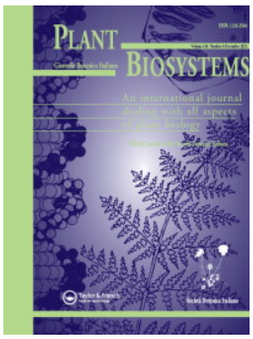


Taylor & Francis
Taylor & Francis Group

Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology

Official Journal of the Societa Botanica Italiana

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tplb20

Phytochemical-rich extracts of *Helianthemum lippii* possess antimicrobial, anticancer, and anti-biofilm activities

Fabiana Plescia, Fabio Venturella, Antonella D'Anneo, Valentina Catania, Maria Letizia Gargano, Giulia Polito, Domenico Schillaci, Antonio Palumbo Piccionello, Marianna Lauricella, Giuseppe Venturella & Demetrio Raffa

To cite this article: Fabiana Plescia, Fabio Venturella, Antonella D'Anneo, Valentina Catania, Maria Letizia Gargano, Giulia Polito, Domenico Schillaci, Antonio Palumbo Piccionello, Marianna Lauricella, Giuseppe Venturella & Demetrio Raffa (2022) Phytochemical-rich extracts of *Helianthemum lippii* possess antimicrobial, anticancer, and anti-biofilm activities, Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology, 156:6, 1314-1324, DOI: 10.1080/11263504.2022.2048274

To link to this article: https://doi.org/10.1080/11263504.2022.2048274

PLANT BIOSYSTEMS - AN INTERNATIONAL JOURNAL DEALING WITH ALL ASPECTS OF PLANT BIOLOGY
2022, VOL. 156, NO. 6, 1314–1324
https://doi.org/10.1080/11263504.2022.2048274

Phytochemical-rich extracts of *Helianthemum lippii* possess antimicrobial, anticancer, and anti-biofilm activities

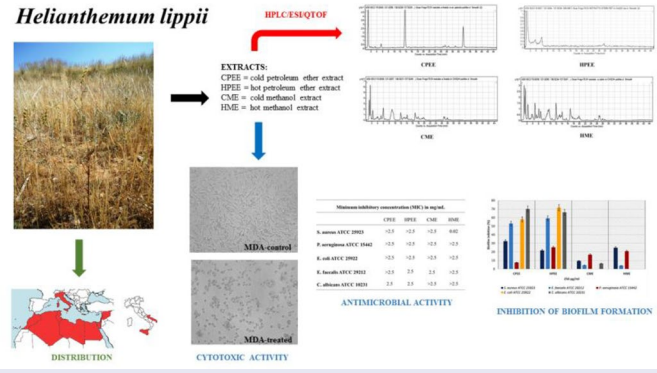
Fabiana Plescia[a], Fabio Venturella[b], Antonella D'Anneo[c], Valentina Catania[d], Maria Letizia Gargano[e], Giulia Polito[f], Domenico Schillaci[a], Antonio Palumbo Piccionello[f], Marianna Lauricella[g], Giuseppe Venturella[h] and Demetrio Raffa[a]

[a]Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF) - Section of Medicinal Chemistry and Technologies, University of Palermo, Palermo, Italy; [b]Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF) – section of Medicinal and Biological Chemistry, Viale delle Scienze, Palermo, Italy; [c]Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), Laboratory of Biochemistry, University of Palermo, Palermo, Italy; [d]Department of Earth and Marine Sciences (DiSTeM), University of Palermo, Palermo, Italy; [e]Department of Agricultural and Environmental Science, University of Bari Aldo Moro, Bari, Italy; [f]Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF) - Section of Chemistry, University of Palermo, Palermo, Italy; [g]Department of Experimental Biomedicine and Clinical Neurosciences, Laboratory of Biochemistry, University of Palermo, Palermo, Italy; [h]Department of Agricultural, Food and Forest Sciences, University of Palermo, Palermo, Italy

ABSTRACT

Helianthemum lippii is a perennial shrubby plant growing in the sandy environments of Italy, Mediterranean countries of North Africa and Middle East. *H. lippii* is used in traditional medicine but there are very few reports referring to the phytochemical characterization, the ethnopharmacology, and the biological activity of *H. lippii*. The goal of this study was to determine the phytochemical composition of different H. lippii extracts, cold (CME) and hot (HME) methanol, cold (CPEE) and hot (HPEE) petroleum ether, as well as to evaluate their anticancer and antimicrobial activities and biofilm formation reduction. Fifty-fours phytocompounds have been determined by HPLC-UV-ESI-QTOF-MS analysis. All the four extracts reduced the viability of human MDA-MB231 and HCT116 cells, being cold and hot methanol extracts the most effective. The antimicrobial activity against *S. aureus*, *P. aeruginosa*, *E. faecalis*, *E. coli*, *C. albicans* was also evaluated. Data showed the greater susceptibility of *S. aureus* to hot methanolic extract. Concerning antifungal activity, *C. albicans* resulted more susceptible to petroleum ether. Moreover, some of the samples exhibited a good antibiofilm activity both on immature and on mature biofilms. The four extracts showed interesting antimicrobial and cytotoxic activities and can be considered good candidates for new therapeutic applications

GRAPHICAL ABSTRACT



Abbreviation: MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM: Dulbecco's modified Eagle medium; MH: Mueller–Hinton broth; BS: Sabouraud broth; TSB: Tryptic Soy Broth; MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; MFC: minimum fungicidal concentration; CPEE: cold petroleum ether extract; HPEE: hot petroleum ether extract; CME: cold methanol extract; HME: hot methanol extract.

ARTICLE HISTORY
Received 2 April 2021
Accepted 21 February 2022

KEYWORDS
Helianthemum lippi; HPLC-UV-ESI-QTOF-MS analysis; cytotoxic effects; antimicrobial activity; antibiofilm activity

CONTACT Demetrio Raffa demetrio.raffa@unipa.it Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF) - Section of Medicinal Chemistry and Technologies, University of Palermo, Via Archirafi 32, 90123 Palermo, Italy

Published online 27 Mar 2022

Introduction

The genus *Helianthemum* Mill. belongs to the family of Cistaceae. The genus includes annual or perennial herbaceous and shrubby distributed species. in the Mediterranean basin and in western Asia. *Helianthemum lippii* (L.) Dum.Cours., a perennial shrubby plant growing in sandy environments, is native in Algeria, Egypt, Greece, Iran, Iraq, Israel, Italy (mainland and Sicily), Jordan, Kuwait, Lebanon, Libya, Malta, Morocco, Oman, Pakistan, Qatar, Syria, Spain, and Tunisia (Fenu et al. 2019). In Italy, the species is restricted to Sicily and Apulia (Figure 1) (Venturella et al. 2015). According to IUCN criteria the species was recently classified as Near Threatened and *in situ* and *ex situ* conservation strategies should be planned to protect this rare plant from further decline (Fenu et al. 2017; 2019). Indeed, the sandy habitats are particularly susceptible to biological invasions that result in habitat degradation and loss of biodiversity in island ecosystems (Celesti-Grapow et al. 2016). Besides, such conservation efforts consider that plant-typed habitats contribute substantially to biodiversity conservation and carbon storage (Burrascano et al. 2016).

It is commonly known with several vernacular names depending on the growth region. Particularly, *H. lippi* is called Al Samhari, Reguig, Tahsowat and Alrjik in Algeria, Alrkaroq in Kuwait, Umm Souika in the Arabian Peninsula and Sun Flower or Sun rose in Jordan (Atef et al. 2015). Also, Eliantemo a spiga, Eliantemo di Lippi, Eliantemo sessilifloro are some of the common names with which *H. lippii* is known in Italy (Fenu et al. 2019).

