

ANTIMICROBIAL AND SYNERGISTIC ACTIVITY OF SOME WHOLE AND SELECTIVE *EPILOBIUM HIRSUTUM* L. (GREAT WILLOWHERB) EXTRACTS TESTED ON STANDARD AND WILD *STAPHYLOCOCCUS AUREUS* STRAINS

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Abstract

This work aimed to establish *Epilobium hirsutum* L. (*Onagraceae* family) aerial part whole and selective extracts antimicrobial activity and possible synergism with some antibiotics (Gentamicin, Tetracycline, Ampicillin, Nalidixic acid and Ciprofloxacin) tested on standard and wild methicillin-resistant *Staphylococcus aureus* (MRSA) strains; the vegetal extracts had identical total phenols content, 5 mg gallic acid (GAE) equivalents 1 mL extract respectively. Thus, there were evidenced stimulatory effects between great willowherb extracts and Ampicillin; statistically significant differences were also obtained for the combination with Gentamicin and Ciprofloxacin, in these cases being recorded smaller inhibition zones compared to those of the antibiotics used alone (inhibitory effects).

Rezumat

Scopul acestui studiu a constat în evaluarea activității antimicrobiene și a unui posibil efect sinergic a unor extracte totale și selective obținute din partea aeriană de *Epilobium hirsutum* L. (fam. *Onagraceae*) în combinație cu substanțe antibacteriene (gentamicină, tetraciclină, ampicilină, acid nalidixic, ciprofloxacin), asupra unor tulpini standard și sălbatice de *Staphylococcus aureus* metilino-rezistent (MRSA); extractele vegetale au prezentat concentrații identice de polifenoli totali, respectiv 5 mg echivalent acid galic (GAE) / 1 mL extract. Rezultatele au indicat efecte stimulatorie între extractele de pufuliță și ampicilină; diferențe semnificative statistic au fost obținute și pentru combinațiile cu gentamicină și ciprofloxacin, respectiv zone de inhibiție mai mici comparativ cu cele ale substanței antimicrobiene singure, deci un efect inhibitor.

Keywords: *Epilobium hirsutum* L., antimicrobial and synergistic activity, *Staphylococcus aureus*

Introduction

It is well known that plant-derived antimicrobials are often more efficient than chemical antimicrobials (common antibiotics), even on resistant pathogens, likely owing to plant compounds synergistic activity. Moreover, it was proved that some phytocompounds possess the ability to act as multidrug resistance modifiers by counteracting the main mechanisms of microbial resistance, respectively active site modification, antibiotic destruction using specific enzymes (such as beta-lactamases), antibiotic influx inhibition or antibiotic efflux stimulation [4].

In view of these, this work aimed at studying the antimicrobial activity of some whole and selective extracts prepared from *Epilobi hirsuti herba* tested on standard and laboratory collection of *Staphylococcus aureus* methicillin resistant *Staphylococcus aureus*

(MRSA), as well as the possible synergism between these extracts and antibiotics (e.g., Gentamicin, Tetracycline, Ampicillin, Nalidixic acid and Ciprofloxacin) tested on the same bacterial strains.

Epilobium hirsutum L. (*Onagraceae* family), commonly known as great willowherb or great hairy willowherb, is a perennial, flowering plant spread in most of Europe, North Africa and parts of Asia, typically growing in wet habitats; in Romania, Transylvania region respectively, there were identified 15 species of *Epilobium*, the most important being *E. hirsutum* L., *E. parviflorum* (Schreb.) Wither, *E. montanum* L., *E. palustre* L., *E. nutans* F.W. Schmidt, *E. alsinifolium* Vill. and *E. (Chamerion) angustifolium* (L.) Holub [17]. *Epilobium* sp. are mainly known for ellagitannins and flavonoids content [13]; up to 5.8% in the case

