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Essential Oils and Plants Extracts with Antibacterial and Anti-Biofilm Activities against Multidrug Resistant Bacteria

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Abstract: The study assessed the efficacy of several extracts and essential oils with less known antimicrobial potential. Antimicrobial activities (microdilutions technique) and antibiofilm activities (crystal violet-based microtiter plate assay) against multiresistant bacterial strains of S. aureus and E. coli were tested for essential oils (Ocimum basilicum, Eugenia caryophyllus/Syzygium aromaticum) and alchoolic extracts of Ocimum basilicum, Robinia pseudocacia, Allium arsinum, Artemisia absinthium, Equisetum arvense previously analyzed by GC-MS. Essential oils elicited inhibitory values of 8 mg/ml on the E. coli growing and biofilm formation. The extracts inhibited bacterial growth, and Equisetum arvense led to a high inhibition rate of S. aureus strains (79.06% and 80.32%). The Equisetum arvense extract, a plant less used for its antibacterial properties, strongly inhibits the S. aureus strains both in culture and biofilms.

Keywords: Essential Oils; Plants Extracts; Anti-Biofilm Activities; Multidrug Resistant Bacteria

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1. Introduction

Antimicrobial resistance is a major global problem and is considered a threat to global public health. WHO reports a worrying increase in the use of antibiotics, which is one of the reasons for the emergence of extremely resistant bacteria [1, 2], along with an insufficient range of antibiotics available on the market, non-discriminatory abuse in treatments and slow rate of new therapeutic agents [3]. At present, the gap between the ability to develop new antibiotics and the rate at which bacteria increase their resistance is deepening, with effects on humans, animals, agriculture, the environment and, consequently, on national economies [4]. Some statistics [5, 6] show that every year, more than 670,000 infections occur in the EU due to resistant bacteria and 33,000 people die as a direct consequence of these infections; the economic impact is also significant, about 1.5 billion euros are spent annually in the EU. It is estimated that the overall use of antibiotics will increase by 200% by 2030 if no effective action is taken. In addition, during this period, the increased non-discriminatory use of antibiotics during the COVID-19 pandemic

will increase bacterial resistance and eventually lead to even more deaths [7]. Unfortunately, Romania ranks first in Europe in terms of germ resistance to antimicrobials, as evidenced by data published by the World Health Organization (WHO) and the European Center for the Control of Communicable Diseases (ECDC) [2]. Over time, a number of measures have been proposed to reduce microbial resistance to antibiotics without much success. Scientific research has recently focused on exploring plant products (essential oils and extracts) as a new source of phytotherapy capable of modifying microbial resistance [5] as molecules with high biological and chemical potential [8]. Biofilms (microbial communities attached to different surfaces in which bacterial cells are embedded in a self-produced extracellular polymeric matrix) are clinically relevant because they protect microorganisms, allowing them to survive in hostile environments by preventing the absorption of antibiotics. They are estimated to cause more than 80% of microbial infections worldwide [3] and are therefore one of the most important challenges in current antibiotic therapy. Plant extracts may have good activity in themselves or may be sources of effective antimicrobial compounds that may act against the biofilm of pathogens.

2. Methods

2.1. Essential oils GC-MS analysis

The sample preparation consisted of the following two steps: 50 µL of each essential oil (Ocimum basilcum and Eugenia caryophyllus/Syzygium aromaticum) [9] were pipetted in two screw top vials (2 mL) and diluted with 950 µL of methanol. The analysis of the essential oils was performed on a 7890A Agilent Technology Gas Chromatograph, coupled with a MSD 5975 Mass Spectrometer and equipped with a HP-5MS capillary column (30 m x 0.25 mm x 0.25 µm). The samples were introduced into the GC-MS injection port using a 5 µL syringe, in the split mode (the split ratio was 100:1) and a volume of 2 µL. Helium was the carrier gas with a flow rate of 1 ml/min. The inlet pressure was 7.5622 psi, the total flow was 104 ml/min and the oven temperature was set at 50°C (3'). The initial temperature was increased with 4°C/min to 120°C and then with 8°C/min to 280°C (4,5'). The total run time was 45'. NIST database was used for the identification of the volatile compounds.

