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In Vitro Antioxidant and Antifungal Properties of *Achillea millefolium* L.

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Abstract

Data regarding antioxidant and antifungal properties of yarrow extracts and essential oils can be found on several other studies; however, due to the fact that the composition of the natural products varies from one geographical area to another and with the extraction procedure, the present study contributes to a better characterisation of natural products obtained from A. millefolium L. The extract was obtained from shade dried plant in 1:1 mixture water-ethanol, while the essential oil was obtained by hydrodistillation. The products were characterized by modern analytical techniques and in terms of phytochemical assays. The antioxidant and antifungal effects of the products were explored. The analytical characterisation revealed the chemical composition (identifying 41 components in the extract and 82 components in the essential oil). The extract and the essential oil present a very good antioxidant effect (determined by two methods). The natural materials analysed revealed a very important in vitro antifungal activity on the studied fungal lines. The antioxidant and antifungal potential of the Achillea millefolium L. extract could find applications in the formulation of herbal products with improved action, both for human consumption and as tools to control mycotoxigenic fungi.

Keywords: *Yarrow, characterization, phytochemicals, antioxidant, antifungal.*

Introduction

The use of medicinal plants was inherited from past generations and used throughout human history, being considered part of the cultural heritage (1). In today's society, when the threats on human health are continuously increasing and the synthetic drugs are becoming less and less effective, the use of vegetal materials can prove a viable alternative for human use (2, 3), as well as for industrial applications (4, 5).

The selected medicinal plant has a very long history in Romanian traditions, being traditionally known as *coada soricelului* (mouse tail) or *iarba soarecelui* (mouse grass). *Achillea millefolium* L. (yarrow), is an herbaceous perennial plant that produces one to several

stems 0.2–1 meter in height, and has a spreading rhizomatous growth form, native to most of Europe (6). It finds applications mainly in traditional medicine, due to its various actions (antipyretic, diaphoretic, anti-inflammatory, antispasmodic, haemostatic, hypotensive, and emmenagogue) (7). The name of the genus, *Achillea* is supposed to be derived from mythical Greek character, Achilles, who used it to stanch the bleeding wounds of soldiers (8).

Data regarding antioxidant and antifungal properties of yarrow extracts and essential oils can be found on several other studies (9, 10). However, due to the fact that the composition of the natural products varies from one geographical area to another and with the extraction procedure (11, 12), the present study contributes to a better characterisation of natural products obtained from *Achillea millefolium* L. Our study is focused on the analytical characterisation of the hydroalcoholic extract and essential oil of yarrow, as well as on their antioxidant and antifungal properties (determined on *Aspergillus niger* and *Penicillium hirsutum* fungal strains). The strains were chosen due to their wide spreading in nature, as well as due to their potential hazardous effects: *Aspergillus niger* causes black mold on different fruits and vegetables (13), being also an agent causing invasive aspergillosis (14), while *Penicillium hirsutum* seems to be the most common species occurring in storage of various flower and vegetable bulbs (15), with possible effects on human health.

The present study aims to contribute to the development of new strategies to use nonchemical plant-derived products to control mycotoxigenic fungi.

Materials and Methods

2.1. Plant materials and natural products

The *Achillea millefolium* L. medicinal plants were collected in June 2014 from Leordeni area, Pitesti hills (N 44°47'30", E 25°8'4", 226 meters above sea level). The plants were identified by a taxonomist from University of Pitesti, Department of Natural Sciences (Associate Professor PhD Cristina Liliana Soare).

The inflorescences of yarrow were shade dried in order to remove the excess moisture (16). The hydroalcoholic extract was obtained from 20 g dried plant in 1:1 mixture water-ethanol (100:100 mL) kept for two hours at 80 °C, method previously demonstrated to be appropriate for obtaining hydroalcoholic extracts (17). In order to obtain the essential oil, 230 g of the dried plant material were firstly ultrasonated in 2000 ml bidistilled water for 30 min. at 40 kHz. The essential oil was subsequently extracted using a Neo-Clevenger installation. The refluxing time was three hours. The yield was approx. 0.5 mL/100 g of dried material.

The ethanol used for all the experiments was analytic grade, purchased from Merck KGaA (Germany), while the bidistilled water was obtained in our laboratory, using a GFL 2102 water still.