of *E. hirsutum* L. according to literature data [17]. Punctually, there were reported [1] some unique ellagitannins as 2-O-galloyl 3-O-valoneoyl dilactone-(α/β)-4C1-glucopyranose, 1'-mono-decarboxyvaloneic acid dilactone and valoneic dilactone dioxine aside common gallic, ellagic and protocatechuic acids, valoneic dilactone and p-coumaric acids, methyl gallate, p-methoxy gallic acid methyl ester as well as flavonoids such as kaempferol, quercetin and myricetin 6-O-galloyl-glucose, 1,6-di-O-galloylglucose, 2,3-di-O-galloyl-glucose, 1,2,6-tri-O-galloylglucose and their 3-O-glucuronides, 3-O-arabinosides, and 3-O-rhamnosides, respectively. Neutral and acidic saponins, anthocyanidins, volatile oils, vitamin C, amino acids (e.g., cysteine, valine, serine, threonine, leucine, phenylalanine, proline, isoleucine, tyrosine, etc.) [13] and microelements (e.g., K, Mg, S, P, Co, Cu, Zn) [8] have also been reported.

Materials and Methods

All the experimental procedures were performed in order to establish the chemical qualitative content of polyphenols in these whole and selective *Epilobi hirsuti herba* extracts and their antimicrobial and possibly synergistic activity in combination with several chemical antibiotics.

Plant material: *Epilobi hirsuti herba* raw material was harvested in August from the wild, Sinaia region, Romania. The taxonomic identification was performed by the botanical department team of the National Institute of Chemical-Pharmaceutical Research & Development (ICCF), Bucharest, Romania and a voucher specimen is deposited in ICCF Plant Material Storing Room. The vegetal raw material was shade dried and ground to a medium-size (3 - 5 mm) powder.

Instruments: UV/Vis spectrophotometer Hélios γ , Thermo Electron Corporation (U.K.) and 1 cm quartz cells were used for the chemical quantitative measurements. CAMAG Linomat5 TLC visualizer (Switzerland) instrument was used for the chemical qualitative analyses.

Reagents and solutions: Reagents (Folin-Ciocalteu and Natural Product) and solvents (methanol, ethanol, ethyl acetate, formic acid, acetic acid and chloroform) were purchased from Sigma-Aldrich Co and Fluka (Germany) as well as quercetin (95%), rutin (min. 95%), chlorogenic (>95%), caffeic (99%) and gallic (95%) acids reference substances.

Bacterial strains, culture media and chemicals: The studied microorganisms included reference (from the American Type Culture Collection - ATCC) and laboratory collection strains of *Staphylococcus aureus*. Nutrient broth (Oxoid CM0001) and Muller-Hinton agar (Oxoid CM0337) were used as culture media. The media were prepared from a

dehydrated base in accordance with the manufacturer's recommendations. All antimicrobials were purchased from Biolab Zrt.: Ampicillin (AM, 10 μ g), Gentamicin (CN, 10 μ g), Tetracycline (TE, 30 μ g), Ciprofloxacin (CIP, 5 μ g) and Nalidixic acid (NA, 30 μ g).

Preparation of plant extracts: 50 g of powdered vegetal material was (twice) heat assisted extracted with 500 mL of 70% (v) ethanol. The two extracts were mixed and filtered through paper filter resulting in 850 $\pm$ 20 mL ethanolic extract (codified E8). 250 mL of (E8) were concentrated at residue and then passed into 20% (v/v) propylene glycol (PG) solution in a manner to obtain a final vegetal extract with exactly 5 mg total phenols (expressed as gallic acid (GAE) equivalents) per 1 mL extract; thus, there were obtained 175 mL whole great willowherb extract (codified P8) further divided into 2 mL Eppendorf tubes and stored at -8°C until microbiological studies. Similarly, 250 mL of (E8) were concentrated at low pressure and the resulted *sicc* product was solved into 100 mL distilled water. The aqueous solution was further (manually) extracted first with (3 x 100 mL) chloroform and then with (3 x 100 mL) ethyl acetate, 24 hours per each solvent. Three fractions resulted: aqueous (P8aq), ethyl acetic (P8ea) and chloroformic (P8chl). The three fractions were further separately concentrated at residue and the residues passed into 20% PG in a manner to obtain the final concentration of 5 mg GAE per 1 mL sample. These products, further called the selective extracts, measuring 120 mL (P8aq), 19.5 mL (P8ea) and 175 mL (P8chl) were also divided in Eppendorf's monodoses. **Note:** Lacking in polyphenols compounds, the chloroformic fraction (P8chl) was passed into 20% PG by means of the whole extract algorithm.

