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Antibacterial Activity of Hydroethanolic Extracts from Stems and Leaves of *Tanacetum Vulgare* L. against Clinical Isolates of Various Infections

Horațiu Roman[1,2], Roua Gabriela Popescu[3,4], Cristina-Elena Găitănaru[3], Cătălin Marinescu[3,4], Luminița Roman[5], Veronica Lazăr[2]

[1]Research Institute of the University of Bucharest, University of Bucharest, ICUB, 050095 Bucharest, Romania.
[2]Faculty of Biology, University of Bucharest, 050095 Bucharest, Romania.
[3]Association Independent Research, 58 Timișului, sector 1, Bucharest 012416, Romania.
[4]Blue Screen SRL, 58 Timișului, Sector 1, 012416 Bucharest, Romania;
[5*]Recsearch Institute of the University of Bucharest (ICUB), 050663

Abstract
Gram-negative bacteria (GNB) have become a major global health problem in the last decade due to their multiple antibiotic resistance. *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* have been identified as critical multidrug-resistant bacteria requiring new effective therapies. The bacteria were isolated from various hospital infections and tested for their resistance to classic antibiotics. *Tanacetum vulgare* in the form of hydroethanolic extract had considerable antibacterial activity, proving once again the strong antibacterial properties of this plant. The minimum inhibitory concentrations had values ranging between 250-500 µg/mL.
The chemical composition of the extract obtained from stems and leaves of *T vulgare* identified by LC-MS/MS method, was an argument for its antibacterial properties. Thus, a number of 37 metabolites with known and very well-studied pharmacological properties were identified. This study is part of a series of several researches carried out on different components of *T vulgare* plant emphasizing its therapeutic qualities and promoting it as a safe drug in infectious therapies.

Keywords: hydroethanolic of *Tanacetum vulgare*; bioactive compounds; Gram-negativ bacteria; MDR.

Introduction

Tanacetum is a genus of over 160 species in the Compositae family, all native to the northern hemisphere, with a restriction to the Eurasian zone. The genus Tanacetum L. is considered the third largest genus of Compositae–Anthemideae, after the species-rich genera Artemisia L. (with 522 spp.) and Anthemis L. (with 175 spp.) [1]. The name Tansy is either used generically to designate any plant in the genus, or is used restrictively only for *Tanacetum vulgare*.
The morphology of the genus also shows diversity in terms of stems, which can be branched or prostrate, sometimes the same plant variety can be recognized in both situations. The plants have a hairy texture, which is often used as an indicator of a source of essential oils sufficient to be exploitable [2]. The plant is not demanding, easily adapting to pedoclimatic conditions. The high content of essential oil could be a condition for the adaptability of plants to the environment [3].
Most studies conducted on *T. vulgare* focus on the exploitation of the essential oil of this plant.
The results published in the specialized literature have highlighted that the main bioactive compounds of *T. vulgare* essential oils are: β-thujone, α- Thujone, Chrysanthenyl acetate, Camphor, Sabinol from different habitats [4].
Studies conducted on hydroalcoholic extracts of *T vulgare* have highlighted the presence of a large number of phenolic acids, flavonoids and their derivatives, such as: Apigenin, Baicalin, Casticin, Eupalitin, Hyperoside, Kaempferol, Luteolin which have pharmacological properties [5].

For the present study, *T. vulgare* flowers were collected during the plant's maximum productivity period, July-August, from the spontaneous flora of the Sohodolului Valley, Gorj County, Romania and preserved according to recommended norms. To demonstrate the antibacterial activity of the hydroethanolic extract from leaves and stems of *T. vulgare*, we selected Gram-negative bacterial strains that showed resistance to all antibiotics, including carbapenems.

Materials and methods

Materials used:

- Reagents: Muller Hinton Agar – purchased from AMEX
- Reference strains Pseudomonas aeruginosa ATCC 27853 – purchased from Merck SA, an affiliate of Merck KGaA, Darmstadt, Germany
- KAPA SYBR® FAST qRT-PCR kits – purchased from Merck SA, an affiliate of Merck KGaA, Darmstadt, Germany
- *Pseudomonas aeruginosa* ATCC 27853 – purchased from Merck SA, an affiliate of Merck KGaA, Darmstadt, Germany)

Equipment:

- Rotary evaporator (Buchi R 210-215) (purchased from AMEX);
- Lyophilizer - Christ Alpha 1-2 / B Braun (purchased from BiotechInternational);
- Biobase BK-EL10C microplate reader, 400 - 750 nm, 96 wells (purchased from AMEX);

-AB Sciex Triple TOF Mass Spectrometer 5600+.With APCI source.- (purchased from Labexchange - Die Laborgerätebörse GmbH, Bruckstr. 58 72393 Burladingen / Germany).