2.2. Analytical methods

UV-Vis analyses were performed using a UV-Vis spectrometer Unicam Helios α Thermo Orion at the resolution of 1 nm, with 1 nm slit width and automatic scan rate. The obtained results were processed using specific data analysis software (Origin Pro 8.0). The Extraction Factor (EF) was determined, from the absorption values ($A_{\lambda max}$), multiplied with the dilution factor (DF) (18).

Gas chromatography–mass spectrometry (GC-MS) analyses were performed with a Varian model 3800 gas chromatograph coupled with a Varian Saturn Ion Trap 2000 MS. The gas chromatograph was equipped with a Factor Four capillary column (30 m x 0.25 mm ID, DF = 0.25 mm). Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. Samples were introduced via split mode in an auto sampler with the injection port at a temperature of

270 °C. The column temperature was initially held at 50°C for 2 min then increased from 50°C to 155 °C at a rate of 8 °C min^{-1} and then from 155 °C to 275 °C at a rate of 40 °C min^{-1} and held at 275 °C for 9 min. The scan range was from 40 to 650 m/z. The GC/MS interface temperature was set at 266 °C. Output files were analyzed using Varian MS workstation version 6 and the NIST98 Mass Spectral Database.

2.3. Phytochemical Analyses

The phytochemical quantification procedures were used for the determination of total monoterpenoids, total flavonoids and total phenolics content in the extract. The assays are presented in Table 1.

Table 1. Phytochemical assays performed on the extracts

No	Assay	Reagents	Conditions	Monitoring and calibration	Ref.
1	Total mono-terpenoids	2 mL extract, 1 mL 2% vanillin-H_2SO_4 reagent in cold;	heated at 60 ºC for 20 min, cooled at 25 ºC for 5 min	Absorbance at 608 nm; Linalool (20 – 100 mg L^{-1})	(19)
2	Total phenolics	1 mL diluted extract, 5 mL Folin-Ciocalteau reagent. After 8 minutes, 4 mL saturated sodium carbonate;	incubated for 60 min at room temperature;	Absorbance at 765 nm; Gallic acid (10-55 µg mL^{-1})	(20)
3	Total flavonoids	0.5 mL extract, 1.5 mL ethanol, 0.1 mL aluminium chloride (10%), 0.1 mL 1 M potassium acetate, 2.8 mL of bidistilled water;	30 minutes of incubation at room temperature;	Absorbance at 415 nm; Rutin (10-55 µg mL^{-1})	(21)

The reagents were analytical grade, as follows: Vanillin (>99%, Fluka AG Switzerland), H_2SO_4 (98%, Merck KGaA Germany), linalool (≥ 97%, Merck KGaA Germany), Folin-Ciocalteu reagent (Merck KGaA Germany), sodium carbonate (≥99.9%, Merck KGaA Germany), gallic acid (99%, Merck KGaA Germany), aluminium chloride (99.999%, Sigma-Aldrich, USA), potassium acetate (≥99%, Sigma-Aldrich, USA), rutin (≥94%, Sigma-Aldrich, USA).

2.4. Antioxidant and antifungal effect

For determination of the *antioxidant activity*, two protocols were established: the DPPH assay and the chemiluminescence assay.

The first protocol was used to determine the free radical scavenging activity of the extract. DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) is a stable free radical (at room temperature) which presents strong absorbance at 517 nm; in the presence of an antioxidant, it is reduced, the solution becomes yellow to colourless and the absorbance decreases.

The protocol followed consists of mixing 0.5mL of the sample with 1 mL of 0.05 mM DPPH solution (Sigma Aldrich, USA). After an incubation period of 30 minutes, solutions were tested by reading the absorbance at 517 nm on the UV-VIS spectrophotometer. For the blank sample, the sample was replaced by bidistilled water.

The antioxidant activity (AA%) percentage was calculated using the formula:

$$AA\ (\%) = [(A_{control} - A_{sample})/A_{control}] \times \qquad [1]$$

where: $A_{control}$ is the absorbance of the DPPH solution without sample, A_{sample} is the absorbance of the extract mixed with the DPPH solution (22).

For the chemiluminescence assay, the extract was diluted in blank (hydroalcoholic) solution at the concentrations of: 1:1, 1:3 and 1:7 (v/v), while the essential oil was diluted in ethanol at the following concentrations: 1:9, 1:19 and 1:39 (v/v). The protocol consists of mixing 200 µL 8mM luminol, 50 µL 5mM hydrogen peroxide and 50 µL of the sample or standard in TRIS-HCl buffer (0.2 M, pH 8.6). The buffer was obtained from TRIS (tris(hydroxymethyl)-aminomethan, ≥99.5, Merck KGaA Germany) and HCl (37%, Merck KGaA Germany). The chemiluminescence (CL) was measured on a Turner Biosystems Modulus.