Quantitative estimation of phenols content in extracts: Total phenols were measured by Folin-Ciocalteu method [16]. Results were expressed as mg gallic acid equivalent (GAE) / 1 mL sample.

Qualitative analysis of polyphenols in extracts: The qualitative analysis was performed by thin layer chromatography (TLC) technique according to Wagner and Bladt [14] and Reich and Schibli [11] recommendations using ethyl acetate-acetic acid-formic acid-water (100:12:12:26) system. Briefly, 2.5 μ L volumes of references, respectively vegetal samples were loaded as 8 mm band length in the 10 x 10 cm Silica gel 60F HPTLC plate (Merck). The loaded plate was then kept in TLC twin developing chamber at 18 -19°C with the respective mobile phase up to 90 mm. The dried, developed plates were immersed into reagent followed by PEG4000 identification reagents and the results compared with literature data.

Test bacteria: The tests were carried out on standard *S. aureus* (ATCC 25923 and ATCC 6538),

and on (five) wild methicillin-resistant *S. aureus* strains (MRSA).

Test extracts: The tests were fulfilled on the great willowherb whole extract (P8) and the corresponding selective extracts, namely the aqueous (P8aq), ethyl acetic (P8ae) and chloroformic (P8chl) fractions, all prepared in 20% PG and identical total phenols content (5 mg GAE / 1 mL extract).

Inoculum preparation: The microorganisms were stored in freezing conditions and activated by cultivation in nutrient broth at 37°C for 24 hours. Bacterial inoculums were prepared from overnight broth culture in demineralised water in order to obtain a bacterial turbidity equivalent to 0.5 McFarland standards. A suspension containing approximately 1.5×10^8 colony forming units (CFU)/mL was obtained [6]. *S. aureus* ATCC 6538 and *S. aureus* ATCC 25923 were used as reference strains.

Antimicrobial assay: Paper disc diffusion method was used to screen the antibacterial activity of plant extracts; it was performed by using Mueller Hinton agar (MHA). The bacterial suspension (0.5 McFarland standards) was swabbed on the surface of MHA and the inoculum was allowed to dry for 5-10 minutes [15]. Sterilized filter paper discs (Whatman, 6 mm in diameter) were placed on the surface of the MHA and soaked with 20 µL of whole great willowherb extract (P8) and selective aqueous (P8aq), ethyl acetic (P8ea), and chloroformic (P8chl) fractions. Propylene glycol (20%) was used as negative control. The inoculated plates were incubated at 37°C for 24 h in the inverted position. The diameters (mm) of the inhibition zones were measured (the diameter of paper disc, 6 mm, was included) [5, 12]. Synergy assay was based on a previously described procedure [10]. The synergistic action of test extracts on the growth of standard *S. aureus* ATCC 25923 and ATCC 6538 in combination with antibacterial substances was assayed by the disc diffusion method on Mueller-Hinton agar.

Statistical analysis: Microbiological results were expressed as means $\pm$ standard deviation (SD) of three measurements. The differences between the means of antimicrobials' effect and means of combination between antimicrobials plant extracts were considered significant at $p < 0.05$ using one-way analysis of variance (ANOVA) [7].