H. lippii is a very important plant from an ecological, economical and pastoral point of view being essential to counteract the desertification and stabilize vulnerable sites (Hamza et al. 2013). It has also a fair energetic and nutritional value that place it among the herbaceous species of the desert able to cover the food needs of dromedaries (Bouallala et al. 2013) especially in Sahara Desert (Volpato and Puri 2014).

Several uses are described in folk medicine for this plant. The powder or the compress of the aerial part is used to treat cutaneous lesion (Hamza et al. 2013; Atef et al. 2015). *H. lippii* is also used traditionally in Libya to treat cutaneous lesions and for antimicrobial treatments (Atef et al. 2015; Alsabri et al. 2013). Libyan population also use it as gastroprotective, like most plants belongs to the family of Cistacee (Ustun et al. 2006). Ethnobotanical study of some plants used in traditional medicine of Algerian Sahara highlighted the use of the leaves of *H. lippii*, in the form of powder or tablets, to treat injury, wounds and skin diseases (Lakhdari et al. 2016).

Finally, leaves decoction is used in Algeria to treat diabetes (Hamza et al. 2019) and roots decoction is used in Saudi Arabia to treat colic problems in camels (Sher and Aldosari 2013).

Despite different *Helianthemum* species are traditionally used in folk medicine as antiseptic, analgesic, and anti-inflammatory to treat burns, respiratory and digestive disorders, hemorrhoids, fever, and diarrhea (Djemam et al. 2020), to our knowledge, there are very few reports referring to the phytochemical characterization, the ethnopharmacology, and the biological activity of *H. lippii*. Particularly, effect on the smooth muscle of the rat distal colon (Djemam et al. 2020), antioxidant and antimicrobial activities (Atef et al. 2015; Belyagoubi-Benhammou et al. 2014; Alsabri et al. 2013), amoebicidal activity (Badria et al. 2014) anti-inflammatory, and antiulcer properties (Alsabri et al. 2013; Ermeli et al. 2012) are reported.

The aim of this work was to estimate and characterize the phytochemical composition of *H. lippii* in petroleum ether and methanol extracts and explore possible biological activities. With this in mind we demonstrated that *H. lippii* extracts possess anticancer and anti-proliferative properties which were strictly related to the different extraction methods.

Materials and methods

Plant materials

Seeds of *H. lippii* were collected at the growth site of Balestrate (Palermo). The seeds were scarified and after germination the seedlings were placed in phytocells. Subsequently, the plants were transplanted in an open-air field located in the Department of Agricultural, Food and

Figure 1. Distribution area of *Helianthemum lippii*.

Forest Sciences (SAAF) of the University of Palermo. Subsequently, the aerial part of the plants was chopped and dried for further analysis (Figure 2). Exsiccata are kept in the Herbarium of the Department of Agricultural, Food and Forest Sciences (SAF PI0145)

Extraction procedure

H. lippii (30 g dry aerial part of the plant) was macerated both with 500 mL of methanol ACS reagent 99.8% (Sigma-Aldrich) or with 500 mL of petroleum ether 40-60° (Macron) for 72 h at room temperature under magnetic stirring. After this time, the mixture was filtered, and the resulting solution was evaporated to dryness under reduced pressure at 55 °C to obtain 2.56 g of brown oil methanol extract and 0.13 g of white solid petroleum ether extracts. Furthermore, the solid part resulting after the filtration of the cold macerates of *H. lippii*, was extracted in a continuous extraction apparatus (Soxhlet) until exhaustion both with 250 mL of methanol or with 250 mL petroleum ether of for 48 h. The solvent of each extract was completely removed to dryness under reduced pressure at 55 °C to obtain g 0.77 of methanol extract and g 0.08 of petroleum ether extract. The extracts were stored at −20 °C until the use.

HPLC-UV-ESI-QTOF analysis

Water and acetonitrile were of HPLC/MS grade. Formic acid was of analytical quality. The HPLC system was an Agilent 1260 Infinity A reversed-phase Agilent Poroshell 120 EC-C_{18}

Figure 2. *Helianthemum lippii* grown in the open-air field.

column (50 mm × 3.0 mm, particle size 2.7 µm) with a Phenomenex C_{18} security guard column (4 mm × 3mm) was used. The flow-rate was 0.5 mL/min and the column temperature was set to 30 °C. The eluents were formic acid-water (0.1:99.9, v/v) (phase A) and formic acid-acetonitrile (0.1:99.9, v/v) (phase B). The following gradient was employed: 0-5 min, 5% B; 5-15 min, from 5% to 15% B; 15-20 min, 15% B; 20-25 min, from 15% to 30% B; 25-35 min, 30% B; 35-42 min, washing and reconditioning of column to 5% B. Injection volume was 10 µL. The eluate was monitored through MS TIC and UV trace at 270 nm. Mass spectra were obtained on an Agilent 6540 UHD accurate-mass Q-TOF spectrometer equipped with a Dual AJS ESI source working in negative ion mode. N_2 was employed as desolvation gas at 300 °C and a flow rate of 9 L/min. The nebulizer was set to 53 psig, the Sheat gas temperature was set at 300 °C and a flow of 12 L/min.

A potential of 2.6 kV was used on the capillary for negative ion mode. The fragmentor was set to 175 V. MS spectra were recorded in the 100-1500 m/z range.

Cell lines and culture conditions

MDA-MB231, a human triple-negative breast cancer cell line, was provided by Istituto Scientifico Tumori (Genoa, Italy) and cultured as monolayers in Dulbecco's modified Eagle medium (DMEM). HCT116 cells, an adenocarcinoma colon cancer cell line, were provided by Interlab Cell Line Collection (ICLC, Genoa, Italy) and cultivated in RPMI 1640 medium. Growth media of both cell lines were supplemented with 10% (v/v) heat inactivated fetal bovine serum, 2 mM L-glutamine, and penicillin–streptomycin antibiotics (50 µg/mL) (Euroclone, Pero, Italy) in a humidified atmosphere of 5% CO_2 in air at 37 °C. When cells reached approximately 80% confluence, they were sub-cultured or harvested using 0.25% trypsin-EDTA (Life Technologies Ltd).

The cell passage number used for the experiments was the fourth and fifth for MDA-MB231 cells and HCT116 cells, respectively.

Cell viability assay

For cytotoxicity studies, cells (8×10^3) were seeded into 96-well plates and after 24 h were incubated with *H. lippii* extracts prepared in different conditions. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay as previously reported (Lauricella et al. 2019; Raffa et al. 2015; Raffa et al. 2017). MTT is a yellow tetrazolium salt that can be reduced to purple formazan by mitochondrial enzymes of living cells. The absorbance of the formazan was read at 570 nm, with 630 nm as a reference wavelength using an ELISA plate reader (Opsys MR; Dynex Technologies, Chantilly, VA, USA). After incubation of cells with extracts, cell survival was calculated as a percentage of the value of the vehicle-treated control.

Antimicrobial activity

The minimum inhibitory concentrations (MICs) were determined by microdilution method. The serial dilutions of each extract were made in Mueller–Hinton broth (MH) (Sigma Aldrich) in a 96-wells plate, starting from a stock solution of 5 mg/mL in Dimethyl Sulfoxide (DMSO). A series of concentrations of each extract, ranging between 25 and 0.035 mg/mL, were tested. The reference bacterial strains used were *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 15442, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922. A bacterial suspension was prepared starting from a culture grown at 37 °C for 24 hours on Tryptic Soy Agar (TSA), in 0.9% NaCl until to 10^6 colony forming units (CFU)/mL and 10 µl of such suspension were added to each well. A positive control to check the bacterial growth, consisting of bacterial strains in the medium without extract, and a negative control to check the medium sterility, represented by the medium without inoculum were also included in the 96-wells plate. Moreover, a substance control to evaluate the absorbance of substance, consisting only of the substance solution without bacterial inoculum, were added. The 96-well plates were incubated at 37 °C for 24 h and MICs were read by a microplate spectrophotometer (GloMax®-Multi Detection System, Promega) as the lowest concentration of extract whose OD (optical density), read at 570 nm, was comparable with the negative control wells (broth without bacterial inoculum). Antifungal activity against *C. albicans* ATCC 10231 were evaluated in terms of MICs by using a micro-method described above, but by employing Sabouraud broth (BS) (Sigma-Aldrich) as growth media.

