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Sardinian plants with antimicrobial potential. Biological screening with multivariate data treatment of thirty-six extracts

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Abstract

In this paper, thirty-six extracts from Sardinian plants were evaluated *in vitro* for their antimicrobial activity towards a panel of reference strains, *Staphylococcus aureus*, *S. epidermidis*, *Klebsiella pneumoniae* and *Escherichia coli*, and for their cytotoxicity on mammalian cells. The biological data, together with total phenolic and flavonoid content of the extracts, were treated by PCA, which highlighted the positive correlation among total phenolic content and increasing antibacterial activities, and a possible involvement of flavonoids in mitigate the cytotoxicity. Thirteen extracts displayed a significant inhibitory effect towards *S. aureus* (IC_{50} from 1.4 to 153.6 µg/mL), ten out of them were active also against *S. epidermidis* (IC_{50} from 3.9 to 150 µg/mL), seven against *K. pneumoniae* (IC_{50} from 28.5 to 97.5 µg/mL), and two against *E. coli* (IC_{50} 74.9 and 156.3 µg/mL). In particular, three extracts obtained from *Pistacia terebinthus ssp. terebinthus*, *Cytinus hypocistis* and *Limonium morisianum* emerged as promising antibacterial candidates. They exhibited remarkable inhibitory activity towards bacterial strains from clinical specimens and presenting different antibiotic-resistance profiles.

Keywords

Antimicrobials; Sardinian plants; *Pistacia terebinthus ssp. terebinthus; Cytinus hypocistis; Limonium morisianum;* multivariate data treatment.

1. Introduction

In the current scenario, the clinical use of antibiotics, and therefore the effective treatment of bacterial infections, is under considerable threat due to the emergence of bacteria that have developed resistance to many classes of generally used antibiotics. Antibiotic-resistant bacterial infections are already widespread across the globe and very high rates of resistance have been ever-increasingly observed in common bacteria (WHO, 2014). Among *Staphylococcus* species, the prevalence of methicillin-resistant *S. aureus* and *S. epidermidis* (MRSA and MRSE, respectively) infections is growing worldwide and epidemiology is changing overtime. Although *S. aureus* and *S. epidermidis* are normal commensals of the skin and mucous membranes, MRSA is a leading cause of nosocomial infections and, more and more frequently, it is associated to community-acquired infections (mainly skin and wound infections) while MRSE has been identified as the most recurrent cause of health-care related bloodstream and device-related infections (Moellering, 2012; Rolo et al., 2012; May et al., 2014). Concerning Gram negative bacteria, high proportions of resistance to cephalosporins and fluoroquinolones have been reported for *Escherichia coli*, a normal inhabitants of the human intestinal microflora, and, of great concern, to carbapenems for *Klebsiella pneumoniae*, a primarily opportunistic bacterium that can be nosocomial or community acquired. These high reported resistances mean limitations to available treatment, which may be common in the population, such as urinary tract infections and pneumonia (Nordman et al., 2011).

Generally, infections by drug-resistant bacteria have an increased risk of worse clinical outcome and death compared to infections by the respective susceptible strains, and treatments must rely on second-line drugs that are more expensive and, sometimes, they have severe side-effects for which monitoring is advisable, increasing costs even further.

All these remarks have hastened and widened the quest for the discovery of novel agents for the treatment of bacterial infections.

In this context, plants represent a very important resource, producing hundreds of diverse metabolites, with medicinal and nutraceutical potential (Cragg & Newman 2013, Toledo et al., 2015; Chen et al.,

2014; Fung et al. 2013). Among their bioactivities, plant metabolites were proved also endowed with antimicrobial potential (Coqueiro et al., 2016; Snene et al., 2017; Dikpınar et al., 2018; Mahadi et al., 2018). In addition to find new antimicrobial molecules, plant extracts resulted interesting to study also for their non-antimicrobial compounds, which might be essential for the total bioactivity of the extract, improving solubility, absorption and stability of the active metabolites. Moreover, some phytochemicals, despite not being antimicrobial by themselves, showed antibiotic adjuvant activity, due to the inhibition of pathogens resistance mechanisms (Abreu et al., 2016, Abreu et al., 2017).

Sardinia (Italy), due to its geographical isolation and high geological and geomorphological diversification, represents a hotspot for biodiversity within the Mediterranean basin (Médail & Quézel, 1997; Médail & Quézel, 1999; Marignani et al., 2017). This Island constitutes an extremely diverse and dynamic environment with wide range of habitats and high degree of endemism (Fois et al., 2017), driving plants to increase and diversify the production of their secondary metabolites in order to adapt, compete and communicate with other species (Jahangir et al., 2008; Wang et al., 2005). In fact, Sardinian plants were found generally endowed with peculiar features, both in respect of the phytochemical and genetic profiles (Bobo-Pinilla et al., 2016; Dettori et al., 2016; Marengo et al., 2017; Sanna et al., 2018a; Venditti et al., 2017; Venditti et al. 2018).

However, despite Sardinian endemic plants resulted interesting for their phytochemical and biological features, yielding also new molecular scaffolds (Cagno et al., 2017; Daino et al., 2018; Mandrone et al., 2015; Mandrone et al., 2017; Maxia et al., 2015; Ornano et al., 2016; Sanna et al., 2018b; Venditti et al., 2016), the majority of them remains still poorly investigated.

On this basis, thirty-six extracts obtained from Sardinian plants, including twelve endemic species, were evaluated *in vitro* for their antibacterial activity against Gram positive and Gram negative reference bacteria, and selected extracts were assayed on a panel of fifteen clinical isolates presenting different antibiotic-resistance profiles. Moreover, cytotoxicity on mammalian epithelial cells was also tested.

The overall biological data, together with phenolic and flavonoid content, were summarized by principal component analysis (PCA).

