ORIGINAL ARTICLE

INVESTIGATION OF ANTIOXIDANT AND ANTIMICROBIAL POTENTIAL OF SOME EXTRACTS FROM *HEDERA HELIX* L.

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Abstract

The aim of this study was the investigation of the antioxidant and the antimicrobial potential of some ethanolic extracts from *Hedera helix* (leaves, flowers, immature and ripe fruits) and the correlation with the profile of polyphenols, using an LC/MS method. In all samples, the chlorogenic acid, isoquercitrin, rutin and quercetin have been identified. The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) were used to evaluate the antioxidant effects and the results showed a better antioxidant activity for *H. helix* ripe fruits ethanolic extract and a positive correlation between antioxidant effects, polyphenolic and flavonoid contents. The antimicrobial effect was tested using six bacterial strains and the microdilution method. The immature fruits extract showed a significant antibacterial activity against *Staphylococcus aureus*, while both immature fruits and flowers extracts possess a good antibacterial activity against *Listeria monocytogenes*.

Rezumat

Scopul acestui studiu a constat în testarea potențialului antioxidant şi antimicrobian ale unor extracte etanolice din frunze, flori, fructe verzi și coapte de *Hedera helix*, precum și corelarea rezultatelor cu profilul polifenolilor determinat prin LC/MS. În toate probele au fost identificați: acidul clorogenic, isoquercitrina, rutozida și quercetolul. Pentru evaluarea efectului antioxidant s-a utilizat metoda DPPH (2,2-difenil-1-picrilhidrazil) și s-a evaluat conținutul total în polifenoli și flavonoide totale. Rezultatele au evidențiat o acțiune antioxidantă mai bună pentru extractul din fructe coapte de *H. helix*, ceea ce a fost în concordanță cu datele experimentale obținute la calculul conținutului de compuși polifenolici și de flavonoide. Efectul antimicrobian a fost testat pe șase tulpini bacteriene, prin tehnica microdiluțiilor. Extractul obținut din fructe imature a avut o activitate antibacteriană semnificativă pe *Staphyloccocus aureus*. De asemenea, extractul din fructe imature și cel din flori au prezentat o activitate antibacteriană bună pe *Listeria monocytogenes*.

Keywords: polyphenols, *Hedera helix*, antioxidant, antimicrobial, LC/MS

Introduction

The structural class of polyphenols comprises more than 8000 compounds, generally identified in higher plants. Throughout the past ten years, researchers have become very interested in plants containing polyphenols, so the literature abounds in scientific publications emphasizing the positive effects associated to these compounds.

Polyphenols are naturally occurring biomolecules, secondary metabolites biosynthesized in plants. Their

role is to defend plants against ultraviolet radiation and aggression of pathogen microorganisms. It is scientifically proved that plants with high content in polyphenols are efficient for the prevention of some diseases associated with oxidative stress such as cancer, neurodegenerative and cardio-vascular diseases [4, 6]. Additionally, the polyphenols also have the capacity to reduce the number of tumours and their growth in some types of cancer and may possess an antidiabetic effect. The polyphenols modulate glycaemia through a complex mechanism that can be reduced to the inhibition of reabsorption of glucose in the gut [4, 6, 11].

Hedera helix L. (ivy or English ivy) is a member of *Araliaceae* family, well known as an ornamental plant, but also for its harmful effects such as contact dermatitis, gastrointestinal irritation, bloody diarrhoea and even death produced by the fresh fruits and leaves [17]. Yet, in the folk medicine it is used to treat the benign warts and for its antioxidant, antispasmodic and antiallergic properties. Various authors have reported the positive effect of dry extracts on respiratory functions of children with chronic bronchial asthma and other therapeutic effects such as: anti-bacterial, antihelmintic, leishmanicidic and antifungal properties [12, 13].