Results and Discussion

Figure 1 shows the chemical qualitative high performance thin layer chromatography (HPTLC) aspects of the four, whole and selective *E. hirsutum* L. extracts comparative to references (ref.). As resulted from the studies [9], great willowherb whole extract (T2-T3 tracks) shows eight major spots (s1-s8, chromatogram A1) amongst that five intense red-orange fluorescent spots likely owing to myricetin derivatives (s2, s3, s5, s6 and s7), two indigo fluorescent (s1, s4) spots, the most probable owing to myricetin-galloyl derivatives [1], and one blue fluorescent (s2) added to one dark blue fluorescent spot (s8) likely owing to chlorogenic and gallic phenolic acids.

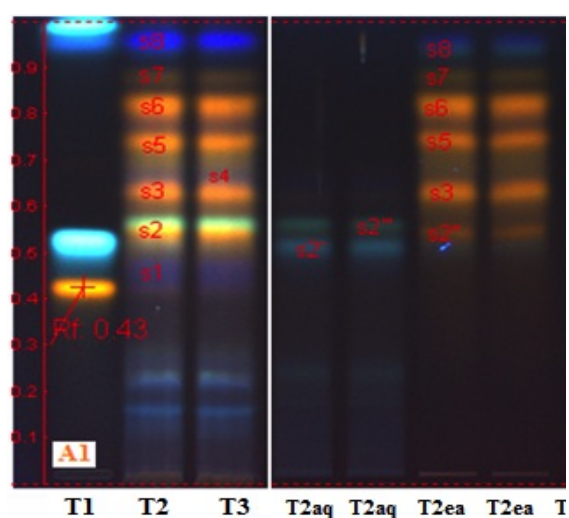


Figure 1.
HPTLC aspects of great willowherb whole (A1)
and selective extracts (A2)

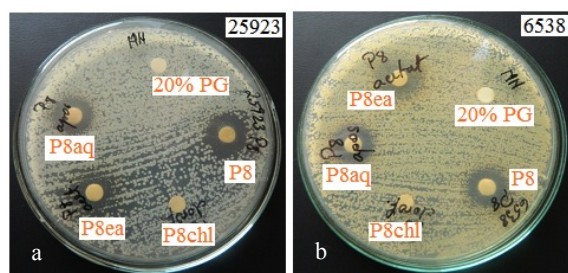
In the case of selective extracts (A2 chromatogram), the aqueous fraction (T2aq tracks) indicated chlorogenic acid (s2') and kaempferol derivatives (s2'') occurrence (previously covered by myricetin derivatives (s2)), while the ethyl acetic fraction (T2ea tracks) revealed myricetin derivatives and gallic acid attendance; chloroform fraction did not show any phenols.

Table I presents the effects of whole great willowherb extracts (P8) and corresponding selective, aqueous (P8aq), ethyl acetic (P8ea), and chloroformic (P8chl) fractions on the growth of standard *Staphylococcus aureus* strains (ATCC 25923 and ATCC 6538) by the paper disc diffusion method.

Table I

Antibacterial properties of the four vegetal extracts (20 μ L vegetal extract representing 100 μ g total phenols content/GAE, *per* each disc) against standard *S. aureus* (ATCC 25923 and ATCC 6538) tested single and in combination with different antibiotics