2. Methods and materials

2.1. Plant material

Wild plants were harvested in Sardinia Island (Italy) during 2017 and 2018 and were identified by Dr. Cinzia Sanna and Prof. Andrea Maxia. Vouchers were deposited at the General Herbarium of the Department of Life and Environmental Sciences, University of Cagliari and reported in Table 1, where plants were listed in alphabetical order using the update nomenclature reported in the new checklist of Italian vascular flora (Bartolucci et al., 2018).

Table 1 The table lists all the plants used in this study. The update botanical names, the plant organ used and their labels, families, places and dates of collection and voucher numbers were reported.

Plant name	Plant organ and sample label in brackets	Family	Location of harvesting	Harvesting date	Voucher
Arbutus unedo L.	Fruits (AuF)	Ericaceae	Jerzu	December 2017	Herbarium CAG 878
	Leaves (AuL)		Jerzu	December 2017	
Asphodelus ramosus L. subsp *ramosus*	Rhizome (ArRh)	Asphodelaceae	Geremeas	April 2017	Herbarium CAG 1405
	Leaves (ArL)		Geremeas	April 2017	
Carlina gummifera (L.) Less.	Leaves (CgL)	Asteraceae	Cala Surya (Cardedu)	July 2018	Herbarium CAG 770
Centaurea calcitrapa L.	Aerial parts (CcA)	Asteraceae	Siliqua	June 2017	Herbarium CAG 781
Centaurea horrida Badarò*	Aerial parts (ChA)	Asteraceae	Capo Falcone	June 2017	Herbarium CAG 777
Centaurea napifolia L.	Aerial parts (CnA)	Asteraceae	Uta	June 2017	Herbarium CAG 784
Cistus monspeliensis L.	Aerial parts (CmA)	Cistaceae	Cala Surya (Cardedu)	April 2018	Herbarium CAG 135

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Cistus salviifolius L.	Aerial parts (CsA)	Cistaceae	Cala Surya (Cardedu)	April 2018	Herbarium CAG 135/C
Cynara cardunculus L.	Aerial parts (CycA)	Asteraceae	Siliqua	April 2017	Herbarium CAG 790
Cytinus hypocistis (L.) L.	Aerial parts (CyhA)	Cytinaceae	Gesturi	May 2017	Herbarium CAG 1200
Ferula arrigonii Bocchieri*	Leaves (FaL)	Apiaceae	Tharros	April 2017	Herbarium CAG 612/A
	Roots (FaR)		Tharros	April 2017	
Galactites tomentosa Moench	Aerial parts (GtA)	Asteraceae	Jerzu	September 2018	Herbarium CAG 789
Genista corsica (Loisel.) DC*	Aerial parts (GcA)	Fabaceae	Seui	May 2017	Herbarium CAG 286
Glechoma sardoa (Bég.) Bég.*	Aerial parts (GsA)	Lamiaceae	Gennargentu	June 2017	Herbarium CAG 1104
Hypericum hircinum L. *ssp hircinum**	Aerial parts (HhA)	Hypericaceae	Jerzu	June 2018	Herbarium CAG 232
Hypericum scruglii Bacch., Brullo & Salmeri*	Aerial parts (HsA)	Hypericaceae	Jerzu	June 2018	Herbarium CAG 239/C
Lavandula stoechas L.	Aerial parts (LsA)	Lamiaceae	Cala Surya (Cardedu)	April 2017	Herbarium CAG 1067
Limonium morisianum Arrigoni*	Aerial parts (LmA)	Plumbaginaceae	Jerzu	December 2017	Herbarium CAG 909/G
Myrtus communis L.	Fruits (McF)	Myrtaceae	Cala Surya (Cardedu)	December 2018	Herbarium CAG 514
	Leaves (McL)		Poggio dei Pini	April 2018	
Pistacia lentiscus L.	Fruits (PlF)	Anacardiaceae	Cala Surya (Cardedu)	December 2017	Herbarium CAG 280
	Leaves (PlL)		Cala Surya (Cardedu)	December 2017	
Pistacia terebinthus L. *ssp. terebinthus*	Leaves (PtL)	Anacardiaceae	Jerzu	June 2018	Herbarium CAG 279
Plagius flosculosus (L.) Alavi & Heywood*	Aerial parts (PfA)	Asteraceae	Iglesias	July 2017	Herbarium CAG 743
Ptilostemon casabonae (L.) Greuter*	Aerial parts (PcA)	Asteraceae	Gairo Taquisara	June 2018	Herbarium CAG 796
Rosmarinus officinalis L.	Aerial parts (RoA)	Lamiaceae	Alghero	May 2017	Herbarium CAG 1091
Santolina corsica Jord. & Fourr*	Aerial parts (ScA)	Asteraceae	Monte Albo	November 2017	Herbarium CAG 732/A
Scolymus hispanicus L. *subsp. hispanicus*	Aerial parts (ShA)	Asteraceae	Sarroch	June 2018	Herbarium CAG 812

Silybum marianum (L.) Gaertn.	Aerial parts (SmA)	Asteraceae	Uta	May 2017	Herbarium CAG 801
Smilax aspera L.	Aerial parts (SaA)	Smilacaceae	Geremeas	May 2017	Herbarium CAG 1414
Stachys glutinosa L.*	Aerial parts (SgA)	Lamiaceae	Gennargentu	June 2017	Herbarium CAG 1099
Tanacetum audibertii (Req.) DC*	Aerial parts (TaA)	Asteraceae	Gennargentu	August 2018	Herbarium CAG 737/A
Thymus herba barona Loisel.	Aerial parts (ThA)	Lamiaceae	Gennargentu	June 2017	Herbarium CAG 1065

*Endemic species of Sardinia

2.2. Chemicals and extracts preparation

All solvents and reagents were purchased from Sigma-Aldrich (Milan, Italy), MeOH was an analytical grade (≥ 99.9%).