There is a direct correlation between the significant number of therapeutic properties and the chemical composition of ivy. Thus, multiple studies have indicated a complex chemical composition for ivy leaves: phenolic acids (caffeic, neochlorogenic, chlorogenic), flavonoids (quercetin, kaempferol, isoquercitrin), phytosterols (stigmasterol, sitosterol), polyacetylenes (falcarinose, falcarinol), hederagenin, oleanolic acid, hederasaponins, etc. [3, 5, 12].

Concerning the chemical composition of ivy fruits and flowers, there is little published data. Determination of fruits composition revealed the presence of triterpene saponins, fatty acids, β-lectins and polyacetylenes [3, 12].

The major goal of the present study is the investigation of LC/MS profile of polyphenols, antioxidant and antimicrobial potentials of *H. helix* ethanolic extracts obtained from leaves, flowers, immature and ripe fruits.

Materials and Methods

Plant material and extraction

Ivy (*H. helix L.*) was collected from the "Alexandru Borza" Botanical Garden of Cluj-Napoca, Romania (46°45′36″N and 23°35′13″E). The material was identified by Dr. M. Pârvu, "Babeş - Bolyai" University of Cluj-Napoca, Romania. A voucher specimen (CL 664210) is deposited at the *Herbarium* of "Babeş - Bolyai" University, Cluj-Napoca, Romania. The plant material was harvested in April 2014 (ripe fruit), in September 2014 (leaves and flowers) and in November 2014 (immature fruits).

Small fragments (0.5 - 1 cm) of fresh *H. helix* L. (ivy) were extracted with 70% ethanol (Merck, Germany) by cold repercolation method [6, 16], at room temperature, for 3 days [16]. The content of ivy extract (w/v; g/mL) was: 1/1.5 (for leaf extract), 1/1 (for immature fruit extract and ripe fruit extract) and 1/1.1 (for flower extract).

Determination of total polyphenols and flavonoid contents

The total phenolic content (TPC) of the extracts was measured using the Folin-Ciocâlteu method, with some modifications [18]. The absorbance was measured at 760 nm, using a JASCO UV-VIS spectrophotometer. Standard curve was prepared using different concentrations of gallic acid. TPC was expressed as mg gallic acid/g dry material plant (mg GAE/g plant material).

The total flavonoid content (TFC) was determined and expressed as rutin equivalents (mg RE/g plant material), using the method described in the Romanian Pharmacopoeia (X^{th} Edition) [20]. The absorbance was measured at 430 nm.

LC/MS analysis of polyphenolic compounds

For the qualitative and quantitative determination of polyphenols we used an HPLC-MS method. The experiment was carried out using an Agilent 1100 Series HPLC system (Agilent, USA) consisting of a G1322A degasser, G1311A binary gradient pump and a G1313A autosampler and a UV detector. The chromatographic separation was achieved using a reversed-phase analytical column (Zorbax SB-C18 100 mm x 3.0 mm i.d., 3.5 µm particle) maintained at 48°C. The mobile phase consisted of a binary gradient: methanol and acetic acid 0.1% (v/v). The mobile phase was delivered with a flow rate of 1mL/min and the injection volume was 5 µL. The detection of polyphenols was performed on UV (330 nm and 370 nm) and MS mode. The MS system operated using an ion trap mass spectrometer with electrospray negative ionization. The chromatographic data were processed using Chemstation and Data Analysis software from Agilent, USA. Also, the calibration curves in the 0.5 - 5 µg/mL range showed good linearity ($R^2 < 0.999$) for a five point plot [7, 8, 10].

DPPH assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used to evaluate the radical scavenging activity, by bleaching of purple methanolic solution of the stable radical. The antioxidant effect implies the disappearance of the DPPH absorption through the action of antioxidants. 20 µL of diluted extracts were added to 980 µL DPPH solution (100 µM). After 30 min incubation period, the decrease in absorbance was measured at 517 nm, using a UV-VIS JASCO V-530 spectrophotometer. Both hydrophilic

and lipophilic synthetic antioxidants, quercetin and butylated hydroxytoluene (BHT) were used as standards. The percentage inhibition of the DPPH radical after adding individual samples was calculated using the following equation: $I = 100 (A_c - A_s)/A_c$, where: I – DPPH inhibition (%), A_c - absorbance of the control sample, A_s - absorbance of the tested sample. The antioxidant activity was also expressed as inhibitory concentration IC_{50}, defined as the concentration of the sample required to cause a 50% decrease in initial DPPH radical absorbance. IC_{50} values in DPPH assay were calculated graphically. All experiments were performed in triplicate [1, 19].