The results were compared with the results obtained for one known antioxidant, citric acid (>99.5%, Sigma Aldrich, USA), at different concentrations. The antioxidant activity of each sample was obtained using the mathematical expression:

$$AA\ (\%) - [(I_0 - I)/I_0] \times \qquad [2]$$

where I_0 is the maximum CL intensity for standard and I is the maximum CL intensity for sample at t =5 s. after reaction initiation (23).

A calibration curve was constructed using Trolox (6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic Acid, ≥98%, Merck KGaA, Germany) in the concentration range 8 to 168 µM and the results were expressed as µM Trolox equivalence.

The antifungal susceptibility of the extracts was evaluated using the disc diffusion or Kirby-Bauer method (24-26). The antifungal activity was tested against two relevant fungal strains *Aspergillus niger* ATCC 15475 and *Penicillium hirsutum* ATCC 52323. The stock culture was maintained at 4 °C. These strains were cultivated onto potato-dextrose agar (abbreviated "PDA" from Sigma-Aldrich with next composition: agar, 15 g L^{-1}, dextrose, 20 g L^{-1} and potato extract, 4 g L^{-1}.

Sterile PDA plates were prepared by pouring the sterilized media in sterile Petri dishes under aseptic conditions. The test organism (1 mL) was spread on agar plates. Using a sterile Durham tube of 6 mm diameter, the wells were made according to the number of samples. The wells were inoculated with 50 µL of hydroalcoholic extract. Similarly, each plate carried a blank well by adding solvent (ethanol:H_2O = 1:1) alone to serve as a negative control. All the plates containing fungal strains were incubated at 37 °C for 24h.

Antifungal activity of the microorganism species to the hydroalcoholic extract was determined by measuring the sizes of inhibition zone (IZ, mm) as clear, distinct zones of inhibition surrounding agar wells, and values <6 mm were considered as not active against microorganisms. The percent inhibition of the target fungi was calculated according to the following formula:

$$I\ (\%) = [(IZ - NC)/IZ] \times \qquad [3]$$

where: IZ - inhibition zone diameter, NC - negative control.

In order to determine the antifungal activity of the essential oils, the same fungal strains were used (*Aspergillus niger* and *Penicillium hirsutum)* and the same culture media.

The base of the Petri dish containing culture medium was inoculated with molds and 5-25 µL mL^{-1} of the essential oil was placed on the cover of the Petri dish.

In order to estimate the radial growth rate of strains the maximum diameter of colonies was measured after 6 days and the ratio diameter/time was calculated. The inhibition ratio was estimated using the following formula (27):

$$Inhibition\ ratio\ (\%) = [(C - E)/C] \times \qquad [4]$$

where C is the diameter of mold colony from control plate and E is the diameter of the mold colony growth in experiment plate which contains the essential oil.

All data were expressed as the mean ± standard deviation SD by measuring three independent replicates. Standard deviation was calculated as the square root of variance using STDEV function in *Excel* 2010.

Results and Discussions

The UV-Vis analysis (figure 1 shows the UV-VIS spectra obtained for the diluted extract-DF=10) identified the maxima wavelengths specific to phenolic acids at 220-280 nm, to flavonoids and quinones at 290-420 nm and chlorophylls at 600-670 nm (18). Table 2 represents the specific absorption values for the plant extracts, as well the calculated extraction efficiency (EF factor).

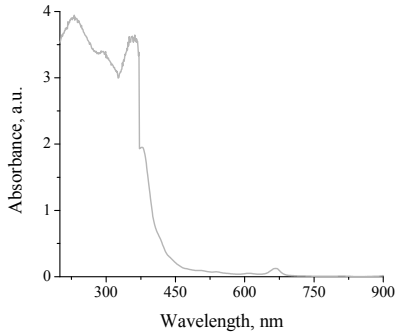

Figure 1. UV-VIS spectrum of the diluted extract (DF=10)