Thirty mg of dried and powdered plant material were extracted by sonication for 30 minutes using 1.5 mL of MeOH/H_2O (1:1). Subsequently, samples were centrifuged (1700 × *g*) for 20 min, the supernatant was separated from the pellet and dried, firstly in vacuum concentrators (speedVac SPD 101b 230, Savant, Italy) for two hours to remove MeOH, then the residual extracts were freeze-dried over night to completely remove the residual H_2O finally yielding the crude extracts. For each sample different extracts were produced, in an adequate number to perform all the biological tests in replicates. This extraction procedure is designed to be performed relatively quickly and to prepare little quantity of extracts for *in vitro* bioactivity tests, been ideal for screenings of high number of plants. Moreover, this procedure allows a minimal waste of both solvents and plant material. The choice of a mid-polar solvent system such as aqueous MeOH and the use of sonication are recommended and used by several metabolomics studies (Kim & Verpoorte, 2010; Verpoorte, R. et al., 2007), where MeOH/H_2O (1:1) turned out as the best choice for a first line extraction procedure for general plant material, since it allows to extract a broad spectrum of compounds. This protocol has been also used to compare biological activities of plants to their phytochemical profile (Mandrone

et al, 2018), resulting also suitable to facilitate further metabolomic studies to identify the active principles of the extracts.

For biological assays, stock solutions were prepared solubilizing extracts in water at 10 mg/mL, centrifuged to remove the pellet if present, and stored at 4°C until use.

2.3. Total flavonoid and phenolic assays

The assays were performed in Spectrophotometer Jasco V-530 as described by Chiocchio et al. (2018). Briefly, for total phenolic content analysis a calibration curve was constructed using 50 µL of different gallic acid stock solutions prepared in MeOH 80% (from 10 to 200 µg/mL) mixed with 250 µL of Folin-Ciocalteau reagent (diluted 1:10) and 500 µL of H_2O. Different stock solutions of extracts were prepared in water (from 0.05 to 0.2 mg/mL) and 50 µL of each stock were mixed with the same reagents as described above. Both calibration curve and samples were incubated at room temperature for 5 min before adding 800 µL of sodium carbonate solution (Na_2CO_3 20%). After 30 min of incubation at 40°C, absorption was recorded at 760 nm. Total phenolic content was calculated by interpolation in the calibration curve and expressed as: mg GAE (gallic acid equivalent)/g of extract (dried weight).

Total flavonoid content was determined using rutin to perform the calibration curve. Different stock solutions of extracts were prepared in water (from 0.05 to 0.2 mg/mL) and 50 µL of each one were mixed with 450 µL of methanol and 500 µL of $AlCl_3$ (2% w/volume of methanol). The absorption at 430 nm was recorded after incubation (15 min) at room temperature. The calibration curve was obtained using 50 µL of different rutin stock solutions prepared in DMSO (from 1 to 100 µg/mL). Total flavonoid content of the extracts was calculated by interpolation in the calibration curve and expressed in terms of mg RE (rutin equivalent)/g of extract (dried weight). Analysis were performed in triplicate.

2.4. Multivariate data analysis

For multivariate analyses (PCA), data were subjected to UV (United Variance) scaling and the model was developed using SIMCA P+ software (v. 15.0, Umetrics, Sweden).

2.5. Bacterial reference strains and clinical isolates

Staphylococcus aureus ATCC 25293, *Staphylococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 9591) were obtained from the American Type Culture Collection. Subsequently, having defined the antibacterial properties of the extracts, the main active were assayed towards 15 clinical isolates recovered from different clinical specimens, and collected at the Microbiology Unit, St Orsola Malpighi University Hospital, Bologna, Italy. Strains included 5 *S. aureus* of which 3 methicillin-resistant (MRSA), 5 *S. epidermidis* of which 3 methicillin-resistant (MRSE) and 5 *K. pneumoniae* of which 2 carbapenemase-producing (KPC-producing *K. pneumoniae*). Species identification and antimicrobial susceptibility testing were performed by Vitek2 semi-automated system (bioMerieux, France), and EUCAST criteria were used for the interpretation of results and for the definition of methicillin and carbapenem resistance.

2.6. Determination of antibacterial activity

The *in vitro* antibacterial activity of the thirty-six extracts was evaluated against four reference strains and some selected extracts towards clinical isolates by a broth microdilution method (Bonvicini et al., 2014; Bonvicini et al., 2017). The bacterial suspension, prepared in Mueller Hinton broth (Sigma-Aldrich, St. Louis, USA) was incubated with the extracts at 200 µg/mL or serially two-fold diluted from 200 µg/mL depending on the assay. A number of wells was reserved in each microplate for negative (no inoculum added) and positive growth controls. The microplate was incubated at 37°C for 24h, and subsequently the $OD_{630\ nm}$ was spectrophotometrically measured (Multiskan Ascent microplate reader, Thermo Fisher Scientific Inc., Waltham, USA). Growth percentage values were determined as relative to the positive control. Extracts demonstrating an inhibitory activity superior to 70% at 200 µg/mL were defined as *active* and their IC_{50} values corresponding to the sample concentrations giving rise to an inhibition of bacterial growth of 50% were obtained by the interpolation on the dose-response curves. Statistical analysis was carried out by nonlinear regression method using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California, USA). A one-way ANOVA was done for comparison between IC_{50} values obtained for the reference

strains and clinical isolates followed by Dunnett's multiple comparison test to detect significant differences among groups.