Antibacterial activity

Microorganisms and culture conditions. For the bioassay, six bacterial strains were used: three Gram positive bacteria: *Staphylococcus aureus* (ATCC 49444), *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19114) and three Gram negative bacteria: *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028) and *Escherichia coli* (ATCC 25922). All of the tested microorganisms were obtained from Food Biotechnology Laboratory, Life Sciences Institute, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania. The bacteria were cultured on Müller-Hinton agar. Cultures were stored at 4°C and sub-cultured once a month. For the antimicrobial activity evaluation, the obtained extract was evaporated to dryness under reduced pressure at 30°C and re-suspended in 1 mL of bi-distilled water.

Microdilution method. The modified microdilution technique was used to evaluate the antimicrobial activity. Anaerobic bacteria were cultured overnight at 37°C on Tryptic Soy Broth (TSB) medium. The bacterial cell suspensions were adjusted with sterile saline to a concentration of approximately 2×10^5 colony-forming unit (CFU)/mL in a final volume of 100 µL per well. The inoculum was stored at 4°C for further use. Dilutions of the inoculums were cultured on solid Müller-Hinton (MH) for bacteria to verify the absence of contamination and to check the validity of the inoculums. Determinations of the minimum inhibitory concentrations (MICs) were performed by a serial dilution technique using 96 - well microtitre plates. Different dilutions of the ethanolic extracts were carried out over the wells containing 100 µL of Müller-Hinton (MH) broth and afterwards, 10 µL of inoculum was added to all the wells. The microplates were incubated for 24 - 48 h at 37°C. The MIC of the samples was detected following the addition of 20 µL (0.2 mg/mL) of resazurin solution to each well and the plates were incubated 2 h at 37°C. A change from blue to pink indicates reduction of resazurin and therefore bacterial growth. The MIC was defined as the lowest drug concentration that prevented this colour change. The minimum bactericidal concentrations (MBCs) were determined by serial sub-cultivation of a 2 µL into microtitre plates containing 100 µL of broth per well and further incubation for 48 h at 37°C. The lowest concentration with no visible growth was defined as MBC, indicating 99.5% killing of the original inoculum. Streptomycin (Sigma P 7794, Santa Clara, CA, USA) (0.05 - 3 mg/mL) was used as positive control for the bacterial growth. A 10% solution of ethanol in water was used as negative control [7, 8].

Results and Discussion

Polyphenols analysis

The total phenolic content and total flavonoid content of the extracts varied considering the natural product, with higher amounts in ripe fruits extract (2.17 mg GAE/g and 1.75 mg RE/g, respectively), followed by flowers extract (1.64 mg GAE/g and 1.33 mg RE/g, respectively), immature fruits extract (1.28 mg GAE/g and 1.02 mg RE/g, respectively), and leaves extract (1.28 mg GAE/g and 1.02 mg RE/g, respectively). The concentrations of the total polyphenols (1.03 - 2.17 mg GAE/g) and flavonoids (0.87 - 1.75 mg RE/g) are presented in Table I.

Table I

TPC and TFC in *H. helix* extracts (± standard deviation (SD))

Extract	TPC (mg GAE/g)	TFC (mg RE/g)
H. helix leaves	1.03 ± 0.09	0.87 ± 0.01
H. helix flowers	1.64 ± 0.11	1.33 ± 0.1
H. helix immature fruits	1.28 ± 0.08	1.02 ± 0.07
H. helix ripe fruits extract	2.17 ± 0.14	1.75 ± 0.12

Previous work on *H. helix* has shown that aqueous methanol extract of ivy stems is related with the presence of triterpenes, flavonoids, tannins and saponins [14]. Other phytochemical investigations of our research group on *H. helix* harvested from Romania showed a high correlation between all anti-oxidant methods used. The extract obtained from the flowers presented a higher content in the total poly-phenols then those from the fruits and leaves, which is in accordance with the results of our study [9].

