

ANTIBACTERIAL ACTIVITY OF ETHANOLIC EXTRACTS FROM *Agrimonia eupatoria* L. AND *Epilobium hirsutum* L. HERBA

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Abstract

Given the growing concern regarding bacterial resistance to antibiotics, it is important to investigate alternative antibacterial compounds, such as phenols and flavones from natural sources. In this context, the aim of the present study was to evaluate the antibacterial activity of two indigenous medicinal plants from Romania against some Gram positive and Gram negative bacteria. Ethanolic extracts (70% v/v) from the aerial parts of *Agrimonia eupatoria* L. and *Epilobium hirsutum* L. were obtained, their total phenols content was determined using Folic-Ciocalteu assay as it is described by the Romanian Pharmacopoeia. The qualitative assay of the two ethanolic extracts was done by high performance thin layer chromatography (HPTLC) and their antibacterial activity was assessed using the agar diffusion method and minimum inhibitory concentration determination against four pathogenic bacteria: *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538 and *Staphylococcus epidermidis* ATCC 12228. Our results showed that both extract contain caffeic acid and some of its derivatives, however the flavones content differs: while *A. eupatoria* contains several quercetin and luteolin derivatives, *E. hirsutum* is rich in myricetin derivatives. The antibacterial activity tests showed better results against the Gram positive bacteria such as *Staphylococcus epidermidis*, especially in the case of great willowherb (*Epilobium hirsutum* L.).

Key words: antibacterial activity, phenols, plant extract.

INTRODUCTION

Although the use of medicinal plant is known for centuries, mainly starting in the second part of the XXth century the scientific research was focused on the mechanisms through which these plants exert their biologic activity. In doing so, it was proven that plant polyphenols can interact with numerous biomolecules, thus leading to different biological activities. In addition, they are non-narcotic, biodegradable, they have very few to none side effects and are not toxic to the environment (Biswas et al., 2013; Shrestha et al., 2013). In a recent study, Farias et al. (2013) showed that between 1981 and 2007 half of the new drugs approved by the FDA contained natural compounds such as flavones, phenols, lactones or saponins. Also, it was estimated that 74% of the plant derived pharmacologically active components now used as therapeutics were discovered by studying plants that were used in traditional medicine (Gibbons, 2003).

Considering this recent increasing interest in medicinal plants, we focused our attention on two indigenous plants from Romania, which are used in traditional medicine. First, *Agrimonia eupatoria* L. (fam. *Rosaceae*), commonly known as agrimony – used primarily as a mild antiseptic and astringent, recommended for sore throat and gastro-intestinal disorders. It also has good antioxidant properties and was proven to help with lipid metabolism in young healthy humans (Ivanova et al., 2013). Agrimoniin, an important flavone found in agrimony has anti-tumor activity, increases the production of interleukin-1 and has antibacterial activity against *Helicobacter pylori* and *Campylobacter jejuni* (Murayama et al., 1992; Funatogawa et al., 2004; Cwikla et al., 2010; Ad’hiah Ali et al., 2013).

Epilobium hirsutum (fam. *Onagraceae*), commonly known as great willowherb is known in the Romanian traditional medicine for its high concentration of flavones (Barakat et al., 1997) and is used for treating fever or pain, but also

for benign prostatic hyperplasia, the extract from this plant being useful for inhibiting the proliferation of PZ-HVP-7 human prostate cell line (Tita et al., 2001; Vitalone et al., 2003; Miano et al., 2008). Further studies determined that *E. hirsutum* contains acidic saponins, anthocyanidines, vitamin C, several minerals and microelements thus having immune-stimulating effects, antimicrobial and anti-cancer properties (Battinelliet al., 2001; Pakravan et al., 2011). A more recent study, Celik et al. (2016) suggests that the extract from the great willowherb may interfere with the activity of CYP P450 enzyme and recommends caution when combining it with other drugs.

Considering all of this, the aim of the present study was to evaluate the antibacterial activity of *A. eupatoria* and *E. hirsutum* and to determine the phytochemical constituents that are responsible for this activity.