Table 2. Specific absorption values for the extract and EF calculated values

Extract /dilution	$A_{220-280nm}$	$EF_{220-280\ nm}$	$A_{290-420\ nm}$	$EF_{290-420\ nm}$	$A_{600-670nm}$	$EF_{600-670nm}$
$E_{DF=10}$	-	-	-	-	A_{665}=0.0651 A_{605}=0.0478	0.65 0.48
$E_{DF=100}$	-	-	A_{416}=0.0384 A_{330}=0.1270 A_{292}=0.1721	3.84 12.7 17.21	-	-
$E_{DF=1000}$	A_{228}=0.6286 A_{278}=0.2499	628.6 249.9	-	-	-	-

The extraction efficiency strongly depends on the polarity of the compounds found in plants and on the solvent polarity. In our case, the solvent used for the extraction (ethanol/water), proved to be a very good solvent for extracting phenolic compounds, as proven by the high values of $EF_{220-280nm}$. The $EF_{290-420\ nm}$ specific to flavonoids and quinones were relatively low (EF=3.84, 12.7 and 17.21), while the low values of $EF_{600-670nm}$ signifies very poor extraction of chlorophylls. The conclusion that can be drawn from these results is that the yarrow extract is rich in phenolic acids (more polar molecules).

The extract and essential oil were also characterized by GC-MS (the essential oil was diluted in alcohol – DF=10). The identified components (based on comparison of the GC-MS spectra and RI with those of internal NIST library) are summarized in Table 3.

Table 3. Compounds identified by GC-MS in *Achillea millefolium* L**.** extract and essential oil.

***Achillea millefolium* L. extract**		***Achillea millefolium* L. oil**	
RT/min.	**Compound**	**RT/min.**	**Compound**
1.934	Amyl ether	1.918	Amyl ether
		1.951	2-methyl-1-Butanol
2.146	Pentanol	2.517	cis-2-Hexen-1-ol
		3.317	2-Cyclohexen-1-ol
2.581	1-Methoxy-2-propanol	3.577	Hexanol
4.044	Ocimene	4.122	Santolina triene
		4.216	Heptanal
4.090	1,5,5-trimethyl-6-methylidenecyclohexene	4.623	α-Pinene
		4.706	α-Phellandrene
4.844	α-Pinene	4.901	3-Carene
		5.338	Camphene
5.271	3 Carene	5.985	Sabinene
5.321	Camphene	6.133	β-Pinene
		6.372	1-hexen-3-ol
5.898	1H-3a,7-Methanoazulene, octahydro-1,4,9,9-tetramethyl	6.538	2,3-dihydro-1,8-Cineole
		6.915	2,2,7-Trimethyl-3-octyne
		7.013	β-Phellandrene
5.979	Myrtenol	7.381	α-Terpinene
6.726	Cyclohexene, 1,5,5-trimethyl-6-methylene-	7.639	ortho-Cymene
		7.770	para-Cymene
7.634	2-Carene epoxide	8.112	Isobornyl acetate
		8.507	Benzyl methyl ether
7.934	Eucalyptol	8.889	2-Carene
		8.970	1,5-heptadien-4-one, 3,3,6-trimethyl
8.856	Isocitronellol	9.443	β-Terpineol
		9.876	(-)-Bornyl isovalerate
9.384	β-Terpineol	9.989	Dibutyl sulfide
		10.400	6-camphenol
		10.512	D-Verbenone
9.712	2,5,5-Trimethyl-2,6-heptadien-4-ol	10.945	α-Thujone
		11.313	β-Thujone
		11.554	cis-2-menthenol
9.946	Dibutyl sulfide	11.636	Campholenic aldehyde
		11.863	γ-Terpineol
10.616	(Z)-sabinene hydrate	12.020	Caryophyllene oxide
		12.099	Myrtenol
10.772	Thujone	12.666	Camphor
		12.763	Bornyl acetate
12.402	Camphor	12.827	Pulegone
		13.172	Pinocarvone
13.441	Borneol	14.350	Isoborneol
		14.968	α-Terpineol
14.459	α-Terpineol	15.388	cis-para-2-Menthen-1-ol
		15.484	2,2,4-Trimethylcyclohex-3-ene-1-carbaldehyde
15.068	cis-para-2-Menthen-1-ol	15.664	Lavandulol
		15.827	trans-Carveol
15.518	trans-Carveol	15.956	5 Caranol
		16.197	Isogeraniol
18.227	(-)-β-Pinene	16.329	cis-Carveol
		16.762	Carvone
20.280	cis-Carveol	17.081	Linalool
		17.194	p-Menth-4-en-3-one