Selection of Gram-negative bacteria

For this study, bacterial strains were selected from the faculty's Microbiology Laboratory Collection, which showed multiple resistance to all antibiotics used.

The strains studied and their sources of isolation are presented in Table 1.

Table 1. Gram-negative bacterial strains isolated from various pathological products.

Acinetobacter baumannii $_{28i}$	Nasal Exudate
Acinetobacter baumannii $_{3997}$	Bronchial Secretions
Pseudomonas aeruginosa $_{3162}$	Nasal Exudate
Pseudomonas aeruginosa $_{11I}$	Urinari Tract Infections
Klebsiella pneumoniae$_{6I}$	Urinary Tract Infections
Klebsiella pneumoniae$_{14I}$	Urinary Tract Infections
Klebsiella pneumoniae$_{15I}$	Urinary Tract Infections
Klebsiella pneumoniae$_{19I}$	Urinary Tract Infections

Phenotypic characterization of Gram-negative bacteria, antibiotic resistance

The antibiotic susceptibility test of the studied strains was performed using the Kirby-Bauer diffusimetric method, standardized, recommended by CLSI (Clinical and Laboratory Standards Institute), 2018 edition, as described [4].

To obtain the extract, the "stock" extract from which we made the dilutions, the macerate obtained from about 200 g of leaves + stems of Tanacetum in 2000 mL of 70% ethanol, was concentrated on a rotary evaporator and then sterilized with a Whatman no. 1 filter, as described [6; 7].

Identification of metabolites of the hydroethanolic extract of *Tanacetum vulgare*

Samples extracted with 70% ethanol of stems and leaves were analyzed by LC-MS/MS on an AB SCIEX TRIPLE TOF 5600+ mass spectrometer. Identification of non-target metabolites from SWATH (Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra) data was performed using MS-DIAL ver.5.3.240719 according to the instructions described [8].

Evaluation of antibacterial activity

To evaluate antimicrobial activity, we calculated the MIC by the serial microdilution method in liquid medium (Mueler-Hinton broth), in 96-well plates with a capacity of 200 µL/well, according to the protocol described by Lazăr et al., [9], wells 11 and 12 corresponded to the controls (positive and negative). Concentrations were obtained with values ranging from 500; 250; 125; 65.2; 31.25; 15.625; 7.831; 3.9; 1.953; 0.976 µg/mL.The MIC was established macroscopically, as the last concentration at which no bacterial growth or turbidity of the medium was observed, and by spectrophotometric reading of the absorbance at 620 nm to determine the optical density of the bacterial cultures.

Results

Antibacterial resistance of selected Gram-negative strains

Tables 2 and 3 show the results obtained from the antibiograms performed for the selected strains. The results were read directly on the culture medium plates inoculated with the pathogenic strains, by measuring the diameter of the clear zones of inhibition of bacterial growth around the applied antibiotic. The interpretation of the results was done according to the size of the inhibition diameter according to CLSI recommendations. The results were expressed using the terms sensitive (S), resistant (R) or intermediate (I), according to the standardized breakpoint tables and corresponding to the working method.

Table 2. Antibiotic resistance profile of A. baumannii and P. aeruginosa strains from different classes. Results are expressed as mean ± SD (n = 3).

Strain	Inhibition diameter (mm)							Resistance profile
	IMP	CAZ	TOB	PRL	CIP	TZP	SAM	
A. baumannii $_{288}$	6 R	6 R	15 R	6 R	6 R	8 R	14-15 R	MDR, C+
P. aeruginosa $_{3162}$	6 R	6 R	15 R	7-8 R	15 R	9 R	8 MLSB	MDR
P. aeruginosa $_{11I}$	8 R	6 R	10 R	12 R	8 R	6 R	10 R	MDR

Abbreviations: IMP = imipenem (beta-lactam - carbapenem); CAZ = ceftazidime (third generation cephalosporin); TOB = tobramycin (aminoglycoside); PRL = piperacillin (broad spectrum beta-lactam); CIP = ciprofloxacin (fluoroquinolone); TPZ = piperacillin + tazobactam (broad spectrum beta-lactam + beta-lactamase inhibitor); SAM = ampicillin + sulbactam (beta-lactam + beta-lactamase inhibitor).

Table 3. Sensitivity/resistance spectrum to antibiotics from different classes of the reference strains *P. aeruginosa* $_{ATCC\ 27853}$, *A. baumannii* and *K. pneumoniae* Results are expressed as mean ± SD (n = 3) and the results were expressed as means ± standard deviation.

