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CHEMICAL COMPOSITION AND ANTIFUNGAL ACTIVITY OF *HEDERA HELIX* LEAF ETHANOLIC EXTRACT

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The 50% ethanol extract obtained from *Hedera helix* leaves was investigated regarding the presence and quantity of polyphenols, sterols and *in vitro* antifungal activity against phytopathogenic fungi. The chemical analysis revealed the presence of rutin, quercetin and kaempferol in the non-hydrolysed sample and quercetin and kaempferol in the hydrolysed sample and stigmasterol in the ivy leaf extract (non-hydrolysed sample). The antifungal activity against phytopathogenic fungi (*Aspergillus niger, Botrytis cinerea, B. tulipae, Fusarium oxysporum* f. sp. *tulipae, Penicillium gladioli,* and *Sclerotinia sclerotiorum*) was assessed using an agar dilution assay. The results are expressed as the minimum inhibitory concentration (MIC = 10–14%) and were compared to a synthetic antifungal drug – fluconazole (MIC = 8–30%). This report presents the first screening of the antifungal activity of the ivy leaf extract on these plant pathogenic fungi species, aiming to use the ivy leaf extract for controlling different diseases of vegetables and ornamental plants, in addition to human disorders.

Keywords: Antifungal – *Hedera helix* – phytopathogenic fungi – polyphenols – sterols

INTRODUCTION

Hedera helix (ivy) is an evergreen woody liana (Araliaceae family) native to most of Europe and Western Asia. The leaves are alternate, evergreen with an accentuated polymorphism; thus, the juvenile leaves are palmate with 3–5 lobes, while the adult ones are cordate, rhomboid to ovate-lanceolate. The flowers produced from summer until late autumn are small, greenish-yellow and come in umbels of 3–5 cm in diameter. The fruits are small black berries that ripen in winter. It is a popular ornamental plant in many countries [13].

In addition to its ornamental value, ivy is used as a medicinal plant. The ivy leaf has been used since ancient times (Dioscorides and Hippocrates), but the phytotherapeutical books of the 16th century would describe very different indications. Steinmetz, in 1961, presumed that although the plant is decidedly poisonous (in large

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doses death can occur by respiratory paralysis), the leaves and berries administered in safe doses have some good uses in therapy [37].

The aim of the present research was to investigate the antifungal properties of the ivy leaf extract against plant pathogenic fungi and to identify and quantify the phenolic compounds and sterols from the leaf extract that are believed to be responsible for the antifungal activity.

MATERIALS AND METHODS

Plant material

Hedera helix was collected from the Alexandru Borza Botanical Garden of Cluj-Napoca (46°45′36″N; 23°35′13″E), and a voucher specimen (CL 664210) is deposited at the Herbarium of Babes-Bolyai University, Cluj-Napoca, Romania.

Plant extract preparation

The plant extract was prepared by a cold repercolation method at room temperature for 3 days [30] by using small fragments (0.5–1 cm) of ivy leaves, which were extracted with 50% ethanol (Merck, Bucuresti, Romania) in the Mycology Laboratory of Babes-Bolyai University, Cluj-Napoca, Romania. The Drug Extract Ratio (DER) was 1:1.

Preparation of fungal colonies

The plant pathogenic fungi chosen for the experiment cause important losses to vegetable and ornamental crops and have never been used to test the antifungal properties of ivy leaf extract. For this purpose, *Aspergillus niger* was isolated from onion bulbs, *Botrytis cinerea* from rose flowers, *B. tulipae* and *Fusarium oxysporum* f. sp. *tulipae* from tulip flowers and bulbs, respectively, *Penicillium gladioli* from gladiolus corms, and *Sclerotinia sclerotiorum* from carrot roots. All the fungal species were identified in the Mycology Laboratory (Babes-Bolyai University, Cluj-Napoca, Romania), by Dr. M. Parvu. The colonies were obtained in Petri dishes on Czapek-agar medium (BD Difco, Budapest, Hungary) by inoculation in the central point with a spore suspension (1×10^5 conidia $\times$ mL^{-1}) and an incubation at 22 °C for 5 days.