The minimum bactericidal and fungicidal concentrations were obtained by subculturing 0.1 mL from each negative well and from the positive control of MIC determination, onto substance-free Mueller-Hinton or Sabouraud agar plates without extract. The plates were incubated at 37 °C for 24 h. The MBC or MFC were defined as the lowest concentration of substance, that enable the microbial growth up to a maximum of three colonies.

Anti-biofilm properties

Microbial strains were incubated in test tubes with 5 mL Tryptic Soy Broth (TSB) containing 2% (w/v) glucose (bacterial strains) or BS containing 2% (w/v) glucose (fungal strain) at 37 °C for 24 h. After that, 2.5 µL of each microbial suspension was placed into each well of a sterile flat-bottom 96-well loaded with 200 µL of the same medium used in the starting suspension (TSB or BS with 2% glucose). Aliquots at sub-MIC concentration of each extract ranging from 250 to 0.5 µg/mL were directly added to the wells. Plates were incubated at 37 °C for 24 h. After biofilm growth, wells were washed twice with sterile NaCl 0.9%, and sessile biomass stained with 100 µL of 0.1% crystal violet solution for 30 min. at 37 °C. Then, surplus solution was discharged, and the plate was washed twice using tap water. A volume of 200 µL of ethanol was added into each stained well to solubilize dye bound to biofilm [Cusimano et al. 2020), for 10 min at room temperature. OD was read at a wavelength of 540 nm using a plate reader (Glomax Multidetection System TM297 Promega, Milano, Italy). BIC50, that is, the concentration at which the percentage of inhibition of biofilm formation (see below) is equal to 50%, was obtained by comparing the ODs of control wells with

that of the sample wells at different concentrations, and the value was calculated using AAT Bioquest, Inc. Quest Graph™ IC50 Calculator (v.1) retrieved from https://www.aatbio.com/tools/ic50-calculator-v1. The experiments were performed at least in triplicates, and three independent experiments were run (Cusimano et al. 2019). The percentage of inhibition was calculated by using the following the formula:

$$\%\ \text{of inhibition} = \left(\text{OD growth control} - \text{OD sample}\right) / \text{OD growth control}) \times 100$$

Antibiofilm activity against a preformed biofilm 24 h old was performed as seen above for inhibition of biofilm formation, concerning the preparation of microbial strains and medium and material used, but with the following differences: the samples were added at a concentration of 2.5 mg/mL on a mature biofilm of 24 h and the plates were incubated at 37 °C for 24 h. After this time each well was washed and stained with crystal violet as above described. Percentages of inhibition were calculated using previously reported formula by comparing treated wells with growth control (not treated) wells.

Statistical analysis

Data were expressed as mean ± standard error. Results of cytotoxicity were analyzed by one-way analysis of variance (ANOVA) test using GraphPad Prism 5.0 software (San Diego, CA, USA). Differences were considered statistically significant for $P < 0.05$.

Results and discussion

Plant extracts and reversed phase HPLC/MS profiles

Fiftyfour phytochemical compounds have been determined by means of HPLC-UV-ESI-QTOF-MS analysis, in hot methanol extract, cold methanol extract, cold and hot extract with petroleum ether (Figure 3 as representative traces in negative ion mode).

Identified products in these four extracts are summarized in Table 1, including molecular formula, retention time (min), calculated and experimental m/z in negative ion mode and relative quantification in terms of percentage area of the compounds identified in the extracts. The qualitative composition of the extracts is quite similar to those previously reported in the literature (Djemam et al. 2020).

Compounds derived from *H. lippii* extracts can be classified in different families such as organic acids and derivatives, phenolic acids and derivatives, flavonoids and other polyphenolic compounds, such as a xanthone, lignans, coumarins and isocoumarins.

Phytochemicals derived from *H. lippii* extracts can be classified in different families such as organic acids and derivatives, phenolic acids and derivatives, flavonoids, a xanthone, lignans, coumarins and isocoumarins.

Among organic acids, the most abundant compounds identified in *H. lippii* extracts are citric acid (peak 1, CME and HME), fumaric acid (peak 2, CME, HME, CPEE, HPEE) and gentisic acid (peak 6, CME and HME). All these compounds showed antimicrobial activity (Shokri 2011) (Laury et al. 2009) (Juurlink et al. 2014) (Vandal et al. 2015) (He et al. 2011).

Among phenolic acids and derivatives, significant compounds for their biological activity identified in *H. lippii* extracts are gallic acid (peak 3, CME and HME), uralenneoside (peak 9, CME and HME), protocatechuic acid (peak 11, CME and HME) and O-methyl gallate (peak 12, CME and HME). Gallic acid (Subramanian et al. 2015) and protocatechuic acid (Masella et al. 2012). possesses anticancer properties, while Uralenneoside (Tomás-Menor et al. 2013) and methyl gallate (Choi et al. 2005) shows antimicrobial activity.

Among flavonoids, relevant compounds for their biological activity identified in *H. lippii* extracts are catechin (peak 15 CME and HME), quercetin-3-β-D-glucoside (peak 33 CME and

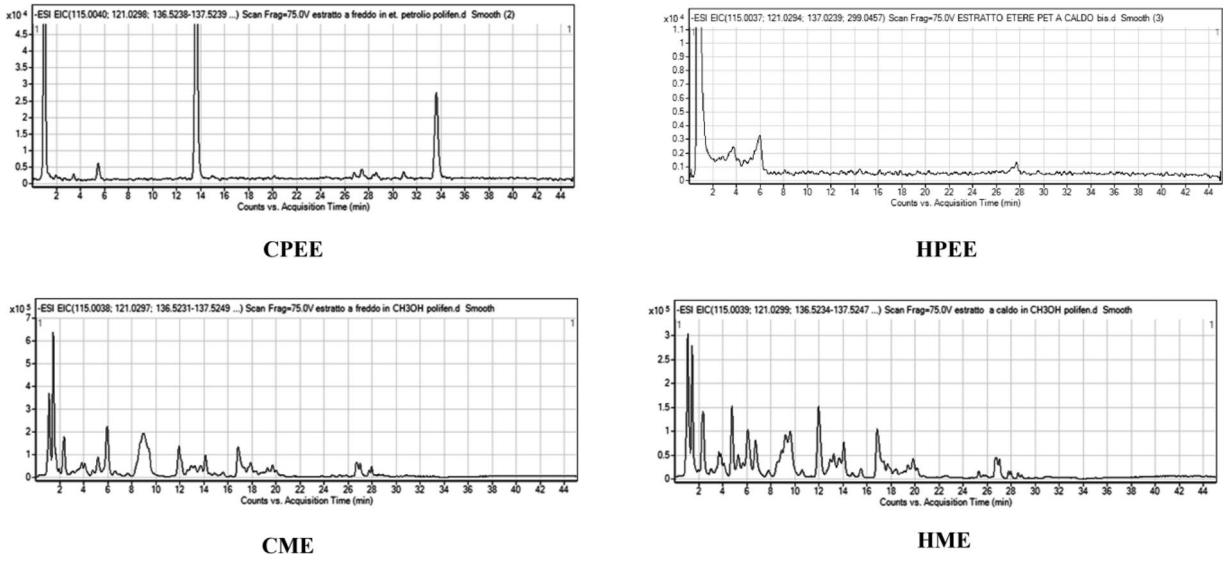


Figure 3. Representative HPLC/ESI/QTOF trace of extract of *Helianthemum lippii* (negative mode) in **CPEE** = cold petroleum ether extract; **HPEE** = hot petroleum ether extract; **CME** = cold methanol extract; **HME** = hot methanol extract.