2.7. Cell viability assay

African green monkey kidney cells (Vero ATCC CCL-81) were cultured in Eagle's Minimal Essential Medium (MEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Carlo Erba Reagents, Milan, Italy), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C with 5 % CO_2. For experiments, cells were seeded into 96-well plates at 10^4 cells/well, and incubated at 37°C for 24h. Cell density and incubation time were previously optimized (Bonvicini et al., 2018). Following washes with PBS (phosphate-buffered saline) to remove floating cells, monolayer was incubated with 100 µL of serially 2-fold dilution of the extract starting from 200 µg/mL, and with standard medium as positive control. The cell viability was assessed by a WST8-based assay according to the manufacturer's instructions (CCK-8, Cell Counting Kit-8, Dojindo Molecular Technologies, Rockville, MD, USA). After 48 h of incubation, culture medium was removed from each well, the monolayer was washed with PBS, and 100 µL of fresh medium containing 10 µL of CCK-8 solution were added and incubated for 2h at 37°C. Cell viability was measured at $OD_{450/630\ nm}$ and expressed as the percentage of the cell viability relative to the untreated controls. The CC_{50} values were obtained by the interpolation of percentage values on the dose-response curves.

3. Results and Discussion

3.1. Screening of biological activities and multivariate data analysis

The thirty-six extracts were assayed *in vitro* at 200 µg/mL to determine their antibacterial activity towards four reference strains and their cytotoxicity on mammalian epithelial cells. Overall data are reported in Tables S1 and S2 in Supplementary Material and Figure 1. Thirteen out of the thirty-six extracts resulted strong inhibitors of one or more bacteria (30% of bacterial growth compared to the extract-free control), as reported in Table 2. In particular, ten extracts inhibited the growth of both *S. aureus* and *S. epidermidis*, while three, PlF, RoA and SaA, showed activity only towards *S. aureus.*

Regarding the effectiveness on Gram negative bacteria, seven extracts were effective against *K. pneumoniae.* Only two extracts, CyhA and PtL were able to reduce the growth of all bacterial strains below the abovementioned threshold of activity (30%), reducing also *E. coli* activity of 34% and 33%, respectively, which were the lowest values obtained out of the thirty-six extracts tested.

Table 2. Bacterial growth of the reference strains treated with the 13 most active extracts at 200 µg/mL. Data are mean values and standard deviation obtained in two independent experiments performed in triplicate. Percentage values are relative to the positive control (100% of growth).

Sample lable	*S. aureus* ATCC 25293	*S. epidermidis* ATCC 12228	*E. coli* ATCC 25292	*K. pneumoniae* ATCC 9591
AuL	16 ± 3	2 ± 3	58 ±5	29 ± 5
CmA	8± 3	5 ± 5	66 ± 6	18 ± 4
CsA	11 ± 6	3 ± 4	47 ± 4	37 ± 10
CyhA	5 ± 4	3 ± 4	34 ± 14	19 ± 1
LmA	9 ± 4	10 ± 5	69 ± 12	44 ± 6
McF	19 ± 5	12 ± 7	69 ± 7	64 ± 6
McL	5 ± 8	4 ± 6	55 ± 8	26 ± 11
PlF	26 ± 9	49 ± 15	77 ± 8	42 ± 3
PlL	9 ± 8	7 ± 13	47 ± 5	24 ± 7
PtL	4 ± 5	3 ± 3	33 ± 6	17 ± 3
RoA	13 ± 6	74 ± 7	97 ± 6	89 ± 2
SaA	30 ± 11	111 ± 15	73 ± 13	76 ± 4
ThA	13 ± 3	21 ± 15	106 ± 10	90 ± 1

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The screening pipeline on the thirty-six extracts included the evaluation of their effects on cell viability and proliferation in order to discriminate between a specific ability to affect bacterial growth or to a general toxic activity on mammalian cells. As depicted in Figure 1, among the thirty-six extracts, eight strongly reduced mammalian cells metabolism below the 30% and, among these

extracts, six were labeled as *active* through the microbiological investigations, thus requiring further evaluations to specify their safety profile.

To gain comprehensive insights on the biological properties of all tested extracts, principal component analysis model (PCA) was build, using as set of x variables: the bioactivity data against the four bacterial strains (expressed as % of inhibition at 200 µg/mL), the cytotoxicity data (expressed as % of cell viability at 200 µg/mL), and total polyphenols and flavonoids content of the extracts, expressed as mg of gallic acid equivalents (GAE)/g of extract and % of rutin equivalents (RE)/g of extract, respectively. These latter phytochemical data are reported in Table S3 of Supplementary Material.

As shown by the PCA scatter plot (Figure 2A), antibacterial activity (against all strains) and phenolic content followed a similar trend. In fact, extracts shifted on the positive side of the component t[1] (PC1) were generally endowed with high value of both antibacterial activity and phenolic content. Phenolic compounds might be involved in the positive effects observed, since they have been recognized as bioactive molecules with pronounced antimicrobial activity (Gomes et al., 2018; Scavo et al., 2019). Conversely, on the negative side of PC1 axis, the extracts showing no activity on bacteria and an extremely low content of phenolic and flavonoid compounds were grouped. On the positive side of the PC1 and along the negative side of the component t[2] (PC2) were placed the extracts with the highest cytotoxicity on mammalian cells, such as CycA and CcA, and showing only a medium activity against *Staphylococci* spp. High level of cytotoxicity on Vero cells was shown also by CyhA, AuL and CsA, which followed, in fact, a similar trend along the PC2, shifting toward the lower-right quadrant of the plot. Nevertheless, their strong antibacterial activities made those extracts still interesting for further investigations (IC_{50} and SI determination), while CycA and CcA were considered not interesting, due to their strong cytotoxicity while scant antibacterial activity.

On the upper part of the plot (positive PC2), the extracts with medium antibacterial activity while very low cytotoxicity were clustered. Interestingly, low toxicity on mammalian cells was associated to high flavonoids content, suggesting a possible cytoprotective role of these compounds, which are

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also renowned antioxidants (Hosseinzadeh & Nassiri-Asl, 2014). Among the samples endowed with high content of flavonoids, a peculiar case was represented by PtL, which, in fact, was identified as an outlier in the PCA model. This extract showed high content of both phenols and flavonoids, high antibacterial activity against all strains tested and very low cytotoxicity.