The polyphenolic profile obtained in this work was similar to that presented by Bahafar *et al.* on other *Hedera* species, *H. pastuchovii* [2]. TPC and TFC of the berry extract (213.5 and 54.2, respectively) were approximately two times higher than those found in the leaf extract (121.4 and 39.3, respectively). Their results were expressed in mg GAE/g of dry extract, which can explain the difference in amounts compared with our study.

In order to determine the LC/MS polyphenolic profile, 18 polyphenolic compounds were used as standards: caffeic, chlorogenic, caftaric, gentisic,

ferulic, sinapic, p-coumaric acids, quercitrin, isoquercitrin, quercetin, rutin, myricetin, hyperoside, fisetin, patuletin, luteolin, kaempferol and apigenin. The results are summarized in Table II. The concentrations of the identified polyphenols are organized in the order of their retention times. MS data and retention times were compared to those of the reference standards.

Table II

The polyphenolic compounds content in the analysed *H. helix* extracts (µg/mL)

Polyphenolic compound	m/z	R_T ± SD (min)	Leaves extract	Flower extract	Fruits extract	
					Immature	Ripe
Chlorogenic acid	353	5.62 ± 0.05	126.400	530.894	118.775	59.740
p-Coumaric acid	163	9.48 ± 0.08	NF	3.571	NF	NF
Ferulic acid	193	12.8 ± 0.10	NF	0.759	2.680	0.810
Hyperoside	463	19.32 ± 0.12	NF	NF	NF	1.440
Isoquercitrin	463	19.60 ± 0.10	1.583	50.898	5.590	33.021
Rutin	609	20.20 ± 0.15	124.663	278.011	48.954	183.746
Quercitrin	447	23.64 ± 0.13	NF	8.404	0.365	NF
Quercetin	301	26.80 ± 0.15	0.284	1.220	0.394	3.862
Kaempferol	285	32.48 ± 0.17	NF	2.937	0.283	0.947

Note: NF-not found, below limit of detection. Values are the mean ± SD (n = 3)

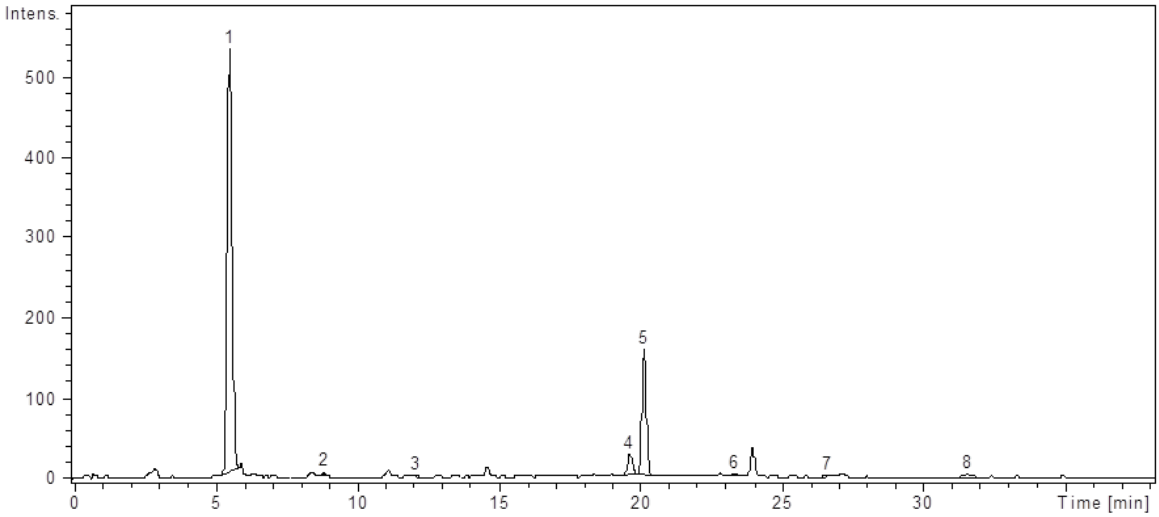

Figure 1.