2.2. Preparation of plant extracts

In order to obtain the extracts, dried plants harvested from the local area were used. 15 grams of the dried and grounded plants have been extracted with a 70% ethanol solution in a ratio of 1:10. The solvent-plant complex was fully mixed (30' at 200 rpm) with a Panasonic MIR-S100-PE Orbital shaker and filtered through a 90 mm filter paper. Before the analysis, the extracts were diluted in a ratio of 1:20.

2.3. Total polyphenol content

For the determination of the total polyphenolic content, the Folin-Ciocalteu micromethod [10, 11] was used, following the protocol described by Tamas-Krumpe Octavia Maria et al. [12]: 25 µL sample mixed with 125 µL Folin-Ciocalteu Reagent and 100 µL Na2CO3. The mixture was kept at room temperature for 30' and the OD was read at 760 nm with a Tecan Infinite M1000 Pro spectrophotometer.

2.4. Total flavonoids content

For total flavonoids content the method described by Zhinsen et al. [13] was adopted. The preparation of the samples was made in 5 ml volumetric flasks. 500 µL of each sample and 150 µL NaNO2 (5%) were mixed and kept at room temperature for 5'. 250 µL AlCl3

(2%) were added and kept for another 6'. 250 μL NaOH were added on the resulted mixture and kept at room temperature for 10'. The OD of the samples was read at 510 nm (Perkin Elmer Lambda 25 spectrophotometer).

2.5. Antibacterial Assay

Antimicrobial and antibiofilm activities of essential oils were tested on two multidrug-resistant bacterial strains S. aureus ES5168 and E. coli ES5649; the effects alcoholic plant extracts were evaluated on 2 strains of S. aureus ES5168, RC0831 and 2 strains of E. coli ES5649, CA0422. All bacterial strains exhibited resistance phenotype by a series of cumulative mechanisms. The antimicrobial activity / MIC of essential oils and plant extracts was determined using the broth microdilution method for bacteria, in a 96-well microplate. The essential oils and plant extracts were dissolved in maximum 1% DMSO solution (in BHI broth) [14]. The plant extracts were also disolved in ethanol 80% and tested at a concentration of 25 mg/mL. Serial dilutions were prepared ranging from 10.0 up to 0.25 mg/mL for the essential oils and from 50 up to 12.5 mg/mL for the plant extracts to a final volume of 50 μL per well. One-hundred microliters of bacterial suspension adjusted to McFarland standard 0.5 were added to each well to obtain a final working volume of 150 μL. Each experiment always had negative control (100 μL BHI broth and 50 μL DMSO 1%, respectively 50 μL ethanol 80% without inoculation), positive/growth control (100 μL bacterial suspension adjusted to McFarland standard 0.5 and 50 μL DMSO 1%, respectively 50 μL ethanol 80% - in order to avoid the potential effects of DMSO 1% and ethanol 80% on bacterial growth and biofilm formation) and blanks (100 μL BHI broth and 50 μL of various concentrations of essential oils and plant extracts). After determining the antimicrobial activity, the microplates were reincubated for another 24 h and crystal violet (CV) assay was performed to assess the biofilm inhibiting activity of essential oils and plant extracts [15, 16]. The medium and planktonic cells were discarded and each well was rinsed twice. Adhered biofilm-biomass was stained with 160 μL CV 0.1% for 30 minutes, at room temperature. The CV was washed out thrice and dye bound to biofilm was re-solubilized in 165 μL of 96% ethanol. The optical density was measured at 540 nm.