The herein described model, providing a graphical overview of all biological data, facilitates also considerations on extracts obtained from plants belonging to the same genus. In particular, samples included three different species of *Centaurea* genus (*C. calcitrapa*, *C. napifolia* and *C. horrida*), and two different species of *Pistacia* (*P. lentiscus* and *P. terebinthus ssp. terebinthus*), *Cistus* (*C. salvifolius* and *C. monspeliensis*) and *Hypericum* (*H. scruglii* and *H. hircinum ssp. hircinum*). Regarding the three *Centaurea* species (CcA, CnA and ChA), they yielded very similar results, namely they were proved not active against all pathogens tested and were also poor in phenols and flavonoids. However, while CnA and ChA were also not cytotoxic on Vero cells, CcA was one of the highly cytotoxic extract of the dataset. Regarding the two *Cistus* species, CsA and CmA, they were placed very close in the PCA plot, since they showed a similar trend in both bioactivities and phenolic/flavonoids content. The same behavior was observed for the two species of *Hypericum* (HsA and HhA), which resulted both rich in flavonoids, not cytotoxic, while endowed with moderate antibacterial activity. Finally, the two *Pistacia*, PlL and PtL, were both strongly active against bacterial strains, even though PtL was more enriched in flavonoids and less cytotoxic than PlL.

As shown in Figure 2B, the majority of the samples studied were plant leaves or aerial parts, one was constituted by rhizomes (ArRh), one by roots (FaR), and three of them were fruits (PlF, McF and AuF). In case of *Myrtus communis* and *Pistacia lentiscus*, both fruits and leaves extracts were tested and proved to be active and characterized by similar features, appearing very close into the PCA scatter plot. Conversely, only leaves of *Arbutus unedo* (AuL) were active, while fruits (AuF), being not active, were placed on the opposite quadrant of the plot.

3.2. Antibacterial activity and selectivity

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The active subset of the thirteen extracts was further assayed *in vitro* towards some selected bacterial strains to obtain IC_{50} values on the specific dose-response curves. Based on data in Table 3, some general remarks can be drawn. Of the thirteen extracts inhibiting *S. aureus*, five displayed potent one-digit µg/mL IC_{50} values and CyhA resulted the most effective *S. aureus* inhibitor (IC_{50} = 1.4 µg/mL); of the ten extracts active towards *S. epidermidis* four exhibited comparable inhibitory effectiveness, and LmA displayed the highest activity (IC_{50} = 3.9 µg/mL). Concerning Gram negative bacteria, according to generally lower inhibition rates, IC_{50} values for the active extracts were superior compared to those obtained for Gram positive strains, however worthy of note for raw plant extracts (Cos et al., 2006). The extracts of CyhA and McL resulted the most potent against *K. pneumoniae* (IC_{50} = 28.5 µg/mL and IC_{50} = 37.0 µg/mL, respectively) and the first one, being active even towards *E. coli* (IC_{50} = 74.9 µg/mL), displayed a broad spectrum antibacterial activity. Differences in susceptibility between Gram positive and Gram negative bacteria are strictly related to the presence of the outer membrane and the lipopolysaccharides in the latter cells; these structures form an additional barrier that account for the Gram negative increased permeability threshold to many molecules.

Table 3. Antibacterial activity of the thirteen selected extracts expressed as IC_{50} (µg/mL of extract), defined as the concentration giving rise to an inhibition of growth of 50% compared to the drug-free control. Data are reported as mean values and 95% confidence interval.

Sample lable	***S. aureus*** **ATCC 25293**	***S. epidermidis*** **ATCC 12228**	***E. coli*** **ATCC 25292**	***K. pneumoniae*** **ATCC 9591**
AuL	31.9 [26.2-38.8]	10.1 [9.3-10.9]	n.d.[§]	93.8 [81.8-107.6]
CmA	5.3 [4.4-6.5]	12.4 [11.1-13.9]	n.d.	64.65 [57.0-73.2]
CsA	9.0 [7.9-10.4]	29.5 [26.4-32.9]	n.d.	97.5 [80.6-118.1]
CyhA	1.4 [0.9-1.9]	8.0 [7.5-8.5]	74.9 [57.9-96.9]	28.5 [22.8-35.6]
LmA	9.2 [6.8-12.3]	3.9 [2.5-6.1]	n.d.	n.d.

McF	15.4 [10.7-21.9]	8.8 [7.5-10.5]	n.d.	n.d.
McL	7.5 [6.0-9.3]	9.7 [8.9-10.9]	n.d.	37.0 [28.3-48.4]
PlF	144.5 [126.0-165.6]	n.d.	n.d.	n.d.
PlL	27.3 [21.6-34.5]	56.8 [48.1-67.2]	n.d.	48.0 [40.6-56.7]
PtL	62.9[48.6-81.4]	103.1 [92.6-109.0]	156.3[138.1-177.0]	49.0 [42.8-56.0]
RoA	99.2 [83.1-118.5]	n.d.	n.d.	n.d.
SaA	153.6 [129.1-182.7]	n.d.	n.d.	n.d.
ThA	63.3 [55.5-72.1]	150.0 [131.0-171.8]	n.d.	n.d.

[§] n.d. = not determined

Dose-effect experiments on Vero cells were finally carried out to establish their safety on non-malignant epithelial cells. Table 4 reports the CC_{50} values and the corresponding selectivity index (SI), calculated as CC_{50}/IC_{50} ratio, for the bacterial strain more susceptible to inhibition. Samples obtained from CyhA, LmA and McL presented very high SI in relation to Vero cells on *Staphylococci* spp. and only moderate values were obtained on *K. pneumoniae*, thus suggesting a preferential inhibitory activity towards bacterial cells with respect to eukaryotic cells.