The UV chromatogram of *H. helix* flowers extract. Identified compounds: 1, Chlorogenic acid; 2, *p*-Coumaric acid; 3, Ferulic acid; 4, Isoquercitrin; 5, Rutin; 6, Quercitrin; 7, Quercetin; 8, Kaempferol.

The only phenolic acid identified and quantified in the ethanolic extract of *H. helix* leaves was chlorogenic acid (126 µg/mL). Two flavonoid glycosides, isoquercitrin and rutin were identified in the same extract, while the class of flavonoid aglycons was represented by quercetin. These results are not in accordance with those obtained by our research group by analysing *H. helix* leaves harvested on September 2011. The amount of rutin was much smaller (34 µg/mL) compared to the amount quantified in the plant material collected in September 2014 (124.663 µg/mL) [9].

The flowers of *H. helix* are the richest in phenolic acids. Thus, among these substances, there were quantified chlorogenic, *p*-coumaric and ferulic acids, with the first being the most abundant (530.894 µg/mL). The dominant flavonoid was rutin (278.011 µg/mL) followed by isoquercitrin. Also, we identified one flavonol, quercetin, but in a minor amount (1.220 µg/mL). For the plant material collected in September 2011, the chlorogenic acid was absent, while the quantity of rutin was inferior (130 µg/mL). Yet, the amount of quercetin and kaempferol was superior, 7.11 µg/mL and 7.90 µg/mL respectively [9], compared to 1.22 µg/mL and 2.937 µg/mL respectively, quantified in *H. helix* flowers harvested in 2014. It is difficult to find an explanation for all these differences, considering that we used the same protocol for extracts preparation, but in the same time we have to take into consideration that environmental factors have a major effect on the polyphenol contents. These factors may be pedoclimatic (ground type, sun exposure, rain fall). Apparently, exposure to light has a considerable effect on most flavonoids [4].

The analysis of ivy fruits extracts revealed interesting results. In both fruits, immature and ripe, were quantified two phenolic acids, chlorogenic and ferulic acids, but the concentrations of these compounds were

higher in immature fruits. Concerning the flavonoid glycosides, isoquercitrin and rutin, the ripe fruits are richer than the immature fruits, while hyperoside was quantified only in ripe fruits. These results support the observations published by Manach *et al.* 2004 [4]. According to them, the degree of ripeness strongly affects the concentration and proportion of polyphenolic compounds. It has been observed that phenolic acid content decreases during ripening and the degree of ripeness at the time of harvest, other environmental factors and also processing and storage of plant material have a major effect on polyphenols concentration.

Antioxidant activity assay

In order to evaluate the ability of ivy extracts and synthetic antioxidants quercetin and BHT to donate the hydrogen atom, the stable free radical DPPH was used. All extracts obtained from *H. helix* were able to reduce DPPH radical with different degrees of scavenging activity. A lower IC_{50} value represents a higher bleaching effect, thus a better antioxidant activity.

The results obtained for the evaluation of the anti-oxidant activity using the DPPH bleaching assay are presented in Table III.

The strongest antioxidant was the positive control quercetin, with IC_{50} value of 5.59 µg/mL. All analysed extracts showed lower DPPH scavenging activity compared to both reference compounds, quercetin and BHT. The highest radical scavenging activity was determined for *H. helix* ripe fruits extract (68.55 ± 4.21 µg/mL), with positive correlation between the scavenging activity on DPPH, the total phenolic content and the total flavonoid content. This might be related to the presence of higher amounts of phenolic compounds in berries and indicates that these compounds contribute to the antioxidant effects of the natural product. The different antioxidant activities between these extracts may be due to the variability of composition and content in various active compounds, and also to the synergy between the natural substances.