MATERIALS AND METHODS

Plant material

Agrimonia eupatoria L. was purchased from a Romanian Plant Product Company, while *Epilobium hirsutum* L. was harvested from the Sinaia region in August. Both specimens were identified by the botanists at the *National Institute for Chemical and Pharmaceutical Research and Development* (ICCF) and voucher specimens are deposited at ICCF *Plant Material Storing Room*.

Ethanolic extracts – obtaining and characterisation

Briefly, 100 g of powdered vegetal material (aerial parts) were twice heat assisted (1 hour, continue stirring) extracted in 1000 ml 70% ethanol. Afterwards, the extracts were filtered through filter paper and used as such for analytical studies. For microbiological investigations, the two extracts were concentrated at residue and then solved in 20% propylene glycol to a final concentration of 5 mg total phenols (gallic acid equivalents) per 1 ml sample (5 mg GAE/ml).

Total phenols content determination

Total phenols content was estimated by the Folin-Ciocalteu assay as described in the *Romanian Pharmacopoeia*. Briefly, 50-100 μ l of vegetal sample was mixed with 200 μ l Folin-Ciocalteu reagents and completed to a final

volume of 5 ml with sodium carbonate 5%. After mixing the solution and 5 min incubation at room temperature in the dark, the optical density was measured at a wavelength of 750 nm. Gallic acid standard calibration curve was used ($r^2=0.9989$) and the results were expressed as gallic acid equivalents/ ml sample (GAE/ml).

HPTLC assay

For the qualitative determination, the HPTLC method was used, as previously described (Nicu et al., 2016). Briefly, volumes measuring from 0.5 to 3 μ l vegetal extract, as well as reference samples were loaded as 8 mm band length in the 10 \times 10 cm silica gel 60F HPTLC plate (Merck, Darmstadt, Germany) using Linomat 5 CAMAG instrument (Muttentz, Switzerland). Afterwards, the plates were kept in a TLC twin developing chamber at 18–19°C with the mobile phase (ethyl acetate–acetic acid–formic acid–water/100:12:12:26) until it reached a length of 90 mm. The developed plate was then dried and immersed in identification reagents (Natural Product followed by PEG4000). Finally, the plate was disposed in a photo-documentation chamber, and the images were taken at UV 366 nm. Spots' assignment was done using reference compounds data and plant product literature (Wagner and Bladt, 1996; Reich and Schibli, 2008)

All chemicals and reagents for these experiments were purchased from Fluka and Sigma-Aldrich Co (Bucharest, Romania).

Antibacterial activity

Four bacterial strains were used in this study: two Gram negative - *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027; two Gram positive – *Staphylococcus aureus* ATCC 6538 and *Staphylococcus epidermidis* ATCC 12228. All strains were purchased from Mecconti (Merck Romania S.R.L.) and were activated by culturing the bacterial cells on casein soya agar medium (Merck Romania S.R.L.) (CaSoA) and incubated for 24 h at 35°C.

The agar diffusion method was performed as described in our previous work (Nicu et al., 2016), briefly 15-20 ml of culture medium inoculated with 10^4 - 10^5 CFU/ml (colony forming units) of the respective test bacteria was poured in a Petri dish with a 90 mm diameter. After the medium solidified at room temperature, 4 stainless steel cylinders (8mm diameter) were placed on the surface of the medium and the

tests samples were added in the cylinders (0.2 ml sample/ cylinder). Finally, the Petri dishes were incubated at 35°C for 24 h and then the growth inhibition zones were measured. The interpretation of the results was made after the *Romanian Pharmacopoeia* as follows: <10 mm – no activity, 10-15 mm – weak activity, 16-20 mm – good activity, >20 mm – certain antibacterial activity.

The minimum inhibitory concentration (MIC) was determined using the microdilution method. In 96-well plates, 80 µl of casein soya broth medium (Merck Romania S.R.L.) (CaSoB) were added, previously inoculated with 10⁴-10⁵ CFU/ml of the respective test bacteria and 80 µl of the test sample. Serial dilutions were obtained for every extract and bacteria combination from 2500 to 156.25 µg GAE/ml. The plates were then incubated for 24 h at 35°C and the optical density was read at 600 nm.