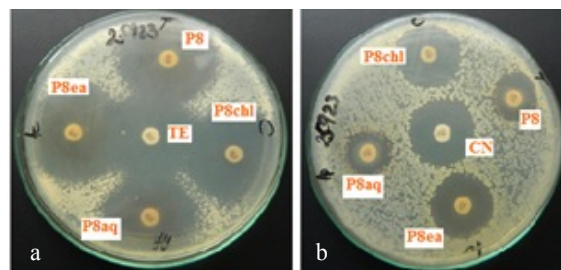
Chemical Agent $\pm$ vegetal extract	Inhibition zone diameter (mm) $\pm$ SD; diameter of the paper disc (6 mm) - included	
	<i>S. aureus</i> ATCC 25923	<i>S. aureus</i> ATCC 6538
Whole Extract (P8)	12 $\pm$ 0.8	12 $\pm$ 1.0
Aqueous fraction (P8aq)	12 $\pm$ 0.9	13 $\pm$ 0.8
Ethyl acetic fraction (P8ea)	9 $\pm$ 0.4	10 $\pm$ 0.4
Chloroformic fraction (P8chl)	6 $\pm$ 0.0	6 $\pm$ 0.0
AM (Ampicilin)	32 $\pm$ 1.2	14 $\pm$ 0.3
AM + P8	32 $\pm$ 1.3	11 $\pm$ 1.0
AM + P8aq	31 $\pm$ 1.2	17 $\pm$ 1.1
AM + P8ea	35 $\pm$ 1.4	14 $\pm$ 1.1
AM + P8chl	35 $\pm$ 1.2	19 $\pm$ 1.1
TE (Tetracyclin)	30 $\pm$ 1.3	23 $\pm$ 0.5
TE + P8	36 $\pm$ 1.3	25 $\pm$ 0.7
TE + P8aq	30 $\pm$ 1.3	24 $\pm$ 0.5
TE + P8ea	36 $\pm$ 1.3	28 $\pm$ 0.9
TE + P8chl	35 $\pm$ 1.1	27 $\pm$ 1.0
CN (Gentamicin)	26 $\pm$ 1.0	21 $\pm$ 1.0
CN + P8	18 $\pm$ 0.6	15 $\pm$ 0.6
CN + P8aq	16 $\pm$ 0.9	17 $\pm$ 0.3
CN + P8ea	24 $\pm$ 1.2	16 $\pm$ 0.1
CN + P8chl	25 $\pm$ 1.1	25 $\pm$ 0.6
NA (Nalidixic acid)	23 $\pm$ 1.0	26 $\pm$ 1.1
NA + P8	21 $\pm$ 1.1	22 $\pm$ 0.9
NA + P8aq	19 $\pm$ 0.6	24 $\pm$ 0.9
NA + P8ea	18 $\pm$ 0.7	24 $\pm$ 1.0
NA + P8chl	22 $\pm$ 0.9	24 $\pm$ 0.9
CIP (Ciprofloxacin)	29 $\pm$ 1.4	31 $\pm$ 1.1
CIP + P8	24 $\pm$ 1.4	26 $\pm$ 1.0
CIP + P8aq	24 $\pm$ 1.3	29 $\pm$ 1.1
CIP + P8ea	28 $\pm$ 1.5	32 $\pm$ 0.9
CIP + P8chl	28 $\pm$ 1.3	30 $\pm$ 0.9

**Figure 2.**

Comparative disc diffusion aspects of the four great willowherb extracts against standard *S. aureus* ATCC 25923 (a) and ATCC 6538 (b) strains

As resulted from Table I, tested alone, the four great willowherb extracts were effective on both standard strains of *S. aureus* ATCC 25923 and ATCC 6538 (Figure 2, a and b), the most active being the whole extract (P8) and the selective aqueous fraction (P8aq). Concerning the combinations antibiotics + vegetal extracts, on *Staphylococcus aureus* ATCC 25923, the most

important stimulatory effects were noticed for the combination with Tetracycline (Figure 3a), mainly in the case of whole extract (P8), ethyl acetic (P8ea) and chloroformic (P8chl) selective fractions; with Gentamicin (CN) there were obtained inhibitory effects (Figure 3b), especially for the aqueous selective fraction (P8aq) and the whole extract (P8).

