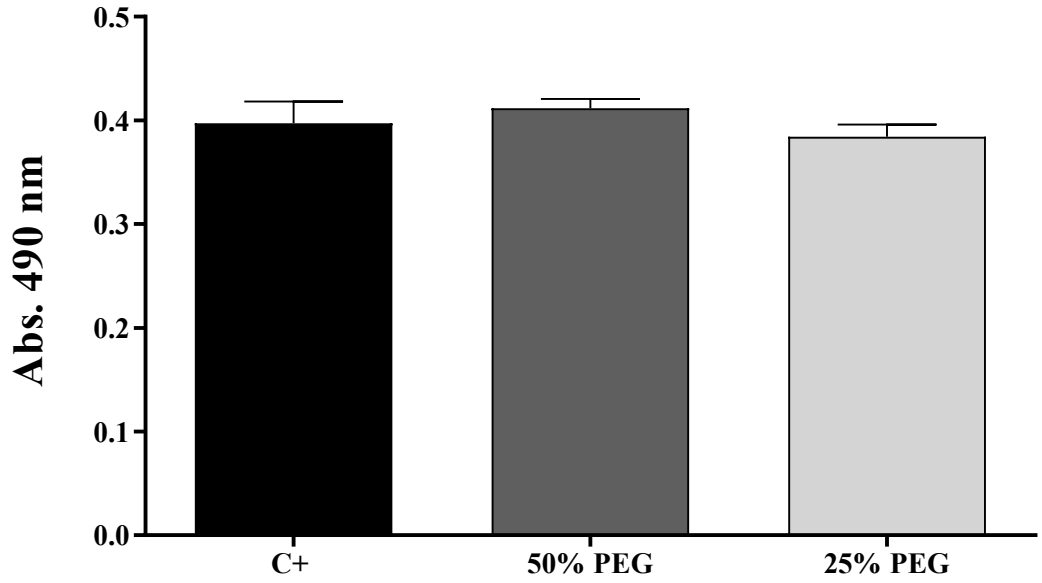


Fig. S5. Lack of PEG antimicrobial activity against *S. aureus* in the AntiBioVol experimental setting. C+ – positive control; 50%, 25% PEG – concentrations (v/v) of polyethylene glycol. No significant difference ($p<0.05$, K-W test, followed by Tukey's analysis) between control and PEGs was detected with relation to *S. aureus* viability.

Tab. S2. Minimal Inhibitory Concentration [**MIC**] and Minimum Biofilm Eradication Concentration [**MBEC**] of eucalyptus, thyme, tea-tree EOs [**E-EO**, **T-EO**, **TT-EO**, respectively] and ethanol [**EtOH**].

Microorganisms	E-EO		T-EO		TT-EO		EtOH
	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC
S. aureus	**12.5**	**n-m**	**0.02**	**0.19**	**6.25**	**12.5**	**12.5**
C. albicans	**1.5**	**n-m**	**0.02**	**0.38**	**0.78**	**n-m**	**3.12**
P. aeruginosa	**25**	**n-m**	**50**	**n-m**	**25**	**50**	**12.5**

"n-m" abbreviation stands for "non-measurable" and it is used when **no** MIC or MBEC values were observed when the highest possible concentration of specific EO was applied. The numbers given in the **Tab. S1** are expressed as the percentage volume of EO within total volume consisting of medium and EO in plate's well.

Description of the results presented in **Tab. S1:** All liquid EOs acted stronger against the planktonic forms of the tested microorganisms than against their biofilmic counterparts. Moreover, liquid EOs acted stronger against thick-walled cells of *S. aureus* and *C. albicans* than against thin-walled *P. aeruginosa*. Liquid E-EO was inactive (within tested range of concentrations) against biofilm of all analyzed microorganisms; liquid TT-EO displayed measurable activity against *S. aureus* and *P. aeruginosa* but not against *C. albicans* biofilm, while T-EO was active against *S. aureus* and *C. albicans* but not against *P. aeruginosa* biofilm.