Table 1. Composition of extracts of *Helianthemum lippii*. **CPEE** = cold petroleum ether extract; **HPEE** = hot petroleum ether extract; **CME** = cold methanol extract; **HME** = hot methanol extract.

						CPEE	HPEE	CME	HME
	Compound	Molecular formula	RT$^-$ (min)	ESI$^-$ [M-H]$^-$ (m/z) (*Calc.*)	ESI$^-$ [M-H]$^-$ (m/z) (*Exp.*)	Relative Area (%)			
1	Citric acid	$C_6H_8O_7$	1.09	191.0197	191.0200			5.53	7.09
2	Fumaric acid	$C_4H_4O_4$	1.11	115.0037	115.0038	36.71	88.48	1.80	2.12
3	Gallic acid	$C_7H_6O_5$	1.44	169.0142	169.0150			13.41	7.31
4	Ovalitenin	$C_{19}H_{14}O_5$	1.67	321.0768	321.0702			1.45	0.52
5	Ovalitenin isomer	$C_{19}H_{14}O_5$	2.25	321.0768	321.0703			2.70	3.94
6	Gentisic acid	$C_7H_6O_4$	2.37	153.0193	153.0177			1.80	1.56
7	Chlorogenic acid	$C_{16}H_{18}O_9$	3.28	353.0878	353.0878			0.09	0.14
8	Hydroxybenzoic acid	$C_7H_6O_3$	3.65	137.0244	137.0241	0.72		0.35	1.42
9	Uralenneoside	$C_{12}H_{14}O_8$	3.82	285.0616	285.0591			1.62	1.82
10	Hydroxybenzoic acid isomer	$C_7H_6O_3$	4.08	137.0244	137.0249	0.18	2.71	1.21	0.80
11	Protocatechuic acid	$C_7H_6O_4$	4.21	153.0193	153.0197			0.34	0.28
12	O-Methyl gallate	$C_8H_8O_5$	4.75	183.0299	183.0281			0.86	6.48
13	4-Hydroxybenzaldehyde	$C_7H_6O_2$	5.61	121.0295	121.0297	2.37	8.12	0.38	0.92
14	Norathyriol	$C_{13}H_8O_6$	5.93	259.0248	259.0256			7.81	7.12
15	Catechin	$C_{15}H_{14}O_6$	6.72	289.0718	289.0692			0.92	4.16
16	3-O-Feruloylquinic acid	$C_{17}H_{20}O_9$	7.65	367.1035	367.1041			0.46	0.71
17	Ethylvanillin glucoside	$C_{15}H_{20}O_8$	8.38	327.1085	327.1075			1.18	1.18
18	Xanthotoxol glucoside	$C_{17}H_{16}O_9$	8.65	363.0722	363.0727			3.04	2.55
19	Epigallocatechin	$C_{15}H_{14}O_7$	8.98	305.0667	305.0677			18.90	8.91
20	Vanillic acid	$C_8H_8O_4$	10.49	167.0350	167.0334			0.05	0.81
21	6-hydroxyferulic acid-6-O-hexoside	$C_{16}H_{20}O_{10}$	11.84	371.0984	371.0954			1.69	2.03
22	Kaempferol 3-(2′′,3′′-diacetylrhamnoside)-7-rhamnoside	$C_{31}H_{34}O_{16}$	11.90	661.1774	661.1716			0.66	1.25
23	Catechin isomer	$C_{15}H_{14}O_6$	11.94	289.0718	289.0692			1.91	4.04
24	Epigallocatechin gallate	$C_{22}H_{18}O_{11}$	12.60	457.0776	457.0798			0.95	0.40
25	Xanthotoxol glucoside isomer	$C_{17}H_{16}O_9$	12.95	363.0722	363.0763			2.19	2.37
26	7-Deshydroxypyrogallin-4-carboxylic acid	$C_{12}H_8O_6$	13.18	247.0248	247.0247			0.99	2.26
27	4′-O-Methylcatechin	$C_{16}H_{16}O_6$	13.29	303.0874	303.0902			1.02	0.62
28	5-O-Feruloylquinic acid	$C_{17}H_{20}O_9$	13.48	367.1035	367.1007			0.06	0.14
29	N-Succinyl-2-amino-6-ketopimelate	$C_{11}H_{15}NO_8$	13.60	288.0725	288.0719	41.57			0.59
30	Salicylic acid	$C_7H_6O_3$	13.70	137.0244	137.0231			2.15	1.92
31	Scutellarin 7-rutinoside	$C_{27}H_{30}O_{15}$	14.11	593.1512	593.1461			2.67	2.21
32	Ellagic acid	$C_{15}H_{10}O_7$	16.80	300.9990	300.9969			7.69	8.36
33	Quercetin-3-β-D-glucoside	$C_{21}H_{20}O_{12}$	17.30	463.0882	463.0850			0.38	0.58
34	Rutin	$C_{27}H_{30}O_{16}$	17.39	609.1461	609.1461			0.53	0.71
35	Quercetin-3-β-D-glucoside isomer	$C_{21}H_{20}O_{12}$	17.67	463.0882	463.0850			0.49	0.58
36	Coriandrone	$C_{16}H_{20}O_5$	17.87	291.1238	291.1245			2.08	0.83
37	Kaempferol 7-galactoside 3-rutinoside	$C_{33}H_{40}O_{20}$	17.98	755.204	755.1966			0.16	0.16
38	Catechin 3-O-rutinoside	$C_{27}H_{34}O_{15}$	18.37	597.1825	597.1838			0.45	0.41
39	Isolariciresinol 9-O-beta-D-glucoside	$C_{26}H_{34}O_{11}$	18.58	521.2028	521.2081			0.04	0.02
40	3,4-Dicaffeoyl-1,5-quinolactone	$C_{25}H_{22}O_{11}$	19.11	497.1089	497.1122			0.39	0.62
41	Kaempferol 3-rhamnosyl-(1->2)-galactoside	$C_{27}H_{30}O_{15}$	19.29	593.1512	593.1507			0.88	1.06
42	Isorhamnetin O-rutinoside	$C_{28}H_{32}O_{16}$	19.48	623.1618	623.1571			0.57	0.65
43	Quercetrin	$C_{21}H_{20}O_{11}$	19.71	447.0933	447.0899			1.90	2.31
44	Isorhamnetin O-rutinoside isomer	$C_{28}H_{32}O_{16}$	20.03	623.1618	623.1637			0.74	0.84
45	Rhamnazin 3-sophoroside	$C_{29}H_{34}O_{17}$	20.26	653.1723	653.1738			0.10	0.06
46	Quercetin 3-methyl ether 4′-glucoside	$C_{22}H_{22}O_{12}$	20.54	477.1039	477.1031			0.10	0.14
47	Rhamnazin 3-sophoroside isomer	$C_{29}H_{34}O_{17}$	20.58	653.1723	653.1742			0.12	0.09
48	Scutellarin	$C_{21}H_{18}O_{12}$	23.43	461.0725	461.0734			0.02	0.04
49	Tiliroside	$C_{30}H_{26}O_{13}$	26.69	593.1301	593.1256	0.56		2.50	2.63
50	Tiliroside isomer	$C_{30}H_{26}O_{13}$	27.00	593.1301	593.1256	0.17		2.00	1.79
51	Diosmetin	$C_{16}H_{12}O_6$	27.43	299.0561	299.0551	1.69	0.69		
52	Kaempferol	$C_{15}H_{10}O_6$	27.75	285.0405	285.0419			0.67	0.44
53	Polydatine	$C_{20}H_{22}O_8$	28.30	389.1242	389.1220	0.47		0.02	
54	Syringaresinol	$C_{22}H_{26}O_8$	33.51	417.1555	417.1579	15.57		0.01	

HME), rutin (peak 34, CME and HME) and tiliroside (peak 49 CPEE, CME and HME). Catechin, (Zanwar et al. 2014), quercetin-3-β-D-glucoside (Razavi et al. 2009), rutin (Gullón et al. 2017) (Imani et al. 2021), tiliroside (Grochowski et al. 2018) possesses different activities including antimicrobial and anticancer.