Determination of antifungal activity

The antifungal activity of the *Hedera helix* leaf extract, expressed as MIC, was assessed by the agar dilution assay and was compared to the antimycotic drug fluconazole (2 mg $\times$ mL^{-1}, Krka, Novo Mesto, Slovenia) and a control (nutritive medium

and ethanol). The results were interpreted by calculating the percentage of mycelial growth inhibition (*P*) at each concentration, by using the formula $P = (C - T) \times 100/C$, where *C* is the diameter of the control colony and *T* is the diameter of the treated colony [21].

Analysis of polyphenols from the ivy leaf extract

Apparatus and chromatographic conditions

The experiment was carried out using an Agilent 1100 HPLC Series system equipped with an autosampler G1311A. For the separation, a reversed-phase Zorbax SB-C18 analytical column (100 × 3.0 mm i.d., 3.5 µm particle) was used. The column was operated at 48 °C in a G1316A oven. For the gradient elution, a degasser G1322A and quaternary gradient pump G1311A were employed. The detection of all of the compounds was performed at 330 and 370 nm using G1316A diode array detector system, and the chromatographic data were processed with the Chemstation software (Agilent, USA). The mobile phase was prepared from methanol and acetic acid 0.1% ($V \times V^{-1}$). The elution began with a linear gradient (started at 5 to 42% methanol) for the first 35 min, followed by an isocratic elution with 42% methanol for the next 3 min.

The flow rate was 1 $mL \times min^{-1}$, and the injection volume was 5 µL. All of the solvents were filtered through 0.5 µm filters (Sartorius) and we degassed in an ultrasonic bath. For the liquid chromatography (LC) electrospray ionization (ESI) mass spectrometry (MS) analysis, an Agilent Ion Trap 1100 SL instrument was used. The MS was equipped with a Turbo-Ionspray (electrospray ionization) interface in negative ion mode. The ESI settings were as follows: negative ionization; ion source temperature: 350 °C; gas: nitrogen at 12 $L \times min^{-1}$; and nebulizer: 70 psi [11, 22, 24, 35].

Chemicals

Standards: chlorogenic acid, *p*-coumaric acid, caffeic acid, rutin, apigenin, quercitrin, isoquercitrin, hyperoside, kaempferol, quercetin, myricetol and fisetin were purchased from Sigma (Germany); ferulic acid, sinapic acid, gentisic acid, patuletin and luteolin from Roth (Germany); and cichoric acid and caftaric acid from Dalton (USA). Methanol of HPLC analytical-grade and hydrochloric acid of analytical-grade were purchased from Merck (Germany). Methanolic stock solutions (1 $mg \times mL^{-1}$) of the above standards were prepared, stored at 4 °C, and were protected from daylight. They were appropriately diluted with double-distilled water before being used as working solutions.

Identification and quantitative determination of the polyphenols

A high-performance liquid chromatographic (HPLC) method was developed for the determination of phenolic compounds from ivy extract [35]. The simultaneous analysis of different classes of polyphenols was performed by a single column pass, and the separation of all of the examined compounds was carried out in 35 min. To obtain more accurate data on the flavonoid glycosides and aglycones concentrations and to estimate the nature of the hydrolysed compounds, each sample was analysed once before and after the acid hydrolysis. The concentrations of the identified polyphenolic compounds were determined in all of the analysed samples before and after the acid hydrolysis. Two millilitres of the extract was treated with 2 mL of 2 M hydrochloric acid and 0.2 mL of the ascorbic acid solution 100 $mg \times mL^{-1}$, and the mixtures were heated at 80 °C in a water bath for 30 min, ultrasonicated for 15 min and heated for another 30 min at 80 °C. During the heating, 1 mL methanol was added to the extraction mixture every 10 min in order to ensure the permanent presence of methanol. The mixtures were centrifuged at 4000 rpm, and the solutions were diluted with distilled water in a 10 mL volumetric flask and were filtered through a 0.45 mm filter before injection.

