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RESEARCH ARTICLE

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Antibacterial, antioxidant and anti-proliferative properties and zinc content of five south Portugal herbs

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ABSTRACT

Context: *Crataegus monogyna* L. (Rosaceae) (CM), *Equisetum telmateia* L. (Equisetaceae) (ET), *Geranium purpureum* Vil. (Geraniaceae) (GP), *Mentha suaveolens* Ehrh. (Lamiaceae) (MS), and *Lavandula stoechas* L. spp. *luisieri* (Lamiaceae) (LS) are all medicinal.

Objective: To evaluate the antioxidant, antiproliferative and antimicrobial activities of plant extracts and quantify individual phenolics and zinc.

Material and methods: Aerial part extracts were prepared with water (W), ethanol (E) and an 80% mixture (80EW). Antioxidant activity was measured with TAA, FRAP and RP methods. Phenolics were quantified with a HPLC. Zinc was quantified using voltammetry. Antibacterial activity (after 48 h) was tested using *Enterococcus faecalis*, *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Listeria monocytogenes*. Antiproliferative activity (after 24 h) was tested using HEP G2 cells and fibroblasts.

Results: Solvents influenced results; the best were E and 80EW. GP had the highest antioxidant activity (TAA and FRAP of 536.90 mg AAE/g dw and 783.48 mg TE/g dw, respectively). CM had the highest zinc concentration (37.21 mg/kg) and phenolic variety, with neochlorogenic acid as the most abundant (92.91 mg/100 g dw). LS was rich in rosmarinic acid (301.71 mg/100 g dw). GP and LS inhibited the most microorganisms: *B. cereus*, *E. coli* and *S. aureus*. GP also inhibited *E. faecalis*. CM had the lowest MIC: 5830 µg/mL. The antibacterial activity is explained by the phenolics present. LS and CM showed the most significant anti-proliferative activity, which is explained by their zinc content.

Conclusion: The most promising plants for further studies are CM, LS and GP.

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HPLC; phenolics; flavonoids; Algarve; traditional medicine; solvent influence



Introduction

Since ancient times, the human race has been exploring the potential of plants to improve its health. From simple infusions to alleviate stomach problems to direct application on the skin to treat wounds, animal bites or other ailments, plants have had an important role in human history. When the modern times arrived, however, the attention turned to the use of synthetic drugs or other chemical substances in high amounts which in part led to bacteria resistance and weak immune system (Fair & Tor 2014; Fauci & Marston 2014). Nowadays the focus is shifting again, this time from synthetic to the natural agents, with a great interest in antioxidant, antibacterial and anti-carcinogenic properties of plants and their extracts. Since some of the plants' strongest antioxidants belong to the group of polyphenols, a preliminary analysis is often made in order to identify the most promising plants. This is important due to the difference in costs, with antioxidant assays being much cheaper and providing quicker results than antibacterial and anti-carcinogenic assays. Many aromatic plants have already been analyzed regarding their essential oil composition (hydrophobic compounds), due to its commercial value. On

this work we analyzed hydrophilic extracts, which are less studied, and determined their zinc, phenolic and antioxidant contents as well as antibacterial and antiproliferative activities. The extracts were obtained from five plants endemic to the Algarve region in Portugal, all used in traditional medicine.

Crataegus monogyna L. (Rosaceae) is an endemic tree in Portugal, which produces edible berries appreciated by shepherds and hunters (Barros et al. 2011). They are considered nutritious due to their content in vitamins and micronutrients and are used to treat gastro-intestinal disorders, rheumatism, heart problems and respiratory infections (Veveris et al. 2004; Jarzycka et al. 2013). Ripened fruits are rich in carbohydrates while unripe ones are rich in polyunsaturated fatty acids. Leaves on the other hand are rich in phenolic compounds, tocopherols and ascorbic acid (Jarzycka et al. 2013).

Plants belonging to genus *Equisetum* (Equisetaceae) are distributed throughout the Mediterranean basin, from Spain to Turkey. In Portugal the most common ones are *Equisetum arvensis* L. and *Equisetum telmateia* L. They are used in the folk medicine of many countries to treat urinary, kidney,

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prostate and gastrointestinal problems (Gurbuz et al. 2009). They are also used to treat inflammation and skin disorders and are believed to possess antimicrobial properties (Milovanovic et al. 2007). Their phytochemical profile revealed the presence of silicic acid, flavonoids and manganese (Veit et al. 1995).