Tulpina bacteriană	Diametrul de inhibiție (mm)							Profilul de rezistență
	IMP	PRL	CIP	TOB	CAZ	ATM	TPZ	
P. aeruginosa $_{ATCC\ 27853}$	20 S	16 R	30 S	20 S	17 I	16 R	20 S	
A. baumannii $_{3997}$	6 R	10 R	6 R	6 R	6 R	6 R	18 I	MDR
K. pneumoniae$_{6I}$	6 R	8 R	6 R	6 R	14 R	6 R	12 R	MDR
K. pneumoniae$_{14I}$	6 R	0 R	0 R	10 R	6 R	6 R	8 R	MDR
K. pneumoniae$_{15I}$	6 R	8 R	6 R	10 R	6 R	14 R	0 R	MDR
K. pneumoniae$_{19I}$	6 R	12 R	6 R	6 R	6 R	10 R	6 R	MDR

Abbreviations: IMP = imipenem (beta-lactam - carbapenem);); PRL = piperacillin (broad-spectrum beta-lactam); CIP = ciprofloxacin (fluoroquinolone); TOB = tobramycin

(aminoglycoside); CAZ = ceftazidime (third-generation cephalosporin); ATM = aztreonam (monobactam); TPZ = piperacillin + tazobactam (broad-spectrum beta-lactam + beta-lactamase inhibitor).

Chemical composition of the hydroethanolic extract of *T. vulgare*

The procedure for identifying bioactive compounds from the hydroethanolic extract obtained from the mixture of T. vulgare stems and leaves by the LC-MS/MS method allowed us to identify compounds of maximum importance with pharmacological value. The main bioactive compounds are listed in the Table 4 and Figure 1.

Table 4. The main metabolites isolated in the hydroethanolic extract obtained from the mix of stems and leaves of *T. vulgare*. The concentration of each metabolite is represented as the value of the average area described by the peak corresponding to the Gaussian curve in the chromatogram. Each peak of a Gaussian curve in the chromatogram corresponds to a chemical compound.

Metabolites	**Area average**
alpha-Cyperone	1896857
Diosmetin	4593673
Emodin	1599758
3',4',7,8-Tetrahydroxyflavona	3194115
Jaceidin	1428229
Casticin	71363
8-(2,3-dihydroxy-2-methylbutyl)-7-methoxychromen-2-one	647369
Luteolin	925616
Apigenin	1240337
Phenylalanine	657979
Myricetin 3,7,3',5'-tetramethyl ether	307437
(3*aR*,5*aS*,9*aS*,9*bR*)-5*a*,9-dimethyl-3-methylidene-4,5,6,7,9*a*,9*b*-hexahydro-3*aH*-benzo[g][1]benzofuran-2-one	1185391
5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)chroman-4-one	406952
Genistein	2181785
Jaceosidin	1274896
3-(benzo[d][1,3]dioxol-5-yl)-6-ethyl-2-methyl-4-oxo-4H-chromen-7-yl acetate	151194
Cinchonine - alcaloid	110224
NCGC00380268-01_C19H30O8_2-Cyclohexen-1-one, 4-[(1E)-3-(beta-D-glucopyranosyloxy)-4-hydroxy-1-buten-1-yl]-3,5,5-trimethyl-,	263437
methyl 3-(3-hydroxy-6-(morpholinomethyl)-4-oxo-4H-pyran-2-yl)-3-(3-(hydroxymethyl)-4-methoxyphenyl)propanoate	21528
(S)-6-Gingerol	469247
Tetrahydroalstonine - alcaloid	71410
Desmotroposantonin - acid carboxilic ester	20385
7-O-Methylaloeresin A - flavonă	16027