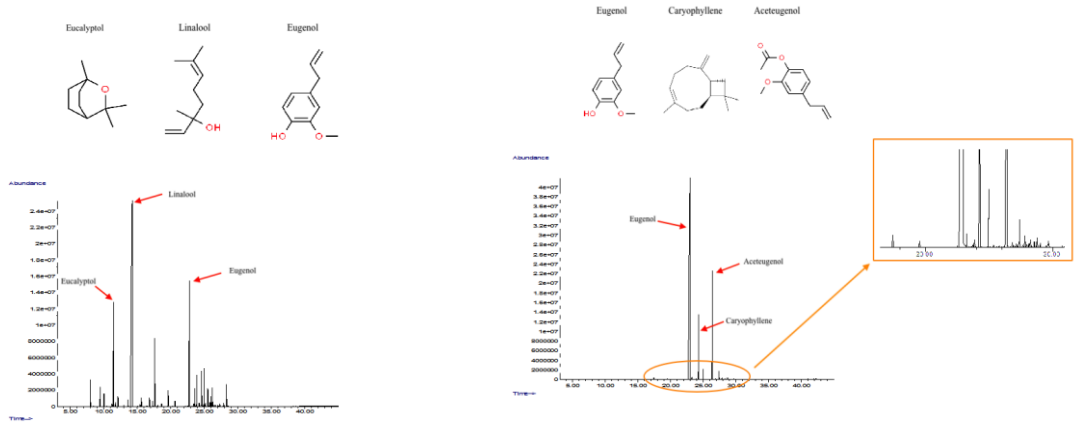
2.6. Statistical analysis

The results were expressed as inhibition rate (IR %): 100 – (treatment x 100)/positive control, for antimicrobial activity and as mean ± standard deviation (SD) (calculated based on the optical density of minimum 12 wells), for anti-biofilm activity. A higher inhibition rate corresponds to a lower OD relative to control and vice-versa. Where applicable, the data were subjected to Mann Whitney U test or Anova Kruskal-Wallis test. P values less than 0.05 were considered statistically significant.

3. Results

3.1. GC-MS Analysis of essential oils

The Ocimum basilicum oil chromatogram showed 3 main peaks for: Eugenol (22.752 min retention time; 10.944% from total compounds), Linallol (14.218 min retention time; 51.159% from total compounds), and Eucalyptol (11.451 min retention time; 8.373% from total compounds) (Figure 1a). The Eugenia caryophyllus/Syzygium aromaticum oil chromatogram showed 3 main peaks for: Eugenol (22.941 min retention time; 78.515% from total compounds), Aceteugenol (26.375 min retention time; 13.071% from total compounds), and Caryophyllene (24.255 min retention time; 5.658% from total compounds) (Figure 1b).



(**a**) (**b**)

Figure 1. GC-MS Analysis Results**:** (**a**) Basil essential oil chromatogram with main detected compounds; (**b**) Eugenia caryophyllus essential oil chromatogram with main detected compounds.

3.2. Total polyphenol and flavonoids content of plant extracts

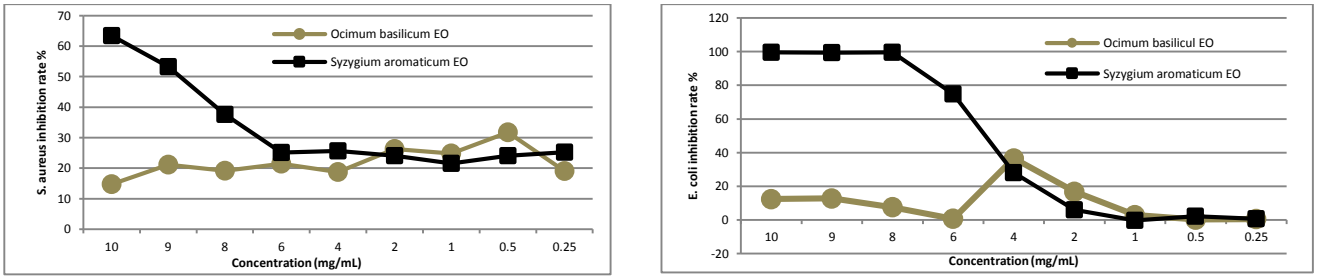
The calibration curve for polyphenol detection was made with Gallic acid at the following concentrations: 3,9 μg/ml, 7,8 μg/ml, 15,62 μg/ml, 31,25 μg/ml, 62,5 μg/ml, 125 μg/ml and 250 μg/ml. For the calibration curve of flavonoids measurements rutin (1mg/ml) was used and 5 standards were prepared (62,5 μg/ml, 125 μg/ml, 250 μg/ml, 500 μg/ml and 1000 μg/ml). The results are showed in the table 1.