Among other compounds identified in *H. lippii* extracts, it's worth mentioning norathyriol, an aglycone of a xanthone C-glycoside mangiferin (peak 14, CME and HME), and coriandrone, an isocoumarin (peak 36, CME and HME). Norathyriol is isolated from several plants and it is known to possess antioxidant, anti-inflammatory, and antitumor properties (Li et al. 2012). Coriandrone A and B exhibit antibacterial, anticancer, hepatoprotective, antimutagenic, antioxidant and antidiabetic properties (Wang et al. 2013).

According to literature, the leaves and flowers of different *Helianthemum* species are traditionally used in folk medicine as antiseptic, analgesic and anti-inflammatory to treat burns, respiratory and digestive disorders, hemorrhoids, and fever (Venturella et al. 2015). Considering the reported activities of identified compounds, we decided to test the obtained extracts for their cytotoxic effects and anti-microbial activity.

Cytotoxic effects of Helianthemum lippii extracts on tumor cells in culture

Preliminary experiments were performed to evaluate a possible anti-proliferative effect of *H. lippii* extracts against two cell lines of breast and colon cancer. These represent the form of cancers with the highest incidence in the world. Thus, the identification of compounds capable of exerting cytotoxic effects on these tumor cells, could open the way to new therapeutic strategy. In particular, we focused our study on MDA-MB231, a very aggressive and poorly differentiated breast cancer cell line and on HCT116, an invasive and highly motile adenocarcinoma colon cancer cells. To this end, we treated MDA-MB231 and HCT116 cells with increasing concentrations of each extract of *H. lippii* for 24 and 48 h. Then, the viability was evaluated by MTT assay as reported in Methods. Different experiments were performed increasing progressively the dose of the extracts. In Figure 4 are reported the data obtained treating both MDA-MB231 and HCT-116 cells with the extracts within the range of 100-500 µg/mL. As shown in Figure 4, all the four extracts reduced the viability of MDA-MB231 cells in a dose and time dependent manner (upper panel in Figure 4). However, the cytotoxic efficacy of the different fractions consistently varied with respect to the extraction conditions. In fact, both cold (CME) and hot (HME) methanol extracts resulted to be more effective in reducing the viability of MDA-MB231 cells than cold (CPEE) and hot (HPEE) petroleum ether extracts. As shown in the lane chart reported in Figure 4, after 48 h of incubation with 100 µg/mL of CME or HME cell viability decreased by 50% of control. Interestingly, increasing the dose of these extracts up to 300 µg/mL, the number of viable cells fell to about 10% of the controls. Instead, HPEE and CPEE reduced the viability of MDA-MB231 cells by 50% only with higher doses (300 µg/mL and 400 µg/mL, respectively) of the extracts.

Studies performed in adenocarcinoma HCT116 colon cancer cells confirmed the better efficacy of methanol extracts (Figure 4 lower panel) in reducing cell viability. Comparing the IC_{50} of the four fractions at 48 h of incubation it appears that a reduction of viability of 50% was obtained for CME and HME with 80 and 100 µg/mL respectively, while the same

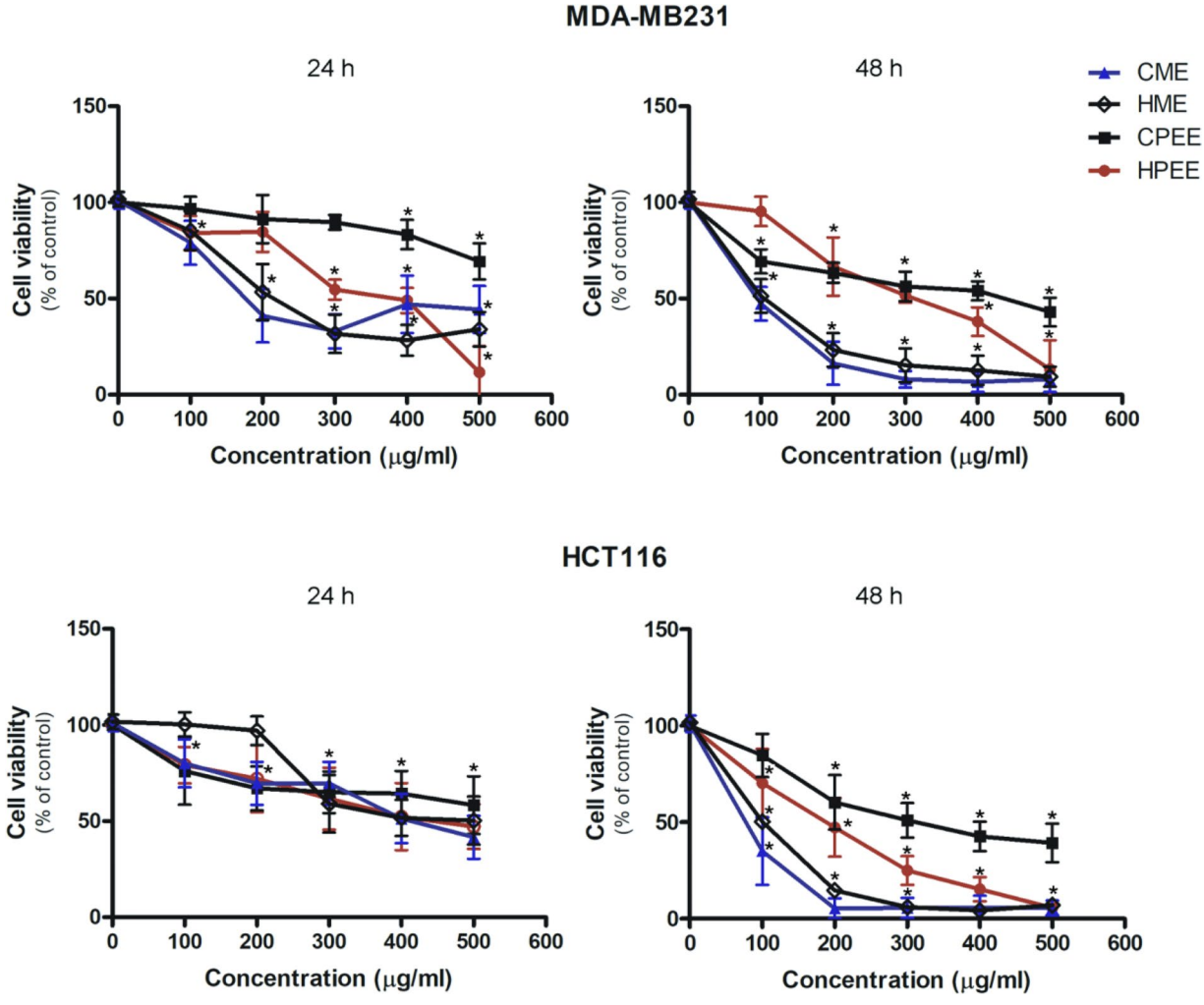


Figure 4. Cytotoxic effect of *Helianthemum lippii* on breast cancer and colon cancer cells. Time-and dose-dependent effects of *Helianthemum lippii* extracts prepared in different conditions were evaluated in two different cancer cell lines (breast cancer MDA-MB231 and adenocarcinoma colon cancer HCT116). Cell viability was assessed by MTT assay as reported in Methods. Data are the mean ± SD of three independent experiments, each performed in triplicate, and expressed as percentage of the vehicle-treated control. Data were considered significant at *$P < 0.05$.**CME** = cold methanol extract; **HME** = hot methanol extract; **CPEE** = cold petroleum ether extract; **HPEE** hot petroleum ether extract.

effect was induced by 200 µg/mL HPEE or 300 µg/mL CPEE, respectively.