Statistical analysis

Results were expressed as mean values of three measurements ± standard deviation (SD).

RESULTS AND DISCUSSIONS

Characterisation of the extracts

As mentioned before, the two extracts were analysed by HPTLC in order to determine the phenolic and flavonoid components. The results for *Agrimonia eupatoria* L. are shown in Figure 1.

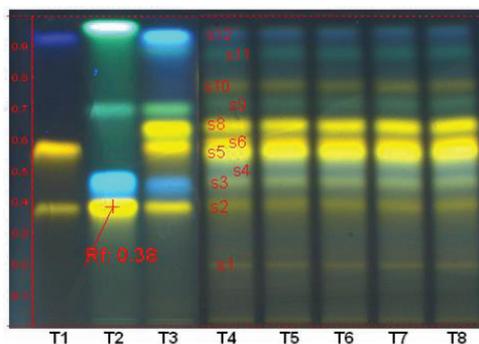


Figure 1: Polyphenols profile of the *Agrimonia eupatoria* ethanolic extract (Pirvu et al., 2016b)

T1 track – quercetin-3-O-rutinoside/ rutin, quercetin-3-O-galactoside/ hyperoside and protocatechuic acid (ref.); T2 track – rutin, chlorogenic acid, hyperoside, luteolin-7-O-glucoside/ cynaroside, apigenin-8-C-glucoside/ vitexine and caffeic acid (ref.);

T3 track – rutin, chlorogenic acid, apigenin-7-O-glucoside/ cosmosiin and kaempferol (ref);

T4-T8 tracks: *Agrimonia eupatoria* ethanolic extract.

As it can be seen in Figure 1, there are several flavonoid compounds observed, such as the yellow-orange fluorescent spots (s2, s6, s10) that were attributed to quercetin derivatives such as rutin, hyperoside and quercetin; the yellow fluorescent spots (s3, s5, s8) attributed to isororientin and orientin and also apigenin (green fluorescent spot s9) or kaempferol (blue-green fluorescent spot s11). As far as phenolic compounds are concerned, they can be seen in smaller quantities in spots 4 and 12 – neochlorogenic (blue fluorescent) and caffeic acid (blue-marine fluorescent).



Figure 2: Polyphenols profile of the *Epilobium hirsutum* ethanolic extract (after Pirvu et al., 2014)

T1 track – rutin, caffeic acid, chlorogenic acid

T2 track – *Epilobium hirsutum* ethanolic extract

Based on Figure 2 and the measured Rf, it can be stated that five red-orange fluorescent spots (s2, s3, s5, s6, s7) were identified as myricetin derivatives, while the indigo fluorescent ones (s1, s4, s8) and the blue fluorescent spot are caffeic and gallic acids derivatives.

Antibacterial activity

All antibacterial activity tests were performed using extracts in 20% propylene glycol at a final concentration of 5 mg GAE/ml vegetal sample. The results for the agar diffusion assay are presented in Table 1.

Table 1: Antibacterial activity of plant extracts

Sample	Bacterial strain	Inhibition zone (mm)
<i>Agrimonia eupatoria</i> extract	<i>Staphylococcus aureus</i> ATCC 6538	<8
	<i>Staphylococcus epidermidis</i> ATCC 12228	15±0.16
	<i>Escherichia coli</i> ATCC 8739	<8
	<i>Pseudomonas aeruginosa</i> ATCC 9027	<8
<i>Epilobium hirsutum</i> extract	<i>Staphylococcus aureus</i> ATCC 6538	17±0.15
	<i>Staphylococcus epidermidis</i> ATCC 12228	17.66±0.577
	<i>Escherichia coli</i> ATCC 8739	17±0.15
	<i>Pseudomonas aeruginosa</i> ATCC 9027	18.33±0.577

Diameter of the inhibition zones is given here as mean ± standard deviation.