Table 1. Measurement of polyphenolic and flavonoids content of the tested plant extracts

	Polyphenols (μg/ml)		**Flavonoids (μg/ml)**	
	$\bar{x}$	±sd	$\bar{x}$	±sd
Basil (Ocimum basilicum)	339,4116	13,39243	308,6633	3,268216
Acacia (Robinia pseudoacacia)	622,9021	28,28998	15,328	0,027495
Wild garlic (Allium ursinum)	681,8131	33,74201	17,417	0,431146
Wormwood (Artemisia absinthium)	742,3959	84,07821	247,4167	0,515202
Common horsetail (Equisetum arvense)	1251,154	45,17416	442,2333	3,510931

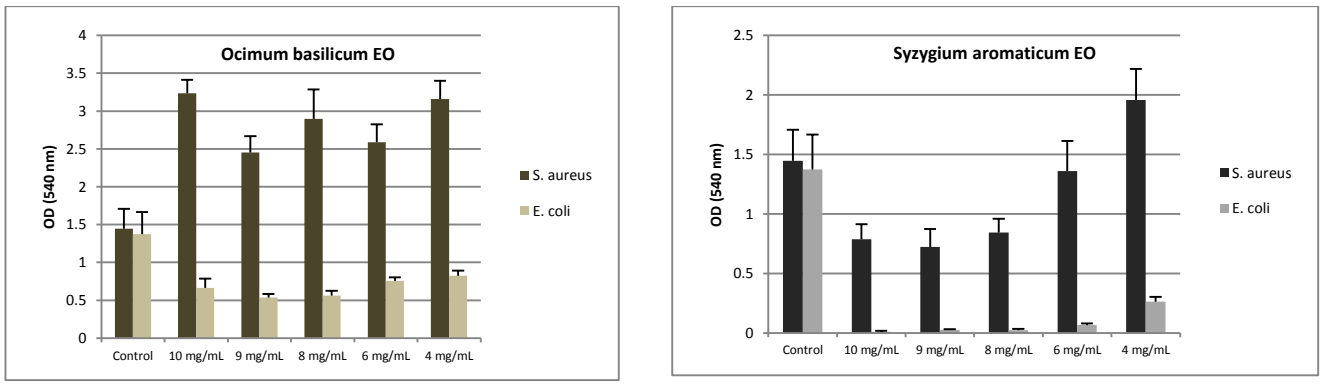
3.3. Antibacterial activity of Ocimum Basilicum and Eugenia caryophyllus/Syzygium aromaticum esential oils

The Ocimum basilicum exhibited low inhibition rates for S. aureus, between 14.77% (10 mg/mL) and 31.71% (0.5 mg/mL). The inhibition rates (IR) for E. coli were even more reduced with an exception for concentration of 4 mg/mL (36.58%). In case of Syzygium aromaticum esential oil was more expressed the tendency of inhibition rates growing according to concentration. For S. aureus MIC_{50} was at 9 mg/mL; starting with 6 mg/mL, IR shows an upward trend, between 0.25 - 4 mg/mL, is 21 - 25%, without significant differences ($p > 0.05$). For E. coli, MIC_{50} was at 8 mg/mL (MIC, minimal inhibitory concentration correspondes to an IR% of 95-100%) (Figure 2). S aureus biofilm inhibition by Ocimum basilicum essential oil was less expressed than E. coli biofilm inhibition. Althought the Syzygium aromaticum essential oil was more potent against biofilm formation, a strong effect was noticed just for E. coli. In that case even reduced concentration of 4 mg/mL impair biofilm formation (Figure 3).