**Figure 3.**

Stimulatory *versus* inhibitory effects of great willowherb extracts in combination with Tetracycline (TE) (a) and Gentamicin (CN) (b) on *S. aureus* ATCC 25923

In the case of *Staphylococcus aureus* ATCC 6538 strain, the most important stimulatory effects were recorded for the combination with Ampicillin (Figure 4a), especially aqueous (P8aq) and chloroformic (P8chl) selective fractions and with Tetracycline (Figure 4b), especially ethyl acetic

(P8ea) and chloroformic selective fractions (P8chl). In combination with Gentamicin (Figure 4c), there were obtained inhibitory effects with whole extract (P8) and aqueous (P8aq) and ethyl acetate (P8ea) selective fractions and stimulatory effects with chloroform selective fraction (P8chl).

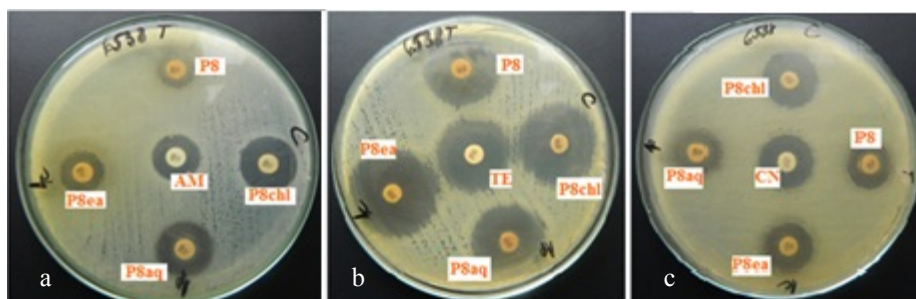


Figure 4.

Stimulatory versus inhibitory effects of great willowherb extracts in combination with Ampicillin (AM) (a), Tetracycline (TE) (b) and Gentamicin (CN) (c) on *S. aureus* ATCC 6538

Subsequently studies on the growth of (five) laboratory collections MRSA strains were roughly in accordance with previous results, on standard strains; there were evidenced stimulatory effects between great willowherb extracts and Ampicillin (AM) ($p = 0.04$). Statistically significant differences were also obtained for the combination of extract / fractions with Gentamicin (CN) ($p = 0.01$) and Ciprofloxacin (CIP) ($p = 0.02$), in these cases being recorded smaller inhibition zones compared to those of the antibacterial substances used alone (inhibitory effects). Although it was noticed the inhibition of Nalidixic acid (NA) antimicrobial effect, the statistical analysis did not reveal significant differences between the mean of the inhibition areas of NA and the mean of the inhibition areas obtained for the combinations of NA with the extract/fractions ($p > 0.05$).

Other studies [2] on *E. (Chamerion) angustifolium* (L.) Holub, *E. hirsutum* L., *E. palustre* L., *E. tetragonum* L. and *E. rosmarinifolium* Haenke extracts indicated that all extracts showed antimicrobial activity in a range of concentrations between 10 and 650 $\mu\text{g/mL}$ of dry extract, *E. (Chamerion) angustifolium* (L.) Holub and *E. rosmarinifolium* Haenke showing the broadest spectrum of action inhibiting bacteria, yeasts and also fungi.

Conclusions

The microbiological screening revealed that great willowherb whole extracts and selective fractions obtained from it have antimicrobial effects, both on *Staphylococcus aureus* standard strains and wild MRSA strains. The combinations between great willowherb extract and fractions and Ampicillin or

Tetracycline led to the increase of the antimicrobial effect against *Staphylococcus aureus* standard strains, while through the combinations with Gentamicin, Nalidixic acid or Ciprofloxacin the effect was contrary, namely the decrease of the antimicrobial effect. Taking also into account the HPTLC data, microbiological results suggest an augmented antimicrobial potency of the combination kaempferol-caffeic acid derivatives (P8aq/aqueous fraction) than myricetin-gallic acid derivatives (P8ea/ethyl acetic fraction); a synergistic activity was also noticed in the case of both fractions, P8aq and P8ea, respectively, but also in the case of non-polar, chloroformic (P8chl) fraction, shown as lacking in polyphenolic compounds.

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