Table 4. Cytotoxicity of active extracts against Vero cells and Selectivity Indexes (SI). CC_{50} is defined as the concentration giving rise to an inhibition of cell metabolism of 50% compared to the drug-free control. Data are reported as mean values and 95% confidence interval. SI = selective index corresponding to the ratio between CC_{50} and IC_{50}.

Sample lable	**CC_{50} (µg/mL)**	**SI**
AuL	41.7 [35.0-49.7]	4.1 (*S. epidermidis*)
CmA	88.2 [69.6-11.7]	16.5 (*S. aureus*)
CsA	53.7 [43.5-66.3]	5.9 (*S. aureus*)
CyhA	90.3 [75.2-108.3]	64.7 (*S. aureus*); 3.2 (*K. pneumoniae*)
LmA	>200	>51.0 (*S. epidermidis*)
McF	>200	>22.6 (*S. epidermidis*)
McL	120.2 [92.9-155.6]	16.1 (*S. aureus*); 3.3 (*K. pneumoniae*)

PlF	>200	>1.4 (*S. aureus*)
PlL	84.2 [74.2-95.5]	3.1 (*S. aureus*)
PtL	>200	4.1 (*K. pneumoniae*)
RoA	>200	>2.0 (*S. aureus*)
SaA	>200	>1.3 (*S. aureus*)
ThA	>200	>3.2 (*S. aureus*)

3.3. Clinical isolates

The three extracts selectively inhibiting bacterial growth were assayed also towards a broad array of relevant multi-resistant pathogens recovered from biological specimens. In particular, CyhA, LmA and PtL were assayed against *S. aureus*, *S. epidermidis* and *K. pneumoniae* strains, respectively. Data are reported in Table 5. Remarkably, the extracts proved to be active towards all the isolates and no statistically significant differences (ANOVA followed by Dunnett's Multiple comparison) were highlighted comparing IC_{50} values of isolates, regardless their antibiotic resistance profile (see Tables S4, S5 and S6 in the Supplementary Material), and reference strains. This is clinically relevant considering that isolates may present phenotypic and genetic heterogeneity compared to laboratory reference strains thus some differences in susceptibility may occur.

Table 5. IC_{50} values of the three selected extracts towards clinical isolates. Data are reported as mean values and 95% confidence interval.

CyhA Vs *S. aureus*	**IC_{50} (µg/mL)**	**Antibiotic-resistance profile**
ATCC 25293	1.4 [0.9-1.9]	
MSSA 1	1.6 [1.3-1.9]	CM^S, E^S, GMN^S, LVX^S, OX^S, P^R, TE^S, SXT^S
MSSA 2	2.8 [2.1-3.9]	CM^R, E^R, GMN^S, LVX^S, OX^S, P^S, TE^S, SXT^S
MRSA 1§	2.6 [1.9-3.6]	GMN^S, LVX^R, OX^R, P^R, TE^S, TEC^S, SXT^S, VA^S
MRSA 2§	3.2 [2.4-4.4]	GMN^S, LVX^R, OX^R, P^R, TE^S, TEC^S, SXT^S, VA^S
MRSA 3§	1.9 [1.6-2.2]	CM^R, E^R, GMN^S, LVX^R, OX^R, P^R, TEC^S, TE^S, SXT^S, VA^S
LmA Vs *S. epidermidis*		
ATCC 12228	3.9 [2.5-6.1]	

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MSSE 1	2.6 [1.0-6.7]	CM^S, E^R, GMN^S, LVX^S, OX^S, TE^S, SXT^S
MSSE 2	4.2 [2.1-8.3]	CM^S, E^S, GMN^S, LVX^S, OX^S, P^R, TE^S, SXT^S
MRSE 1§	3.0 [2.1-8.4]	CM^S, E^R, GMN^S, LVX^S, OX^R, P^R, TE^S, TEC^S, SXT^R
MRSE 2§	6.7 [3.9-11.5]	CM^S, E^S, GMN^S, LVX^S, OX^R, TE^S, SXT^R, VA^S, TEC^S
MRSE 3§	3.7 [1.8-7.8]	CM^S, DA^S, E^I, GMN^S, LVX^R, OX^R, TE^S, SXT^S, VA^S, TEC^S
PtL Vs *K. pneumoniae*		
ATCC 9591	49.0 [42.8-56.0]	
Kp 1	48.7 [42.0-56.5]	AK^S, AMC^R, CTX^R, CFZ^R, CIP^R, FOS^S, GMN^S, TZP^S, SXT^R
Kp 2	46.1 [37.5-56.6]	AK^S, AMC^S, CTX^S, CFZ^S, CIP^S, FOS^S, GMN^S, TZP^S, SXT^S
Kp 3	45.5 [34.7-59.7]	AK^S, AMC^S, CTX^S, CFZ^S, CIP^S, FOS^R, GMN^S, TZP^S, SXT^S
*KPC-Kp 1**	53.0 [42.2-66.5]	AK^R, AMC^R, AMP^R, CFZ^R, CIP^R, EPM^R, GMN^S, MEM^R, TZP^R, SXT^R, TGC^I, CS^S
*KPC-Kp 2**	47.3 [44.0-56.9]	AK^S, AMC^R, AMP^R, CFZ^R, CIP^R, EPM^R, GMN^R, MEM^I, TZP^R, SXT^R, TGC^S, CS^S

AK = Amikacin; AMC = Amoxicillin/Clavulanic Acid; AMP = Ampicillin; CM = Clindamicyn; CTX = Cefotaxime; CFZ = Ceftazidime; CIP = Ciprofloxacin; CS = Colistin; EPM = Ertapenem; E = Erythromycin; FOS = Fosfomycin; GMN = Gentamicin; LVX = Levofloxacin; MEM = Meropenem; OX = Oxacillin; P = Penicillin; SXT = Trimethoprim/Sulfamethoxazole; TE = Tetracycline; TEC = Teicoplanin; TZP = Piperacillin/Tazobactam, TGC = Tigecycline; VA = Vancomycin

R = Resistant; S = Susceptible; I = Intermediate, as defined following the EUCAST guidelines

§*Staphylococcus* species resistant to oxacillin were declared, by convention, methicillin-resistant.