(**a**) (**b**)

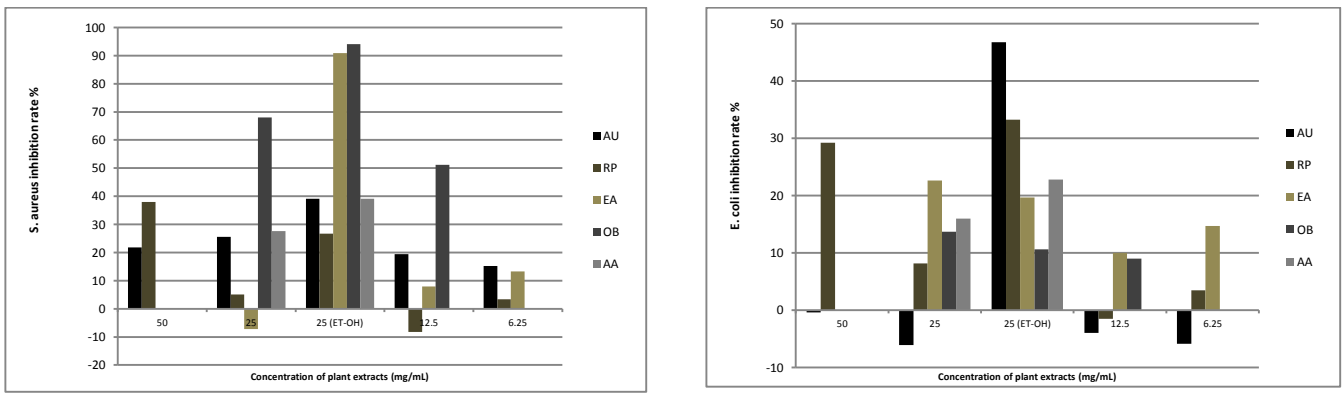
Figure 2. Antibacterial activity of the Ocimum basilicum and Syzygium aromaticum essential oils against the growth rate of S. aureus (a) and E. coli (b). Data are presented as inhibition rate %, calculated based on the mean OD.



(**a**) (**b**)

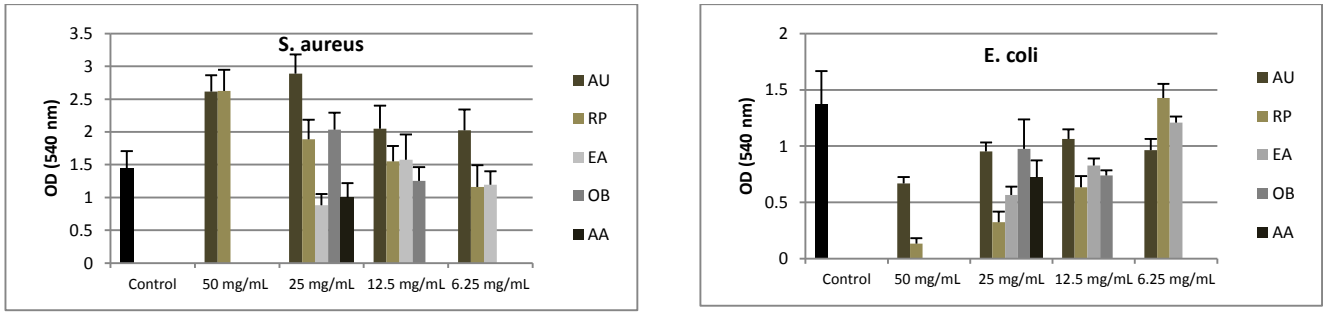
Figure 3. Biofilm inhibition activity of the Ocimum basilicum (a) and Syzygium aromaticum (b) essential oils against S. aureus (dark bars) and E. coli (light bars). Data are presented as mean ± SD of optical density (at 540 nm).