Identification and quantitative determination of sterols

The LC-MS technique was also used to analyse the sterols from the ivy leaf extract. Thus, a Zorbax SB-C18 reversed-phase analytical column (100 × 3.0 mm i.d., 3.5 μm particle) operated at 40 °C was used. The separation was achieved using a mobile phase consisting of 10:90 (V/V) methanol and acetonitrile with a flow rate of 1 mL/min and an injection volume of 5 μL. Mass spectrometry analysis was performed on an Agilent Ion Trap 1100 VL mass spectrometer with atmospheric pressure chemical ionization (APCI) interface. The instrument was operated in a positive ion mode. The gas temperature (nitrogen) was 325 °C at a flow rate of 7 L/min, nebulizer pressure 60 psi and capillary voltage –4000 V. Three standards were used for the quantitative analysis, namely β-sitosterol, stigmasterol, and cholesterol.

Statistical analysis

The statistical analyses of the experimental data were performed in the R environment (version 2.14.1) using a one-way ANOVA and the post hoc Tukey HSD test for the significance of differences between treatments. P values ≤ 0.05 were considered to be statistically significant. The correlation analysis was performed by the Pearson test.

RESULTS

The analysis of polyphenols from the *Hedera helix* leaf extract revealed the presence of rutin, quercetin and kaempferol in the non-hydrolysed sample (Fig. 1) and quercetin and kaempferol in the hydrolysed sample (Fig. 2). The quantification of the polyphenols in the ivy leaf extract displayed a quantity of 120.655 ± 6.032 $\mu g \times mL^{-1}$ rutin, 1.550 ± 0.069 $\mu g \times mL^{-1}$ quercetin, and 1.278 ± 0.063 $\mu g \times mL^{-1}$ kaempferol in the non-hydrolysed sample, and 24.068 ± 1.203 $\mu g \times mL^{-1}$ quercetin, 7.513 ± 0.338 $\mu g \times mL^{-1}$ kaempferol in the hydrolysed sample, whereas the analysed sample of the ivy leaf extract contained 0.373 ± 0.018 $ng \times mL^{-1}$ stigmasterol.

Taking into consideration some of the most frequent diseases of vegetables and ornamentals, 6 phytopathogenic fungi (*Aspergillus niger, Botrytis cinerea, B. tulipae, Fusarium oxysporum* f. sp. *tulipae, Penicillium gladioli,* and *Sclerotinia sclerotiorum*) were selected for testing the antifungal properties of the *Hedera helix* leaf extract. The extract inhibited the germination and growth of the plant pathogenic fungi in a concentration-dependent manner. The minimum inhibitory concentration (MIC) varied between 10% and 14% ($P \leq 0.05$) for the leaf extract compared to 8% and 30% ($P \leq 0.05$) for fluconazole (Table 1). These results are comparable with those obtained in our previous study for the ivy flower extract (MIC = 8–12%) and fruit

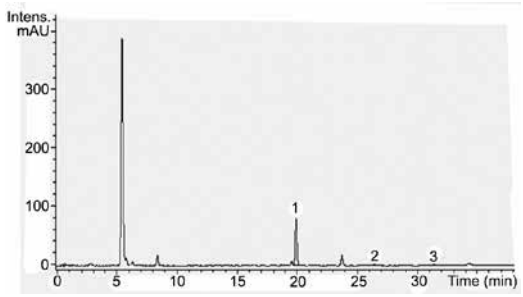

Fig. 1. Chromatogram of the polyphenols from the non-hydrolysed sample of the *Hedera helix* leaf extract. The peaks are marked: "1" rutin; "2" quercetin; "3" kaempferol