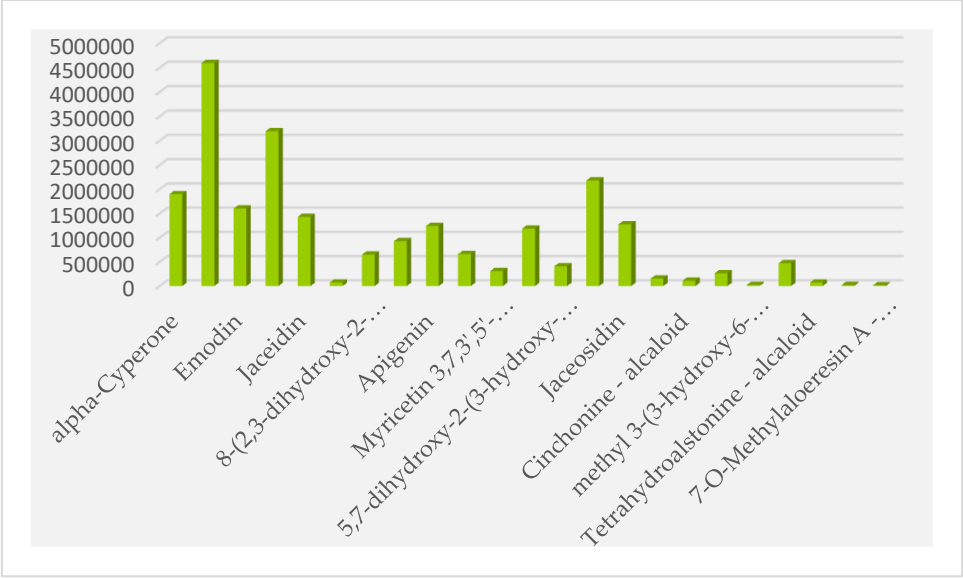

Figure 1. Graphical representation of the main compounds identified in the hydroethanolic extract obtained from stems and leaves of T. vulgare. The predominant bioactive compound is Diosmetin. A high concentration was also found for 3',4',7,8-Tetrahydroxyflavone (fisetin), Genistein, Jaceosidin, Apigenin and Luteolin, and the other compounds were in moderate but remarkable concentrations, being of pharmacological importance.

Quantitative evaluation of the antibacterial activity of *T. vulgare* extract against Gram-negative bacteria by the serial dilution method

The minimum inhibitory concentrations (MIC) of the hydroethanolic extract obtained from the mixture of leaves and stems of *T. vulgare* for the tested bacterial strains obtained by the standard method using miniplates with 96 wells with the reading of the results using the spectrophotometer are shown in Figure 2 and Table 5.

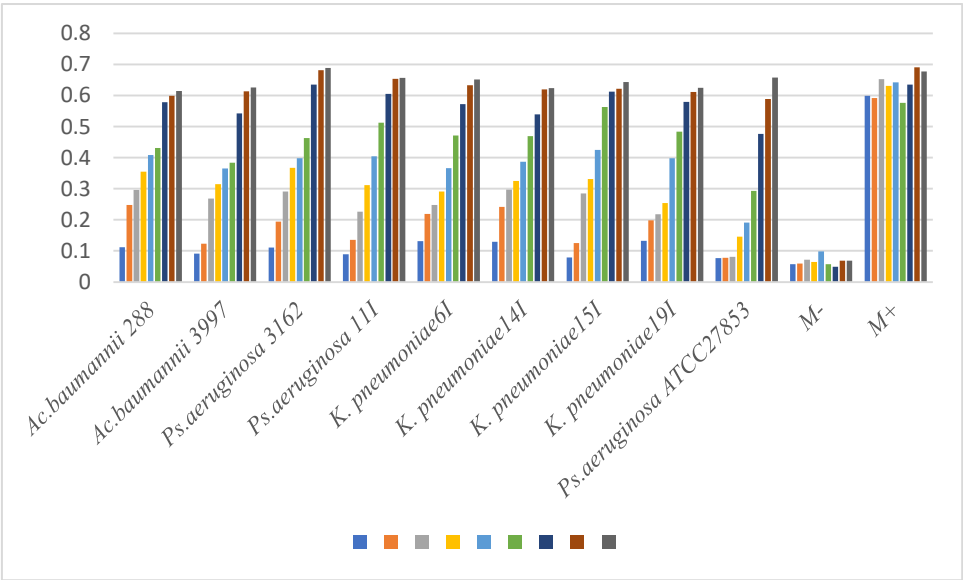

Figure 2. Graphical representation of the minimum inhibitory concentration of *T. vulgare* extract against MDR bacterial strains. The ordinate shows the optical density value of the cultures read spectrophotometrically at 620 nm and the abscisse shows the concentration of the *T. vulgare* extract (µg/mL). M+ positive control (bacterial culture without extract addition) and M− (sterile culture medium). Results are expressed as mean ± SD (n = 3) and the results were expressed as means ± standard deviation.

Table 5. Optical density values read at 620 nm of the bacterial concentration corresponding to the inhibition of bacterial growth of the *T. vulgare* extract. Results are expressed as mean ± SD (n = 3) and the results were expressed as means ± standard deviation.