These preliminary results suggest that *H. Lippii* possesses cytotoxic effects against both breast and colon cancer cells in culture. Subsequent studies will be performed to clarify the underlying mechanisms.

Antimicrobial and antibiofilm activity of Helianthemum lippii extracts

Experiments to evaluate whether *H.Lippii* extracts also possess antimicrobial and antibiofilm activity were performed. To this end, the *H. lippii* extracts, subjected to in vitro microbiological experimentation, were initially solubilized in DMSO or H_2O at the following concentrations: cold petroleum ether extract (CPEE) (40 mg/mL in DMSO); hot petroleum ether extract (HPEE) (26 mg/mL in DMSO); cold methanol extract (CME) (170 mg/mL in H_2O) and hot methanol extract (HME) (154 mg/mL in DMSO).

Table 2. Antimicrobial activity of the four substances tested against reference bacterial and fungal free-living strains.

	Minimum inhibitory concentration (MIC) in mg/mL			
	CPEE	HPEE	CME	HME
S. aureus ATCC 25923	>2.5	>2.5	>2.5	0.02
P. aeruginosa ATCC 15442	>2.5	>2.5	>2.5	>2.5
E. coli ATCC 25922	>2.5	>2.5	>2.5	>2.5
E. faecalis ATCC 29212	>2.5	2.5	2.5	>2.5
C. albicans ATCC 10231	2.5	2.5	>2.5	>2.5

The MIC determination is expressed in mg/mL. **CPEE** = cold petroleum ether extract; **HPEE** = hot petroleum ether extract; **CME** = cold methanol extract; **HME** = hot methanol extract.

A starting solution of each substance (5 mg/mL) in MH medium for all bacteria or BS medium for *C. albicans* was prepared and sterilized by filtration. The samples were subjected to evaluation of the antimicrobial activity against important human pathogenic bacteria and fungi. The following bacterial reference strains were used in the *in vitro* microbiological experimentation: *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 15442, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922; and a reference fungal strain *Candida albicans* ATCC 10231.

The antimicrobial activity against free-living microbiological strains is expressed in terms of Minimum inhibitory concentration (MIC) in mg/mL and is showed in Table 2.

HME showed an interesting activity against important Gram positive pathogen *S. aureus* with a MIC value of 0.02 mg/mL, instead HPEE and CME were active at 2.5 mg/mL against the other Gram positive E. faecalis. All four extracts showed a lack of antibacterial activity at the highest concentration against tested Gram-negative pathogens *P. aeruginosa* and *E. coli*. Moreover, the minimum bactericidal concentration (MBC) has been determined for all extracts which showed some antibacterial activity (HPEE and CME against *E. faecalis* and HME against *S. aureus*), but no bactericidal activity was detected.

Concerning the antifungal activity, the CPEE and HPEE were active against *C. albicans* at a MIC value of 2.5 mg/mL, instead both methanol extracts showed no activity at the screening concentration. No fungicidal activities were detected at the maximum tested concentrations for all extracts.

Surface-attached microbial communities (biofilms) able to adhere to biotic (host tissues) or abiotic surfaces (medical devices) are of clinical relevance and it is estimated that

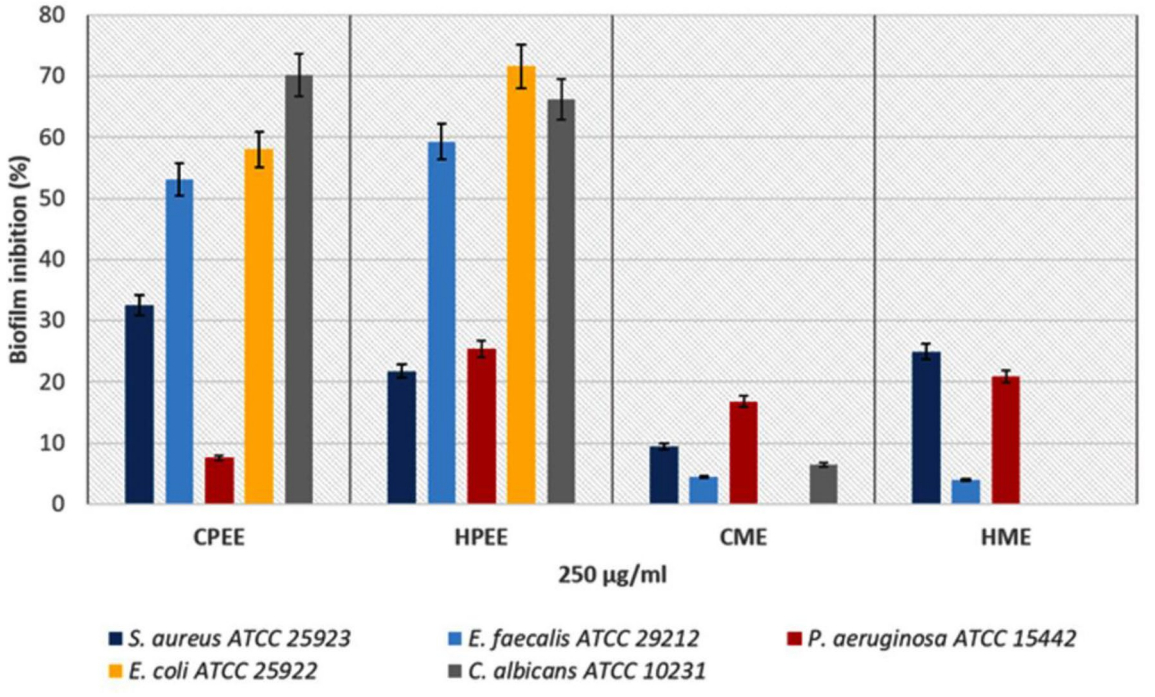


Figure 5. Inhibition of biofilm formation, data are the mean ± SD of two independent experiments, each performed at least in quadruplicate, and expressed as inhibition percentage respect to the growth control. Data were considered significant at $P < 0.05$. **CPEE** = cold petroleum ether extract; **HPEE** = hot petroleum ether extract; **CME** = cold methanol extract; **HME** = hot methanol extract.

pathogens capable of forming biofilms are responsible at least 60% of all human infections (Mihai et al. 2015). Therefore, novel antibiofilm agents are needed and the four extracts CPEE, HPEE, CME and HME were tested for their ability to inhibit biofilm formation and tackle preformed biofilms of previously mentioned reference strains. Sub-MIC concentrations ranging from 250 to 0.5 µg/mL were tested and inhibition of biofilm formation at the maximum tested concentration is reported in Figure 5.

CPEE and HPEE showed at the maximum tested concentration of 250 µg/mL a good activity in inhibiting biofilm formation of *E. faecalis*, *E. coli* and *C. albicans*. Biofilm inhibition concentration 50% (BIC_{50}), that is the concentration at which the percentage of inhibition of biofilm formation is equal to 50%, was also calculated.

The sample CPEE with a BIC_{50} of 174.2 µg/mL was the most effective in inhibiting biofilm formation of *C. albicans*, other data are reported in Table 3. The presence of fumarate as component could explain the anti-biofilm activity of this extracts (Barnes and Karatzas 2020).