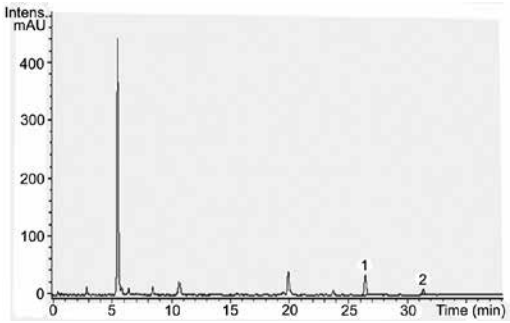

Fig. 2. Chromatogram of the polyphenols from the hydrolysed sample of the *Hedera helix* leaf extract. The peaks are marked: "1" quercetin; "2" kaempferol

Table 1
In vitro effect of the *Hedera helix* leaf extract

Phyto-pathogenic fungus	*Hedera helix* leaf extract (1:1) concentration (%)	Colony's diameter[a] (mm)	P[a] (%)	Fluconazole (2 mg × mL^{-1}) concentration		Colony's diameter[b] (mm)	P[b] (%)
				(%)	(mg × mL^{-1})		
Aspergillus niger	C	22	0	C		22	0
	6	18	18.18±0.81	10	0.2	11	54.13±0.50
	8	15	31.81±0.50	20	0.4	7	68.18±0.50
	10	8	63.63±0.57	25	0.5	3	86.36±0.57
	12	2	90.90±0.57	30	0.6	0	100
	14	0	100				
Botrytis cinerea	C	65	0	C		65	0
	3	44	32.30±0.50	2	0.04	40	38.46±0.50
	6	11	83.07±0.81	6	0.12	20	69.23±0.95
	8	4	93.84±0.95	10	0.20	3	95.38±0.50
	10	0	100	12	0.24	0	100
Botrytis tulipae	C	62	0	C		62	0
	3	41	33.87±0.50	2	0.04	50	19.35±0.50
	6	10	83.87±0.50	6	0.12	24	61.29±0.81
	8	3	95.16±0.57	10	0.20	3	95.16±0.50
	10	0	100	12	0.24	0	100
Fusarium oxysporum f. sp. *tulipae*	C	32	0	C		32	0
	3	29	9.37±0.57	2	0.04	20	37.5±0.50
	6	17	46.87±0.50	6	0.12	8	75±0.50
	8	12	62.5±0.50	8	0.16	2	93.75±0.57
	10	4	87.5±0.81	10	0.20	0	100
	12	0	100				
Penicillium gladioli	C	15	0	C		15	0
	3	11	26.66±0.50	10	0.2	11	26.66±0.50
	6	7	53.33±0.81	16	0.32	9	40±0.50
	8	3	80±0.57	20	0.4	6	60±0.50
	10	0	100	25	0.5	3	80±0.50
				30	0.6	0	100
Sclerotinia sclerotiorum	C	64	0	C		64	0
	3	31	51.56±0.50	2	0.04	30	53.12±0.50
	6	15	76.56±0.57	4	0.08	15	76.56±0.50
	8	3	95.31±0.81	6	0.12	4	93.75±0.57
	10	0	100	8	0.16	0	100

Legend: [a]the effect of the *Hedera helix* leaf extract; [b]the effect of fluconazole; P = mycelial growth inhibition; C = control (50% aq. EtOH); results are the mean±SD of 4 replicates.