20.394	γ-Terpinene	18.081	Isocyclocitral
		18.288	Isobornyl propionate
21.200	Eugenol	18.450	Terpinyl propionate
		18.563	Myrtenol
24.597	Isocaryophyllene	18.855	Thymol
		19.196	Carvacrol
25.876	α-selinene	20.406	Carvyl acetate
		21.258	Eugenol
27.562	2,4-Di-tert-butylphenol	22.237	Thujopsene
		22.957	(Z)-Jasmone
30.011	Spathulenol	23.695	Caryophyllene
		25.290	beta-Chamigrene
30.171	Caryophyllene oxide	25.968	γ-selinene
32.118	γ-Eudesmol	26.227	Germacrene D
		26.357	α-Curcumene
32.230	Cubenol	26.506	Eremophilene
		27.095	α Cedrene oxide
32.986	α-Eudesmol	30.239	α-guaiene
		30.352	(-)-Alloisolongifolene
38.112	α-Cedrene oxide	31.307	Longifolenaldehyde
		31.949	γ-Gurgujenepoxide
38.259	Longifolenaldehyde	32.486	d Selinene
		33.463	α-Eudesmol
44.752	Ethyl palmitate	34.380	Lanceol
		35.468	Zierone
49.453	6,9,12,15-Docosatetraenoic acid methyl ester	36.421	Ledene alcohol
		39.622	Methyl palmitoleate
		39.754	Phytone
49.828	Ethyl linoleate	48.161	γ-palmitolactone
		53.495	N-Tetratetracontane

Phytochemical evaluations results, performed by spectrophotometric methods presented in the Materials and methods chapter, are presented in Table 4.

Table 4. Phytochemical characterization of the extracts

No	Assay	Calibration curve	Units	Results
1	Total mono-terpenoids	y=0.0016x+00168, R^2=0.993	linalool equivalence mg g^{-1} dried weight	61.95±3.24
2	Total phenolics	y=0.01122x+0.00804, R^2= 0.9979	gallic acid equivalence mg $100g^{-1}$ dried weight	76.1±3.5
3	Total flavonoids	y=0.0067x-0.0401, R^2= 0.996	rutin equivalent mg g^{-1} dried weight	17.79±0.99

The results summarised in Table 4 confirms the findings from the UV-Vis analysis. The extract is richer in phenolic compounds that in flavonoid.

The antioxidant activity of the natural products was evaluated following two methods: the DPPH radical scavenging assay and a chemiluminescence method, as presented in the Materials and Methods chapter.

The results obtained for the DPPH assay, calculated according eq. 1 (82.14% ± 0.35) reveal a good antioxidant activity of the crude extract tested. The essential oil shows a high DPPH radical scavenging activity with an IC_{50} of 1.83 ± 0.11 mg mL^{-1}; positive control (ascorbic acid) showed an IC_{50} of 2.96 ± 0.16 mg L^{-1}.

For the chemiluminescence (CL) assay, a calibration curve was constructed using Trolox (y=0.2847x+21.467, R^2=0.9911). The results obtained by the chemiluminescence assay (calculated acc. eq. 2 and as Trolox equivalence) are presented in Table 5, compared with one known antioxidant (citric acid).

Table 5. Antioxidant activity of the samples determined by CL assay

Sample		Antioxidant activity (%)	Antioxidant activity (µM Trolox eq)
Extract	Undiluted	89.29±0.16	238.23±0.43
	DF=2	89.02±0.25	237.28±0.67
	DF=4	88.19±0.27	234.36±0.72
	DF=8	88.08±0.13	233.98±0.35
Essential Oil	DF=10	96.11±0.43	262.18±1.17
	DF=20	93.74±0.51	253.86±1.39
	DF=40	89.61±0.71	239.35±1.89
Citric acid (mM)	5.6	94.1±0.29	255.12±0.79
	1.12	80.8±0.34	208.41±0.88
	0.28	51.16±0.5	104.29±1.02

The analyzed samples (extract and essential oil) present significant total antioxidant capacity at all tested concentrations, even when compared with the known antioxidant.

The results of the two assays (DPPH & CL) revealed that the natural products (extract and essential oil) have a very interesting antioxidant activity. The differences in results between the two tested methods are most probably due to mechanisms of reactions and to different times at which the antioxidant action is estimated.