The experiments regarding antibacterial activity of the plant extracts revealed high IR values for alcoholic extracts in comparison with DMSO extracts, comparable results were obtaind against S. aureus and E. coli as well (Figure 4). The biofilm inhibition activity for extract solubilized in DMSO or ethanol was more pronounced against E. coli for all extracts (Figure 5).



(**a**) (**b**)

Figure 4. Antibacterial activity of the Allium ursinum (AU), Robinia pseudoacacia (RP), Equisetum arvense (EA), Ocimum basilicum (OB) and Artemisia absithium (AA) plant extracts (disolved in DMSO 1% and ethanol 80% - 25 ET-OH) against the growth rate of S. aureus (a) and E. coli (b). Data are presented as inhibition rate %, calculated based on the mean OD.



(**a**) (**b**)

Figure 5. Biofilm inhibition activity of the Allium ursinum (AU), Robinia pseudoacacia (RP), Equisetum arvense (EA), Ocimum basilicum (OB) and Artemisia absithium (AA) plant extracts disolved in DMSO 1% against S. aureus (a) and E. coli (b). Data are presented as mean ± SD of optical density (at 540 nm).

4. Discussion and conclusions

The experiments clearly highlighted that Syzygium aromaticum essential oil had a significantly grated inhibitory effect on bacterial growth than Ocimum basilicum essential oil, both in the case of S. aureus and in the case of E. coli ($p < 0.001$). Various mechanisms of antibacterial activity of essential oils have been proposed. Essential oils primarily destabilize cellular architecture, leading to decomposition of membrane integrity and increased permeability, which disrupts many cellular activities, including energy production, membrane transport, and other metabolic regulation functions. Essential oils can affect both the outer shell of the cell and the cytoplasm. Due to their lipophilic nature, essential oils are easily penetrated through bacterial cell membranes. The variability of the antimicrobial activity of the essential oils towards the investigated microorganism can be attributed to the qualitative and quantitative differences in the constituents of the individual oils. It has been observed that the composition of essential oils varies depending on local climatic and environmental conditions; consequently, they have different bioactivities. Some essential oils and their components are very active against bacteria but not against fungi and vice versa, while some essential oils stimulate the growth of microorganisms [17]. We should test whether the main component of the Syzygium aromaticum essential oil (in our case Eugenol) is responsible for the antibacterial effects.

The tests using plant extracts gave more efficient inhibitory effects when ethanol was used as the solvent while solubilisation in DMSO did not expressed the potent antibacterial results. Most of the ethanol soluble extracts used had inhibitory effects in both strain types, S. aureus and E. coli. Robinia pseudoacacia (RP) extract showed an IR below 40%, with no significant differences between strains ($p > 0.05$). The most powerful effect was induced by Equisetum arvense (EA) ethanol extract which had over 90% IR for S. aureus. Essential oils are volatile, natural, complex compounds produced by plants as secondary metabolites. The use of plant extracts in the treatment of mastitis, in the murine model, is not a topic addressed by many researchers. Magnolol is a polyphenolic compound extracted from the bark of the magnolia stem (Magnolia sp.), has been shown to have anti-inflammatory activity [18]. Alcoholic extract from the fruits of a plant in the Myrtaceae family has shown surprising antibacterial properties in vitro, but after inoculation in a murine mammalian model, the antibacterial effect has been greatly diminished, probably due to the neutralization of active compounds by casein in milk [19].

In conclusion, even though the antimicrobial effect of plant extracts on the multidrug-resistant bacteria is not very high in all cases, the *Equisetum arvense* extract, a plant less used for its antibacterial properties, strongly inhibits the S. aureus strains both in culture and biofilms. This could also be explained by the highest polyphenols and flavonoids content of all the extracts tested as shown in the table 1.

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Conflicts of Interest: "The authors declare no conflict of interest."

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