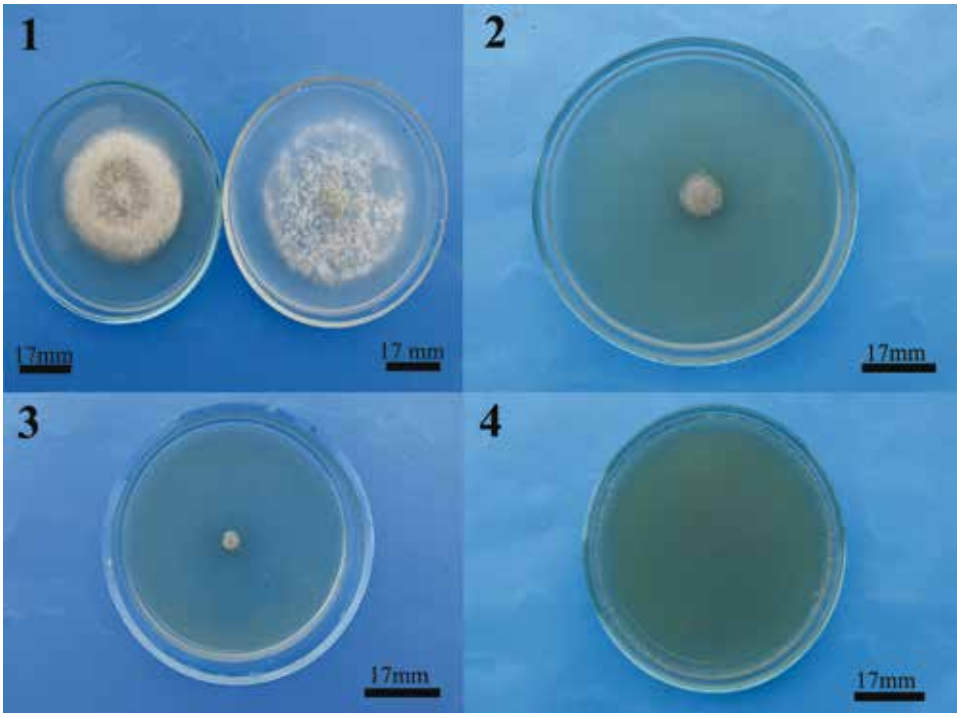

Fig. 3. The antifungal activity of the *Hedera helix* leaf extract on the germination and growth of the *Botrytis cinerea* fungus: **1** – control colony (left) and colony treated with 3% ivy leaf extract (right), 5 days after inoculation; **2** – colony treated with 6% ivy leaf extract, 5 days after inoculation; **3** – colony treated with 8% ivy leaf extract, 5 days after inoculation; **4** – colony treated with 10% ivy leaf extract, 5 days after inoculation (total inhibition)

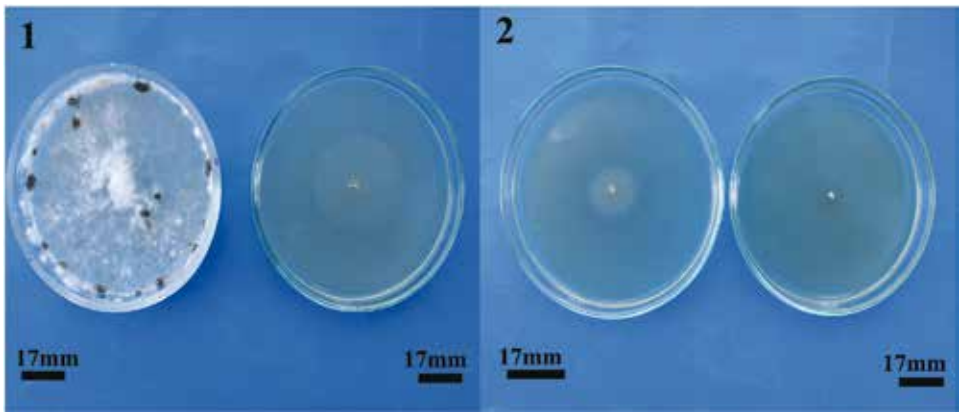

Fig. 4. The antifungal activity of the *Hedera helix* leaf extract on the germination and growth of the *Sclerotinia sclerotiorum* fungus: **1** – control colony, 14 days from inoculation (left) and colony treated with 2% ivy leaf extract, 5 days after inoculation (right); **2** – Colony treated with 6% ivy leaf extract (left) and colony treated with 10% ivy leaf extract (right), 5 days after inoculation (total inhibition)

extract (MIC = 10–14%) [23]. These findings suggest that ivy flowers contain principles with higher antifungal activities and might be more efficient in treating plant mycoses.