Table III

Antioxidant activity for *H. helix* extracts

Sample	IC_{50} (µg/mL)
H. helix leaves extract	122.47 ± 9.31
H. helix flowers extract	82.59 ± 6.44
H. helix immature fruits extract	94.72 ± 7.68
H. helix ripe fruits extract	68.55 ± 4.21
Quercetin	5.59 ± 0.13
BHT	15.88 ± 1.06

Note: Values are the mean ± SD (n = 3).

Considering the obtained results, the following order regarding the antioxidant activities was established: *H. helix* leaves extract < *H. helix* immature fruits extract < *H. helix* flowers extract < *H. helix* ripe fruits extract < BHT < quercetin. According to this method, the ethanolic extract of *H. helix* ripe berries showed a good antioxidant activity (IC_{50} = 68.55 µg/mL).

These findings are in agreement with Bahafar *et al.* [2], who reported IC_{50} values of 87.10 µg/mL and 139.02 µg/mL for berry and respectively leaf extracts obtained from *H. pastuchovii*.

Antimicrobial activity assays

The *in vitro* antibacterial potential of *H. helix* extracts against both Gram-positive and Gram-negative bacteria is presented in Table IV.

Table IV

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *H. helix* extracts and streptomycin against bacterial strains tested with the microdilution method

Bacterial strain	Standard antibiotic	Minimum Inhibitory Concentration (mg/mL) / Minimum Bactericidal Concentration (mg/mL)			
	Streptomycin	Leaves	Flowers	Immature fruits	Ripe fruits
Staphylococcus aureus	0.03	1.25	0.15	0.078	1.25
	0.06	2.5	0.3	0.15	2.5
Bacillus cereus	0.015	1.25	1.25	1.25	1.25
	0.03	2.5	2.5	2.5	2.5
Listeria monocytogenes	0.015	0.62	0.15	0.15	0.62
	0.03	1.25	0.3	0.3	1.25
Pseudomonas aeruginosa	0.06	5	2.5	2.5	1.25
	0.12	10	5	5	2.5
Salmonella typhimurium	0.06	2.5	2.5	0.62	0.62
	0.12	5	5	1.25	1.25
Escherichia coli	0.12	2.5	1.25	1.25	1.25
	0.24	5	2.5	2.5	2.5

Note: Each value is the mean ± SD of three independent measurements.

As shown in Table IV, different plant parts exhibited antibacterial activity, but to varying extents (MIC values 0.078 - 5 mg/mL). The results indicate that the bacterial strain *Staphylococcus aureus* was the most sensitive to both *H. helix* immature fruits and flower extracts, with MIC values of 0.078 mg/mL and 0.15 mg/mL, respectively and MBC values of 0.15 mg/mL and 0.3 mg/mL, respectively. Also, these

two extracts present a good activity against *Listeria monocytogenes* with the same MIC value of 0.15 mg/mL, and the identical MBC value of 0.3 mg/mL. According to Salvat *et al.* [15], if MIC value of the plant extracts is less than/or around 0.5 mg/mL, the antibacterial activity is appropriate, so we conclude that *H. helix* immature fruits and flower extracts exhibited good antimicrobial activity against *S. aureus* and *L. monocytogenes*, while the growth inhibitory effect of all the other plant parts tested showed low activity against all evaluated bacterial strains.

Conclusions

This study revealed the antioxidant and antimicrobial activities of some ethanolic extracts from leaves, flowers, immature and ripe fruits of *H. helix*. The highest amount of phenolic and flavonoid compounds was identified in the ripe fruits extract; also, the same extract exhibited the best antioxidant activity. Regarding the results of the antibacterial test, the immature fruits extract showed a significant activity against *S. aureus*, followed by the flower extract with a good growth inhibitory effect against the same bacterial strain. Both immature fruits and flowers extracts possess appropiate antibacterial capacity against *L. monocytogenes*.

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