The *Kirby-Bauer* diffusion method was used as antifungal susceptibility testing method. The diameters of inhibition zones (in millimetres) of the extract against test strains are shown in figure 2.

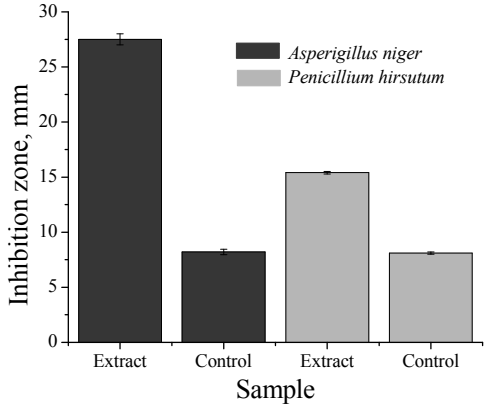

Figure 2. Antibacterial activity of the extract against *A. niger* and *P. Hirsutum*

The *Achillea millefolium* L. extract strongly affected the growth of all target fungi. The inhibition percent, calculated acc. eq. 3 was 70.19% for *Aspergillus niger* and, respectively, 47.40% for *Penicillium hirsutum*, compared with negative control.

Volatile oils show a significant effect on morphological structure of molds. Several studies (28-30) have demonstrated the action of essential oils on plasma membrane whose structure and function are altered and the transport of nutrients is modified.

For the essential oil experiments, the diameter of cultures was measured in control dishes and in the experimental plates containing the essential oil and there were calculated the average of growth rates (figure 3).

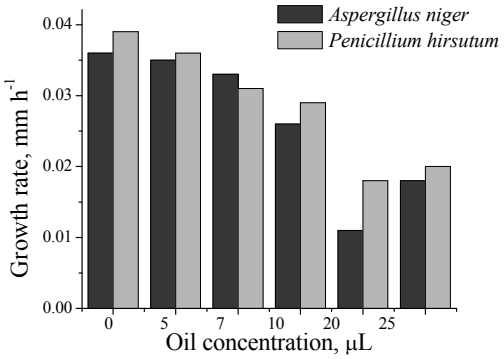

Figure 3. The average of growth rates for the two fungal strains

As can be seen in figure 3, the essential oil exhibited high antifungal activity. All essential oils were found to inhibit the growth of fungal strains of *Aspergillus* and *Penicillium*; they induce the important changes in the macroscopic appearance of fungal colonies and the addition of 20 µL mL^{-1} dramatically inhibits the growth of mold and the colonies appear white and adherent to the medium surface. In all cases is observed a lag phase of few days and the colonies have the smallest diameters.

The inhibition ratio shows a strong action of the essential oil against the two tested mold strains, with values greater than 85 % in all plates with 20 µL mL^{-1} of added oil. For all the fungal strains the inhibition growth depends on the amount of oil applied to Petri dish.

Conclusions

The present study represents a contribution to the development of new strategies to use nonchemical plant-derived products to control mycotoxigenic fungi. In the present work we report the analytical characterisation of the Romanian native *Achillea millefolium* L. extract and essential oil (41 components in the hydroalcoholic extract and 82 components in the essential oil were identified).

The high antioxidant potential of the extract ranges between 88.08 – 89.29% measured by CL method and 82.14% ± 0.35 for DPPH method, dependent on the dilution factor. The essential oil shows a high DPPH radical scavenging activity with an IC_{50} of 1.83 ± 0.11 mg mL^{-1}; positive control (ascorbic acid) showed an IC_{50} of 2.96 ± 0.16 mg L^{-1}. The values obtained by CL ranges between 89.61-96.11%, dependent on the dilution factor.

We also investigated the antifungal activity of the extract. The hydroalcoholic extract of *Achillea millefolium* L. revealed a very important *in vitro* antifungal activity on the studied fungal lines. It can therefore be used as a natural antifungal agent for the treatment of several infectious diseases affecting fruits, vegetables and humans. Natural products (extracts and essential oils) could prove to be an important alternative to classical, synthesized fungicides.

Further investigations will be done for developing commercial formulation based on field trail and toxicological experiment.

The ultimate conclusion of this study supports the traditional medicinal use of *Achillea millefolium* L. in treating different infections. The antioxidant and antifungal potential of the

Achillea millefolium L. extract could find applications in the formulation of herbal products with improved action, both for human consumption and as tools to control mycotoxigenic fungi.

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