Ivy leaf extract inhibited, in a similar manner, the fungi causing grey mould (*Botrytis cinerea* – Fig. 3, *B. tulipae*), blue mould (*Penicillium gladioli*) and white rot (*Sclerotinia sclerotiorum* – Fig. 4) of plants. The MIC for all of these fungi was 10%. Less of an inhibitory effect was recorded in the case of *Fusarium oxysporum* f. sp. *tulipae*, with a MIC of 12%. The most resistant of all of the selected fungi was *Aspergillus niger*, with a MIC of 14%. At any rate, the obtained results are better than those for fluconazole (Table 1), which makes the ivy leaf extract a promising biocontrol agent for plant pathogenic fungi.

DISCUSSION

The antifungal activity of plant extracts is due to chemical constituents, such as alkaloids, polyphenols, saponins, sterols and essential oils [31]. Therefore, it is important to identify and quantify such bioactive compounds using adequate methods [3].

The phytochemical screening of *Hedera helix* indicated the presence of different classes of secondary metabolites, such as alkaloids, terpenoids, saponins and tannins. These compounds vary in type in different parts of the plant and are dependent on the type of the solvent used for the extraction. The chloroform and methanolic extracts revealed the presence of alkaloids, while terpenoids, saponins, and tannins were present in the *n*-hexane, chloroform, ethyl acetate and methanol extracts, respectively [33]. In the methylene chloride extract, from the leaves of *Hedera helix,* the following active components were found: β-amyrin; stigmasterol and hexadecanoic acid [20].

Using a new solid-liquid extraction technique highlighted the presence of active ingredients in the aqueous leaf extract of *Hedera helix*. Thus, flavonoids, proteins, amino acids and small amounts of triterpene saponins were recorded. In the extract, the flavonoids rutin, quercetin, kaempferol, and apigenin were identified, and the amino acids asparagine, glycine, isoleucine, leucine, phenylalanine, proline, tyrosine, and valine were identified by means of chromatographic methods [10].

Numerous studies have investigated the bioactive compounds of *Hedera helix*. Such studies used different methods to identify the compounds. Thus, triterpene saponins, such as hederasaponins E(1), F(2), H(7) and I(8) and cauloside F, were isolated from the leaves of *Hedera helix* and were characterised by chemical and spectroscopic methods [9]. The saponins hederacoside C and α-hederin, from different ivy leaf extracts, were detected using reversed-phase high-HPLC [6].

A thorough investigation of the phenolic constituents from the dry extract of *Hedera helix* revealed the presence of rutin, kaempferol 3-*O*-rutinoside, quercetin 3-*O*-glucoside, kaempferol 3-*O*-glucoside, quercetin, kaempferol, chlorogenic acid, neochlorogenic acid, 4,5- and 3,5-*O*-dicaffeoyl-quinic acids, as well as rosmarinic, caffeic, and protocatechuic acids. These compounds were isolated and identified by spectroscopic methods [32].

Furthermore, data from the literature mention the presence of emetine alkaloid in the ivy plant. Emetine is a natural product alkaloid found in several plant species. Its structure was elucidated, and its biological properties were studied. Thus, the antiviral, anticancer, antiparasitic and contraceptive activities were assigned. The major problem discouraging the medicinal use of this alkaloid is represented by cardiotoxicity and cytotoxicity, but these are dose-dependent [1]. Emetine alkaloid was separated for the first time from the alcoholic extract of the four varieties of *Hedera helix* growing in Egypt. The presence of the alkaloid was investigated by applying IR, UV, NMR and MS analyses [17]. Another innovative, sensitive and accurate method for the isolation and quantification of emetine alkaloid is the RP-HPLC [15].