*Carbapenemase-producing *K. pneumoniae.*

3.3 Traditional uses, bioactivities and phytochemical data of the three selected plants.

The effectiveness of these selected extracts validates the Sardinian plants *Cytinus hypocistis, Pistacia terebinthus ssp. terebinthus* and *Limonium morisianum* as important source of antimicrobial compounds. These plants might be interesting for the development of food supplements and herbal products with antibacterial activity. Moreover, since *Limonium morisianum* is an endemic plant of Sardinia, the obtained results might contribute also to valorize the biodiversity of the territory and the development of local industries.

Cytinus hypocistis is a parasitic plant belonging to Cytinaceae family that grows on roots of *Cistus spp*. It has been used in Sardinian traditional medicine as astringent, tonic and haemostatic (Loi et al.,

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2002), to soften corns and hard skin, and to sooth epidermal inflammations (Ballero et al., 1997). Despite this wealth of traditional uses, its chemical composition is largely unknown. Hydrolysable tannins were previously identified as the main components (Magiatis et al., 2001), confirming the high phenolic content of CyhA extract observed in this study, and among them, isoterchebin, belonging to the ellagitannin class, was characterized (Schildknecht et al., 1985).

Given the well-known antimicrobial properties of hydrolysable tannins (Buzzini et al., 2008) it is likely that these compounds might be responsible for the observed antibacterial activity of CyhA. Recently, Zucca et al. (2015) found antimicrobial activity of *C. hypocistis* but using an extraction procedure different from the one performed in this work. Chiocchio et al. (2018) reported also the anti-elastase and anti-tyrosinase activities of this plant. Moreover, antimalarial and antitumor properties of this plant have also been described (Fokialakis et al., 2007; Magiatis et al., 2001).

Pistacia terebinthus ssp. terebinthus (Anacardiaceae), commonly known as terebinth or turpentine tree, is a small deciduous tree widely distributed in the Middle East and Southern Europe. In Sardinia, it grows only on a calcareous restricted area of east coast (Usai et al. 2006). The consumption of *P. terebinthus ssp. terebinthus* in the Mediterranean countries traced back to ancient times. For instance, leaves of this plant have been used for the treatment of burns and the branch resin for bronchitis and other respiratory afflictions, as well as for anti-inflammatory and antipyretic properties (Topcu et al., 2007). The mature fruits were used as a diuretic and for urinary inflammations, stomachache (Cakilcioglu et al., 2010), stomach ulcers (Polat et al., 2013), antiseptic, hypotensive and for headache (Agelet and Vallès 2003). The resin is used as a chewing gum and as food additive (Schoina et al., 2015). In Sardinia the decoction has been used to treat catarrhal cough (Bruni et a., 1997), while the resin as expectorant, diaphoretic, analgesic, tonic and to obtain an ointment used for the treatment of bladders (Atzei 2003). *P. terebinthus ssp. terebinthus* has been reported to be rich in essential oil, proteins, organic acids, sugars, flavonoids, tannins and resinous substances (Couladis et al., 2003; Marengo et al., 2018; Ozcan, 2004; Ozcan et al., 2009; Piras et al., 2017; Pulaj et a., 2016; Usai et al., 2006). Several studies highlighted remarkable differences in the essential oil composition of this

plant, attributable to geographic and climatic features (Couladis et al., 2003; Dhifi et al., 2013; Duru et al., 2003; Ismail et al., 2013; Marengo et al., 2018; Piras et al., 2017; Ulukanli et al., 2014; Pulaj et al., 2016). *P. terebinthus ssp. terebinthus* is reported to be active as: antibacterial, antifungal, antioxidant, cytotoxic, neuroprotective, antinflammatory and insecticidal agent (Dhifi et al., 2013; Duru et al., 2003; Orhan et al., 2012; Ismail et al., 2013; Kavak et al., 2010; Kordali et al., 2003; Piras et al., 2017; Ulukanli et al., 2014; Pulaj et al., 2016; Topcu et al., 2007).

Limonium morisianum (Plumbaginaceae) is a dwarf frutex endemic and exclusive of calcareous mountains of Sardinia. To the best of our knowledge, no information on its use in Sardinian traditional medicine is available, since it is a very rare species. *Limonium* spp. are reported to contain several classes of active components, such as hydrolysable and condensed tannins, alkaloids, flavonoids, sterols, terpenes, saponins, coumarins, and amino acids (Blainski et al. 2013; Medini et al. 2014; Gadetskaya et al. 2015; Medini et al. 2015; de Oliveira Caleare et al. 2017). Moreover, myricetin, myricetin 3-*O*-rutinoside, myricetin-3-*O*-(6"-galloyl)-*β*-D-galactopyranoside, (-)-epigallocatechin 3-*O*-gallate, tryptamine, ferulic and phloretic acids have been identified from its aerial parts (Sanna et al., 2018. Definitely, *L. morisianum* has been slightly studied both phytochemically and biologically. Recently, the antiviral activity has been reported against HIV-1 and Ebola viruses (Sanna et al., 2018c; Daino et al., 2018), as well as the ability to inhibit tyrosinase and elastase enzymes (Chiocchio et al., 2018). No information on antimicrobial and cytotoxic activities has been previously reported for any extract of this plant.