A **B**

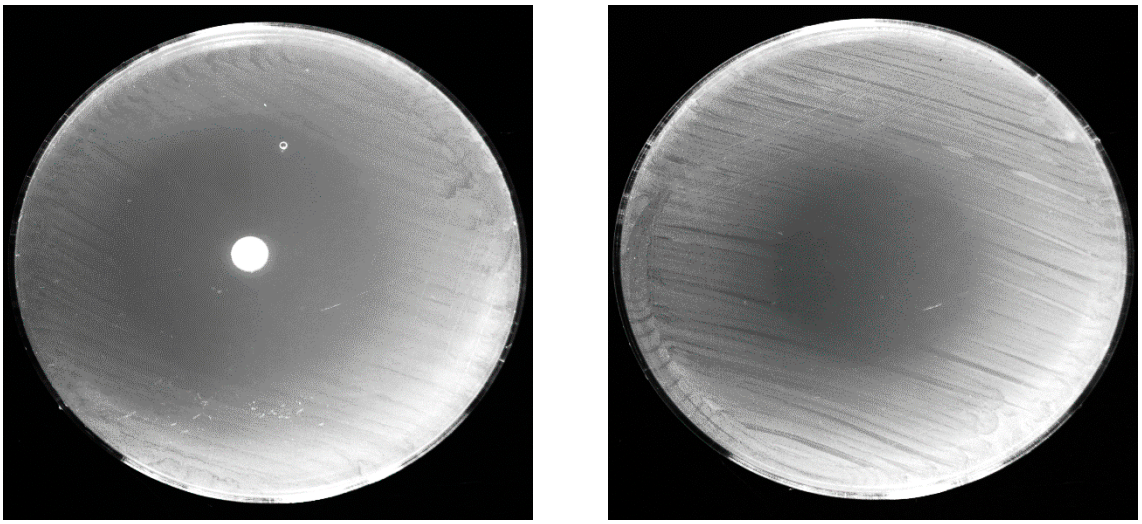


Fig. S6. Antibacterial activity of thyme essential oil liquid against *S. aureus* determined by disc diffusion method **(A)** and inverted Petri plate method **(B).**

A **B**

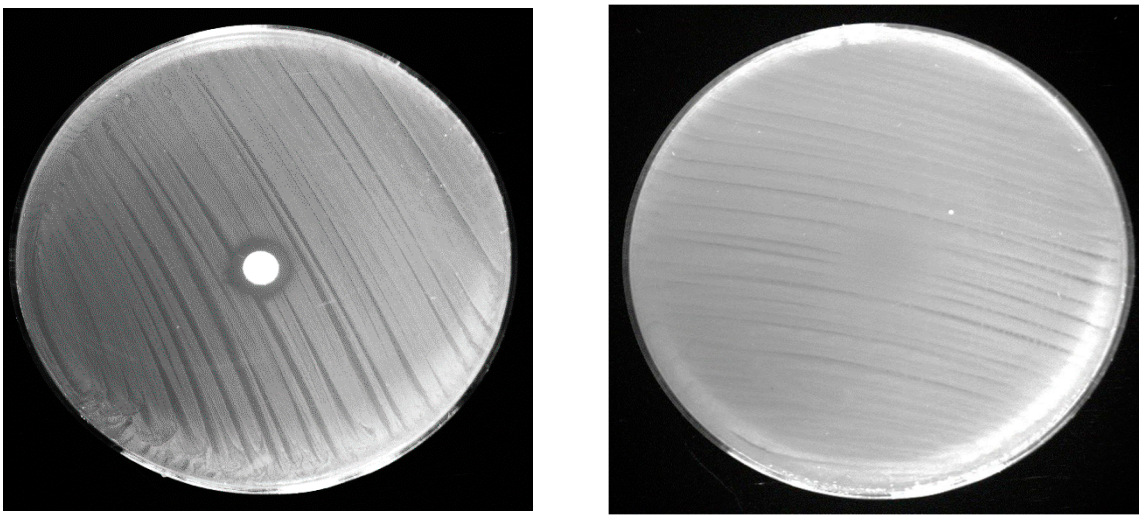


Fig. S7. Antibacterial activity of thyme essential oil liquid against *P. areuginosa* determined by disc diffusion method **(A)** and inverted Petri plate method **(B).**

Tab. S3. Antibiofilm activity of volatile fractions of EOs and ethanol against *S. aureus, P. aeruginosa*, *C. albicans* biofilm.

Volatile fractions	***S. aureus***		***P. areuginosa***		***C. albicans***	
	Mean	SEM	Mean	SEM	Mean	SEM
T-EO	0.377	0.048	0.572	0.109	4.555	0.592
TT-EO	0.193	0.049	0.338	0.057	5.190	0.618
E-EO	0.062	0.007	0.357	0.076	4.134	0.522
EtOH	0.157	0.029	0.199	0.036	3.917	0.657
C+	0.549	0.112	1.048	0.129	7.928	0.501

Data are presented as a mean ± standard error of the mean (SEM) of absorbance value (TTC assay) for *S. aureus*, *P. aeruginosa* and $\log_{10}$ CFU/mL for *C. albicans*. **C+** – positive control of growth. **EtOH** – usability control.

Tab. S4. Comparison of antibiofilm activity of 3 concentrations of TT-EO against *S. aureus* biofilm.

Volatile fractions	**Single plate**		**Separate plate**	
	Mean	SEM	Mean	SEM
TT-EO 25%	0.357	0.028	0.403	0.063
TT-EO 50%	0.312	0.036	0.299	0.069
TT-EO 100%	0.203	0.052	0.228	0.022

Data are presented as a mean of absorbance ± standard error of the mean (SEM) of absorbance value (TTC assay).