Even if most of the studies elucidate the chemical composition of plant extracts, we believe that it is equally important to quantify the bioactive substances, because they might be very different depending on the plant organ, the solvent, and the method used for extraction.

The goal after identifying and isolating biologically active compounds from plants is to study them as possible curing agents. These kinds of studies are related especially to the therapeutic properties of the bioactive compounds, which are mainly used to treat human disorders. Such studies showed the anti-inflammatory effect of ivy, specifically the saponins extracted from the leaves of *Hedera helix* [7] and the anti-inflammatory and antiarthritic effects of the ethanolic extract of *Hedera helix,* which were found to be comparable to that of diclofenac [26]. Additionally, the methanolic extract of *Hedera helix* leaves and the saponins (hederacolchiside-E and -F) have dose-dependent analgesic and anti-inflammatory activities [12, 19].

A number of studies regarding ivy properties have demonstrated its benefits in respiratory diseases. Such studies confirmed that ivy leaf extract, in the form of syrup and in cough drops, is an effective and safe treatment of cough in children [28], in acute bronchitis therapy [4], and for acute upper respiratory tract infections, in general [16]. The traditional use of ivy for medicinal purposes is confirmed by its secretolytic, expectorant, and bronchospasmolytic effects, mainly due to the triterpene saponins, which are the main ingredients and are pharmacologically the most important substances from the ivy leaf extracts [29]. Moreoever, the *Hedera helix* extract was reported to have antitumour [25] and antimutagenic [34] effects, antioxidant properties due to the saponins (α-hederin and hederasaponin-C) [14], hypoglycemic activity [36], antileishmanial [18] and antihelmintic [8] properties.

Studies regarding the antimicrobial properties of the *Hedera helix* extracts are incomparably fewer. Such studies mention that the aqueous extract of *Hedera helix* has antibacterial, antimycobacterial and antifungal activities [5]. The ethyl acetate and methanol extracts were active against two strains of Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*) and three strains of Gram-positive bacteria (*Staphylococcus aureus*, *S. epidermidis*, *Bacillus subtilis*) at concentration of 22 $mg \times mL^{-1}$ [33].

In addition to its therapeutic properties for human disorders, there are a few studies related to the antibacterial and antifungal activity of the ivy extract against plant pathogenic agents. Interesting results were obtained when testing the antibacterial

effect of the *Hedera helix* extract against *Erwinia amylovora*. The ivy extract caused a pronounced increase in peroxidase and chitinase activity and induced the activation of defence genes, leading to the accumulation of structural and biochemical activities at strategic sites, which can be associated with induction of resistance against fire-blight [2]. More specifically, the ethanolic extract of *Hedera helix* exhibited a high degree of antifungal activity against late blight (*Phytophthora infestans*) of tomato and downy mildew (*Pseudoperonospora cubensis*) of cucumber [27].

Previous studies attest the antifungal properties of ivy extract against plant pathogenic fungi, while our results contribute the data in the literature on this topic with useful information regarding the possibility of being able to control plant pathogenic fungi that cause important and frequent diseases to vegetables and ornamental plants. Further studies should consider the assessment of the specific bioactive compound with the highest antifungal activity.

CONCLUSIONS

The ivy leaf ethanolic extract exhibited antifungal activity against the tested phytopathogenic fungi (*Aspergillus niger, Botrytis cinerea, B. tulipae, Fusarium oxysporum* f. sp. *tulipae, Penicillium gladioli* and *Sclerotinia sclerotiorum*) in a dose-dependent manner with an MIC of 10% to 14%. The results were comparable to those obtained for a synthetic drug (fluconazole), which makes ivy leaf extract a cost-effective and a potent herbal control agent for the treatment of plant diseases, such as grey mould, plant wilt and white rot. We believe that the antifungal activity is highly due to the polyphenols (rutin, quercetin, kaempferol) and the sterol (stigmasterol) that were detected in considerable amounts in the ivy leaf extract. Also, the role of the saponins with proven antifungal activity cannot be overlooked.

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