Strain	Bacterial density read at different concentrations of *T. vulgare* extract [µg/mL]								
	500	**250**	**125**	**62.5**	**31.25**	**15.125**	**7.831**	**3.906**	**1.953**
Ac.baumannii $_{288}$	0.112	0.248	0.296	0.355	0.408	0.431	0.578	0.599	0.614
Ac.baumannii $_{3997}$	0.091	0.123	0.268	0.315	0.365	0.384	0.542	0.613	0.626
Ps.aeruginosa $_{3162}$	0.111	0.194	0.291	0.367	0.398	0.463	0.635	0.681	0.688
Ps.aeruginosa $_{11I}$	0.089	0.135	0.226	0.312	0.404	0.512	0.605	0.653	0.657
K. pneumoniae $_{6I}$	0.131	0.219	0.248	0.291	0.366	0.471	0.572	0.633	0.651
K. pneumoniae $_{14I}$	0.129	0.241	0.297	0.325	0.387	0.469	0.539	0.619	0.624
K. pneumoniae $_{15I}$	0.079	0.125	0.285	0.331	0.425	0.563	0.612	0.621	0.643
K. pneumoniae $_{19I}$	0.132	0.198	0.218	0.254	0.398	0.483	0.579	0,611	0.625
Ps.aeruginosa $_{ATCC27853}$	0.077	0.078	0.081	0.146	0.191	0.293	0.476	0.589	0.658
M-	0.057	0.059	0.072	0.064	0.098	0,057	0.049	0.069	0.068
M+	0.599	0.592	0.652	0.631	0.642	0.576	0.635	0.691	0.677

Discussions

The treatment of infectious diseases is facing a major crisis due to the alarming scale of antibiotic resistance (AR). Designing new (classes of) antibiotics is difficult, so efforts are being made to stop and reduce the phenomenon, however, monitoring studies do not report the effects of these policies [10].

The evaluation of resistance profiles for *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* strains showed a high level of resistance to the tested antibiotic classes: β-lactams (100% - imipenem, ceftazidime, piperacillin, aztreonam (10%) - 100% and aminoglycosides (tobramycin) and β-lactamase inhibitors (piperacillin + tazobactam).

Excessive use of antibiotics, insufficient monitoring of infections, and the rapid spread of bacterial resistance are important factors contributing to the increasing ineffectiveness of drugs [11].

Regarding the metabolites identified from the hydroethanolic extract obtained from the mix of leaves and stems of *T. vulgare*, by LC-MS/MS method, a total of 37 compounds with pharmacological importance were identified (Table 4 and Figure 1). We managed to identify more bioactive compounds with relatively high concentrations, compared to other authors [5]. This is mainly due to the different pedoclimatic conditions.

Diosmetin was the bioactive compound with the highest concentration, followed by Fisetin, Genistein, Jaceosidin, Apigenin, Luteolin, Gingerol, Casticin, which had considerable concentrations, important in the treatment of various bacterial infections. Thus, Diosmetin is a natural flavonoid, with varied pharmacological importance, being a product with antitumor, antioxidant, anti-inflammatory, antibacterial and metabolic regulator action. Numerous studies published in the literature have highlighted the importance of this metabolite isolated especially from citrus fruits [12]. Fisetin is a bioflavonoid recognized as a commercial product, being identified especially in Butea frondosa, Gleditschia triacanthos, Quebracho Colorado and Cotinus coggygria used for its anti-inflammatory, antibacterial, antioxidant and antitumor properties [13; 14; 15].

Our results indicated a strong antibacterial activity of the hydroethanolic extract obtained from the mix of leaves and stems of *T. vulgare* against MDR Gram negative bacteria from different infections. A higher antibacterial activity was observed against the strains *Ac.baumannii*$_{3997}$ isolated from bronchial secretions and *Ps.aeruginosa*$_{11I}$ and *K. pneumoniae*$_{15I}$ isolated from urinary tract infections with a concentration of 250 µg/mL compared to the other strains with a concentration of 500 µg/mL.

Conclusions

Although there are currently few studies conducted on hydroethanolic extracts of *T. vulgare* that would motivate and inspire us in a more in-depth study, we managed to demonstrate with convincing arguments that they can come to our aid in critical situations, such as infections with MDR bacteria. The advantage of

hydroethanolic extracts lies in their ease of obtaining, being within everyone's reach, without involving excessive costs. The strong antibacterial activity against MDR Gram-negative bacteria is primarily due to the high content of bioactive compounds with especially antibacterial properties, which we identified by LC-MS/MS method, which are otherwise very well studied, such as: Diosmetin, Fisetin, Genistein, Jaceosidin, Apigenin, Gingerol, having higher concentrations of the 37 compounds identified.

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Conflict of interest statement

The authors declare no conflict of interest.

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