4. Conclusions

This work reports the antimicrobial activity of some plants growing spontaneously in Sardinia (Italy). Thirty-six extracts were assayed *in vitro* towards four reference bacterial strains and evaluated for their cytotoxicity on mammalian epithelial cells.

The results of the biological screening, together with total phenolic and flavonoid content of the extracts, were processed through Principal Component Analysis (PCA), which highlighted the

positive correlation among total phenolic content and increasing antibacterial activities, and a possible involvement of flavonoids in mitigate the cytotoxicity against eukaryotic cells.

A significant activity was observed for thirteen extracts at non-cytotoxic concentration, and among them three emerged for their selective and potent inhibitory effect on bacterial growth; *Cytinus hypocistis* proved to be a broad spectrum antibacterial extract, mainly active towards *S. aureus* (IC_{50} 1.4 µg/mL), *Limonium morisianum* exhibited a potent anti-staphylococcal properties and *Pistacia terebinthus ssp. terebinthus* resulted the extracts with the highest SI on *K. pneumoniae*. These extracts, when tested towards isolates obtained from biological specimens and with different antibiotic-resistance profiles, confirmed their effectiveness to inhibit bacterial growth, thus validating their potential as antimicrobial agents.

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Declarations of interest

None

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Fig. 1

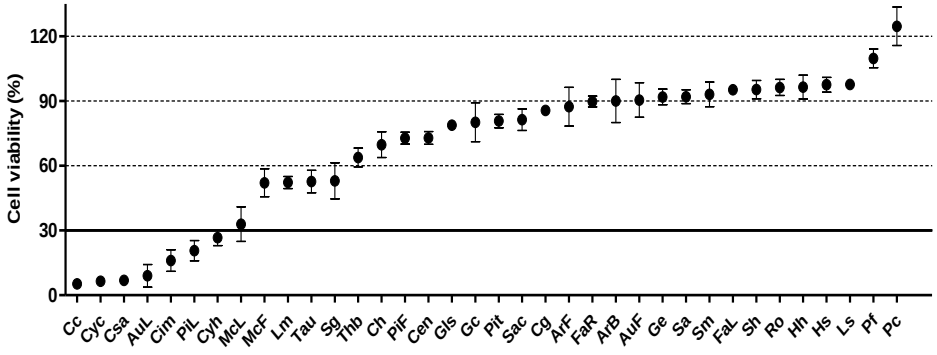
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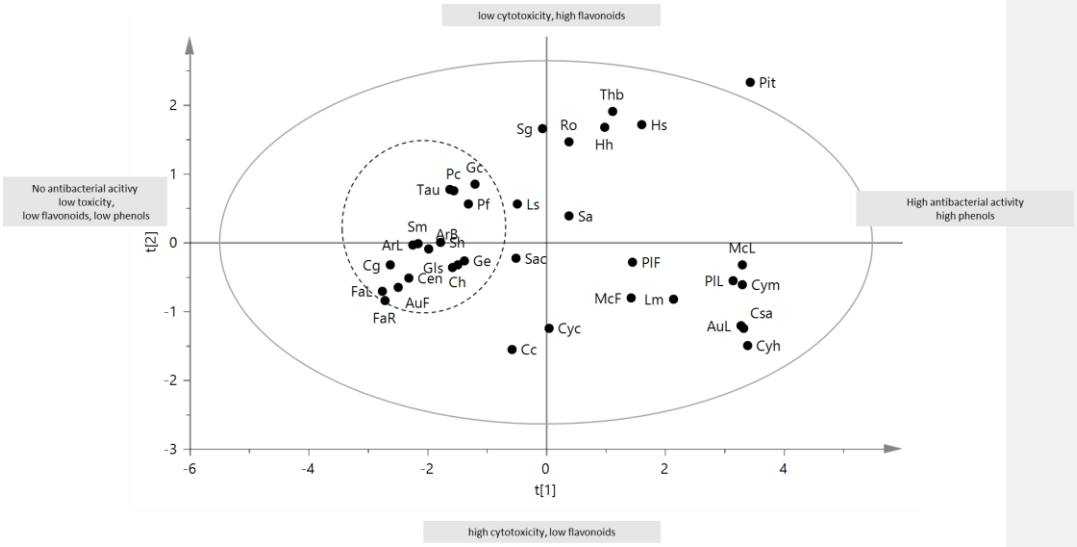
Fig 2.

A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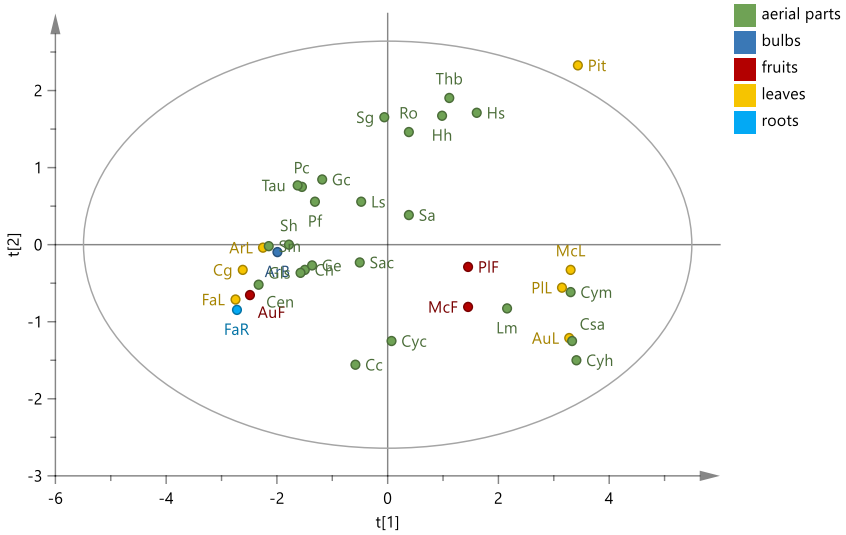
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