Concerning the activity against mature biofilms 24 h old, we screened the extracts at 2.5 mg/mL (see Figure 6) for their ability to destroy a preformed biofilm. CPEE showed activities ranging from 32 to 14 percentages of inhibition against respectively *S.aureus* (32%) *E. faecalis* (21%) and *C. albicans* (14%). HPEE showed an interesting activity (48% inhibition) against a preformed biofilm of *C.albicans*. CME and HME displayed a similar percentage of inhibition against *C.albicans*, with values equal to 43.7 and 44.2% .None of tested extracts was active against *P. aeruginosa*.

Table 3. BIC_{50} in µg/mL of **CPEE** = cold petroleum ether extract; **HPEE** = hot petroleum ether extract.

	BIC50		
	E. faecalis ATCC 29212	*E. coli* ATCC 25922	*C. albicans* ATCC 10231
CPEE	194.8	180.4	174.2
HPEE	187.4	177.9	178.3

The value of BIC_{50} was calculated using AAT Bioquest, Inc. (2021, February 12). *Quest Graph™ IC_{50} Calculator (v.1)*. Retrieved from https://www.aatbio.com/tools/ic50-calculator-v1.

Conclusion

In this work, the phytochemical composition as well as the anticancer and antimicrobial activities of *H. lippii* in both petroleum ether and methanol extracts was determined. In the first instance, among all the analyzed extracts, the cold and hot methanol ones resulted the most interesting. They showed the best phytochemical composition and the highest biological activity. Particularly, cold, and hot methanol extracts were found to be the richest in phytochemicals with 54 phytochemicals found versus only ten in the petroleum ether extracts; this could explain the reduced cytotoxic efficacy showed by the petroleum ether extracts. Instead, the petroleum ether extracts were active in inhibiting free living cells and biofilm formation of *C. albicans*. Alternatively, cold, and hot methanol extracts of *H. lippii*, showing good both cytotoxic and antimicrobial activities, can be considered good candidates for new therapeutic applications.

Finally, more detailed studies are necessary to validate the chemical composition and to identify new possible active compounds as a new source of antiproliferative as well as antimicrobial agents.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

Financial support from "European Union 2014-2020 PON Ricerca e Innovazione grant from the Italian Ministry of Education, University and Research, entitled "PROGEMA-Processi Green per l'estrazione di principi attivi e la depurazione di matrici di scarto e non" (ARS01_00432) to P.D." is gratefully acknowledged.

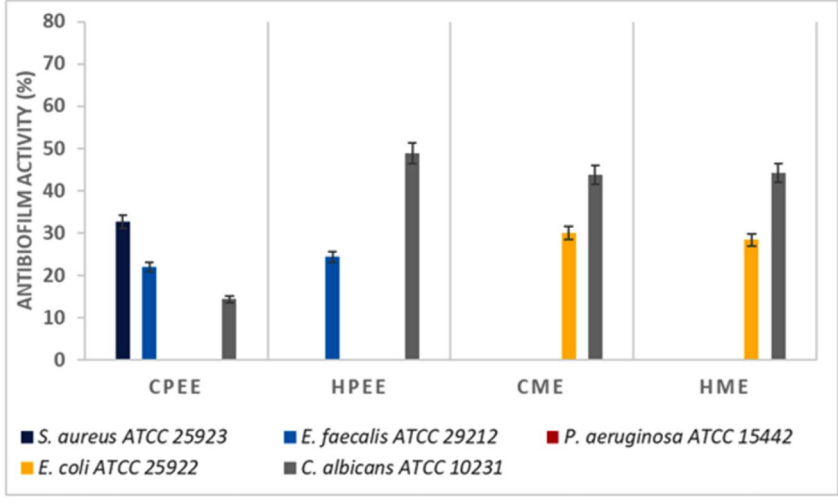


Figure 6. Activity against preformed biofilm 24 h old at 2.5 mg/mL concentration. Data are the mean ± SD of two independent experiments, each performed at least in quadruplicate, and expressed as inhibition percentage respect to the growth control. Data were considered significant at $P < 0.05$. **CPEE** = cold petroleum ether extract; **HPEE** = hot petroleum ether extract; **CME** = cold methanol extract; **HME** = hot methanol extract.

ORCID

Valentina Catania http://orcid.org/0000-0002-9439-0980
Demetrio Raffa http://orcid.org/0000-0002-1073-7706

References

Alsabri SG, Rmeli NB, Zetrini AA, Mohamed SB, Meshri MI, Aburas KM, Bensaber SM, Mrema IA, Mosbah AA, Allahresh KA, Hermann A, Gbaj A. 2013. Phytochemical, anti-oxidant, anti-microbial, antiinflammatory, and anti-ulcer properties of *Helianthemum lippii*. J Pharmacogn Phytochem. 2:86–96.

Atef C, Anouar F, El Hadda A, Azzedine A. 2015. Antimicrobial activities of *Helianthemum lippii* (L.) Pers in different stages of growth (somatic, flowering and fruiting). World J. Pharm Pharm Sci. 4:337–349.

Badria FA, Hetta MH, Sarhan RM, Ezz El-Din MH. 2014. Ezz El-Din HM 2014. Lethal effects of *Helianthemum lippii* (L.) on acanthamoeba castellanii cysts in vitro. Korean J Parasitol. 52(3):243–249.

Barnes RH, Karatzas KAG. 2020. Investigation into the antimicrobial activity of fumarate against Listeria monocytogenes and its mode of action under acidic conditions. Int J Food Microbiol. 324:108614.

Belyagoubi-Benhammou N, Belyagoubi L, Bekkara FA. 2014. Phenolic contents and antioxidant activities in vitro of some selected Algerian plants. J Med Plant Res. 8:1198–1207.

Bouallala M, Chehma A, Hamel F. 2013. Évaluation de la valeur nutritive de quelques plantes herbacées broutées par le dromadaire dans le sahara nord-occidental algérien. Leban Sci J. 14:33–39.

Burrascano S, Chytrý M, Kuemmerle T, Giarrizzo E, Luyssaert S, Sabatini FM, Blasi C. 2016. Current European policies are unlikely to jointly foster carbon sequestration and protect biodiversity. Biol Conserv. 201:370–376.

Celesti-Grapow L, Bassi L, Brundu G, Camarda I, Carli E, D'Auria G, Del Guacchio E, Domina G, Ferretti G, Foggi B, et al. 2016. Plant invasions on small Mediterranean islands: An overview. Plant Biosystems. 150(5):1119–1133.

Choi YH, Han SS, Lee HO, Baek SH. 2005. Biological activity of bioactive components from acer ginnala max. Bull Korean Chem Soc. 26:1450–1452.

Cusimano MG, Ardizzone F, Nasillo G, Gallo M, Sfriso A, Martino-Chillura D, Schillaci D, Baldi F, Gallo G. 2020. Biogenic iron-silver nanoparticles inhibit bacterial biofilm formation due to Ag+release as determined by a novel phycoerythrin-based assay. Appl Microbiol Biotechnol. 104(14):6325–6336.

Cusimano MG, Spinello A, Barone B, Schillaci D, Cascioferro S, Magistrato A, Parrino B, Arizza V, Vitale M. 2019. A synthetic derivative of antimicrobial peptide Holothuroidin 2 from Mediterranean Sea Cucumber (*Holothuria tubulosa*) in the Control of Listeria monocytogenes. Mar Drugs. 17(3):159.

Djemam N, Lassed S, Gül F, Altun M, Monteiro M, Menezes-Pinto D, Benayache S, Benayache F, Zama D, Demirtas I, et al. 2020. Characterization of ethyl acetate and n-butanol extracts of Cymbopogon schoenanthus and *Helianthemum lippii* and their effect on the smooth muscle of the rat distal colon. J Ethnopharmacol. 252:112613. and references therein cited.