As it can be seen in Table 1, the polyphenolic extract from *Epilobium hirsutum* has a more potent activity against all four bacterial strains tested than *Agrimonia eupatoria*. It is interesting to mention that although in general Gram positive bacteria tend to be more susceptible to antibacterial agents due to the lack of an outer membrane that can act as a barrier, in this case *E. hirsutum* shows virtually the same potency against both Gram positive and negative bacteria.

Due to the fact that the agar diffusion assay is known to have some limitations, such as the very high probability that the components of a mixture exhibit different diffusion rates and therefore can give uncertain results (Silva et al., 2005), this method is recommended only as a preliminary screening and so MIC determination was also carried out in this study. It is important to mention that some of this results were previously published before (Pirvu et al., 2014; Pirvu et al., 2016b), however the results are shown here for an easier comparison between the two plants in discussion and for an easier transition to the MIC determination part of the study, the results of which are shown in Table 2. We chose MIC determination because is a more sensitive method than the diffusion assay and it allows the use of small quantities of extract (Langfield et al., 2004).

Table 2: MIC determination for plant extracts, expressed as phenolic compounds value

Sample	Bacterial strain	MIC (µg/ml)
<i>Agrimonia eupatoria</i> extract	<i>Staphylococcus aureus</i> ATCC 6538	625
	<i>Staphylococcus epidermidis</i> ATCC 12228	625
	<i>Escherichia coli</i> ATCC 8739	1250
	<i>Pseudomonas aeruginosa</i> ATCC 9027	312.5
<i>Epilobium hirsutum</i> extract	<i>Staphylococcus aureus</i> ATCC 6538	156.25
	<i>Staphylococcus epidermidis</i> ATCC 12228	625
	<i>Escherichia coli</i> ATCC 8739	625
	<i>Pseudomonas aeruginosa</i> ATCC 9027	312.5

Regarding MIC determination, it can be easily seen from Table 2 that the lower values were determined also in the case of *E. hirsutum*, with emphasis on *S. aureus* and *P. aeruginosa*, proving once again that this extract has good antibacterial activity against both Gram positive and Gram negative bacteria.

What is surprising is that *A. eupatoria* presents a MIC value of 312.5 µg/ml against *P. aeruginosa* although it showed no activity when the agar diffusion method was used. This could probably be due to some compounds that cannot migrate in the agar medium, however further studies are necessary in order to test this theory. The results obtained against the other three bacterial strains are in correlation with the first results, showed in Table 1.

Other study reports confirm our findings, in the sense that low MIC values are not always correlated with high activity in the agar diffusion assay (Lourens et al., 2004). Furthermore, another recent study obtained slightly different results in the MIC determination of *Epilobium hirsutum* ethanolic extract against some of the same bacteria, such as *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228 or *Escherichia coli* ATCC 8739 (Pirvu et al., 2016a). However, the differences are not major and they may be due to the different method used for MIC determination, the conclusion being the same, that the great willow herb is a promising source for natural antibacterial products.

CONCLUSIONS

The present study aimed at investigating the antibacterial activity of two indigenous species of medicinal herbs from Romania: *Agrimonia eupatoria* L. and *Epilobium hirsutum* L. We proved that the ethanolic extracts from these two species contain several different phenolic compounds, some which are common such as caffeic acid and its derivatives, while some others are specific to just one plant, such as the myricetin derivatives from the great willowherb or the quercetin and apigenin derivatives found in agrimony.

The qualitative assay showed some differences in the chemical composition of the two extracts, differences that were observed also in the second part of the study, determining the antibacterial activity. While agrimony only showed weak activity against *Pseudomonas aeruginosa* and a MIC of 312.5 µg/ml, the great willowherb had moderate activity against all four bacterial strains used for testing and lower MIC values, especially against *Staphylococcus aureus* (156.25 µg/ml) and *Pseudomonas aeruginosa* (312.5 µg/ml), thus proving efficient on Gram negative and Gram positive bacteria.

These results are a good basis for justifying the need for further studies in order to better understand the mechanism through which these two plants exert their antibacterial activity and how to better use them in treating infections.

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