Ermeli NB, Alsabri SG, Bensaber SM, Mohamed SB, Zetrini AA, Aburas KM, Fitouri SR, Jaeda MI, Mrema IA, Hermann A, Gbaj AM. 2012. Screening of analgesic and anti-inflammatory activities for two Libyan medicinal plants: *Helianthemum lippii* and *Launaea residifolia*. J Chem Pharm Res. 4:4201–4205.

Fenu G, Bacchetta G, Giacanelli V, Gargano D, Montagnani C, Orsenigo S, Cogoni D, Rossi G, Conti F, Santangelo A, et al. 2017. Conserving plant diversity in Europe: outcomes, criticisms and perspectives of the Habitats Directive application in Italy. Biodivers Conserv. 26(2):309–328.

Fenu G, Bernardo L, Calvo R, Cortis P, De Agostini A, Gangale C, Gargano D, Gargano ML, Lussu M, Medagli P, et al. 2019. Global and regional IUCN red list assessments: 8. IB. 8:17–33.

Grochowski DM, Locatelli M, Granica S, Cacciagrano F, Tomczyk M. 2018. A review on the dietary flavonoid tiliroside. Compr Rev Food Sci F. 17(5):1395–1421.

Gullón B, Lú-Chau TA, Moreira MT, Lema JM, Eibes G. 2017. Rutin: a review on extraction, identification and purification methods, biological activities and approaches to enhance its bioavailability. Trends Food Sci Technol. 67:220–235.

Hamza A, Gtari M, Mohamed N. 2013. Micropropagation of *Helianthemum lippii* L. var *Sessiliforuim* (Cistaceae) an important pastoral plant of North African arid areas. Afr J Biotechnol. 12:6468–6473.

Hamza N, Berke B, Umar A, Cheze C, Gin H, Moore N. 2019. A review of Algerian medicinal plants used in the treatment of diabetes. J Ethnopharmacol. 238:111841.

He CL, Fu BD, Shen HQ, Jiang XL, Wei XB. 2011. Fumaric acid, an antibacterial component of *Aloe vera* L. Afr J Biotechnol. 10:2973–2977.

Imani A, Maleki N, Bohlouli S, Kouhsoltani M, Sharifi S, Dizaj SM. 2021. Molecular mechanisms of anticancer effect of rutin. Phytother. Res. 35:2500–2513.

Juurlink BH, Azouz HJ, Aldalati AM, AlTinawi BMH, Ganguly P. 2014. Hydroxybenzoic acid isomers and the cardiovascular system. Nutr J. 13:63.

Lakhdari W, Dehliz A, Acheuk F, Mlik R, Hammi H, Doumandji-Mitiche B, Gheriani S, Berrekbia M, Guermit K, Chergui S. 2016. Ethnobotanical study of some plants used in traditional medicine in the region of Oued Righ (Algerian Sahara). J Med Plants Stud. 4:204–211.

Lauricella M, Lo Galbo V, Cernigliaro C, Maggio A, Palumbo Piccionello A, Calvaruso G, Carlisi D, Emanuele S, Giuliano M, D'Anneo A. 2019. The anti-cancer effect of *Mangifera indica* L. peel extract is associated toγh2ax-mediated apoptosisin colon cancer cells. Antioxidant. 8(10):422.

Laury AM, Alvarado MV, Nace G, Alvarado CZ, Brooks JC, Echeverry A, Brashears MM. 2009. Validation of a lactic acid– and citric acid–based antimicrobial product for the reduction of Escherichia coli O157: H7 and Salmonella on beef tips and whole chicken carcasses. J. Food Protect. 72:2208–2211.

Li J, Malakhova M, Mottamal M, Reddy K, Kurinov I, Carper A, Langfald A, Oi N, Ok Kim M, Zhu F, Sosa CP, Zhou K, Bode AM, Dong Z. 2012. Norathyriol suppresses solar UVinduced skin cancer by targeting ERKs. Cancer Res. 72:260–270.

Masella R, Santangelo C, D'Archivio M, Li Volti G, Giovannini C, Galvano F. 2012. Protocatechuic acid and human disease prevention: biological activities and molecular mechanisms. Curr Med Chem. 19(18):2901–2917.

Mihai MM, Holban AM, Giurcaneanu C, Popa LG, Oanea RM, Lazar V, Chifiriuc MC, Popa M, Popa MI. 2015. Microbial biofilms: impact on the pathogenesis of periodontitis, cystic fibrosis, chronic wounds and medical device-related infections. Curr Top Med Chem. 15(16):1552–1576.

Raffa D, Maggio B, Plescia F, Cascioferro S, Raimondi MV, Cancemi G, D'Anneo A, Lauricella M, Cusimano MG, Bai R, et al. 2015. Synthesis, antiproliferative activity and possible mechanism of action of novel 2-acetamidobenzamides bearing the 2-phenoxy functionality. Bioorg Med Chem. 23(19):6305–6316.

Raffa D, Plescia F, Maggio B, Raimondi MV, D'Anneo A, Lauricella M, Daidone G. 2017. Anthranilamide-based 2-phenylcyclopropane-1-carboxamides, 1,1′-biphenyl-4-carboxamides and 1,1′-biphenyl-2-carboxamides: Synthesis biological evaluation and mechanism of action. Eur J Med Chem. 132:262–273.

Razavi SM, Zahri S, Zarrini G, Nazemiyeh H, Mohammadi S. 2009. Biological activity of quercetin-3-O-glucoside, a known plant flavonoid. Russ J Bioorg Chem. 35(3):376–378.

Sher H, Aldosari A. 2013. Ethnobotanical survey on plants of veterinary importance around Al-Riyadh (Saudi Arabia). Afr J Pharm Pharmacol. 7(21):1404–1410.

Shokri H. 2011. Evaluation of inhibitory effects of citric and tartaric acids and their combination on the growth of *Trichophyton mentagrophytes, Aspergillus fumigatus, Candida albicans, and Malassezia furfur*. Comp. Clin. Pathol. 20:543–545.

Subramanian AP, John AA, Vellayappan MV, Balaji A, Jaganathan SK, Supriyanto E, Yusofa M. 2015. Gallic acid: prospects and molecular mechanisms of its anticancer activity. RSC Adv. 45:35608–35621.

Tomás-Menor L, Morales-Soto A, Barrajón-Catalán E, Roldán-Segura C, Segura-Carretero A, Micol V. 2013. Correlation between the antibacterial activity and the composition of extracts derived from various Spanish *Cistus* species. Food Chem Toxicol. 55:313–322.

Ustun O, Ozcelik B, Akyon Y, Abbasoglu U, Yesilada E. 2006. Flavonoids with anti-Helicobacter pylori activity from *Cistus laurifolius* leaves. J Ethnopharmacol. 108(3):457–461.

Vandal J, Abou-Zaid MM, Ferroni G, Leduc LG. 2015. Antimicrobial activity of natural products from the flora of Northern Ontario, Canada. Pharm Biol. 53:800–806.

Venturella G, Gargano LM, Compagno R, La Rosa A, La Bella S, Leto C, Tuttolomondo T. 2015. Up-to-date report on the distribution of *Helianthemum lippii* (Cistaceae) in Italy. J Plant Taxon Geog. 70(1): 151–154.

Volpato G, Puri RK. 2014. Dormancy and Revitalization: The fate of ethnobotanical knowledge of camel forage among Sahrawi nomads and refugees of Western Sahara. Ethnobot Res App. 12:183–210.

Wang W, Xue J, Tian T, Jiao Y, Li Y. 2013. The first asymmetric total synthesis of (+)-coriandrone A and B. Org Biomol Chem. 11(39):6686–6690.

Zanwar AA, Badole SL, Shende PS, Hegde MV, Bodhankar SL. 2014. Chapter 21 - Antioxidant role of catechin in health and disease polyphenols. Human Health Dis. 1:267–271.