Lavandula stoechas L. ssp. *luisieri* belongs to the Lamiaceae family, is endemic in the Iberian Peninsula and common in Portugal. Lavandula oils, well known for their scent and aroma, have been used in perfumery and food industry for many years (Da Porto et al. 2009). Its uses in traditional medicine include cold and headache therapy, an expectorant, stimulant and disinfectant. They are also believed to have antifungal and anti-depressive effects, and can be used to alleviate insect bites and burns (Cavanagh & Wilkinson 2002). The oil is composed by irregular monoterpenoids such as necrodane derivatives (Baldovini et al. 2005) and is known to possess antifeedant and antibacterial activities (Baldovini et al. 2005; Gonzalez-Coloma et al. 2006). Although the oil is well studied, there is little research concerning on its hydrophilic extract composition (Cavanagh & Wilkinson 2002).

The genus *Geranium* (Geraniaceae) is distributed throughout the northern hemisphere and is constituted by approximately 250 different species including *Geranium purpureum* Vil. which can be found in Portugal. Its aromatic oils are used in perfumery and the plant itself is used in gardens (Camacho-Luis et al. 2008). Its uses in traditional medicine include the treatment of inflammatory diseases, gastric disorders, fever, gall bladder, gastritis and hemorrhages (Neagu et al. 2010). Both kaempferol and quercetin derivatives are reported to be present in the *Geranium* genus which may explain some of the uses in traditional medicine (Sohretoglu et al. 2011).

Mentha suaveolens Ehrh. belongs to the Lamiaceae family and is highly present in the Mediterranean area. Its leaves, flowers and stems are used frequently in herbal teas or as food additives in many countries for their aroma and flavour (Diaz-Maroto et al. 2003). Moreover, the consumption of teas made from parts of *Mentha* spp. has many benefits on human health. In folk medicine the plant is used to treat nausea bronchitis, anorexia, ulcerative colitis and liver problems (El-Kashoury et al. 2013). This plant is known to possess anti-inflammatory, antiemetic, antispasmodic and analgesic properties, (Gulluce et al. 2007). It is also known that the essential oil from *Mentha suaveolens* has antimicrobial and antioxidant properties. According to its phytochemical analyses, the plant is rich in piperitenone, piperitone oxides, terpenic alcohol and other derivate compounds (Civitelli et al. 2014).

Material and methods

Plant material

Samples were randomly selected and collected during March 2013 in the Algarve region of Querença, Fonte Benémola, GPS coordinates 37.198946, -8.004309 and were identified by naturalist José Manuel Rosa Pinto, responsible for University of Algarve's herbarium, where a voucher specimen was deposited. The collected samples were *C. monogyna* (13501), *L. stoechas* spp. *luisieri* (13499), *G. purpureum* (13490), *E. telmateia* (13496) and *M. suaveolens* (13489). Plants were stored on a dry place, protected from sunlight and naturally air dried (ambient temperature of approximately 20 °C) for about one week, after which they were stored in plastic vials at -20 °C until extraction.

Biological material

Bacteria strains were gently provided by "Stress by Antibiotics and Virulence of Enterococci" laboratory from IBET (Instituto de Biologia Experimental e Tecnológica) and consisted of: *Enterococcus faecalis* - DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) (Brunswick, Germany) 20478, *Bacillus cereus* - ATCC (American Type Culture Collection) (Manassas, VA) 11778, *Escherichia coli* - ATCC 8739, *Staphylococcus aureus* - ATCC 6538, *Listeria monocytogenes* - DSMZ 7644.

Extraction procedure

A 0.2 g mass of plant material (in powder form) was extracted with 20 mL of solvent for 30 min in an electro thermal heating mantle. Stirring speed was set to 200 rpm, solvent temperature was 90 °C for water and 70 °C for both ethanol and 80% ethanol: water 1:1(v:v). For antibacterial assays the solvent was evaporated (Nahita serie 503, Navarra, Spain) and the extracted material was re-suspended. The solvent chosen for the resuspension was distilled water (which was able to resuspend the extracts after slightly heating them to 50 °C) instead of DMSO due to the latter capability of inhibiting bacterial growth, even at relatively low concentration (Wadhvani et al. 2009). Extracts and resuspended extracts were transferred to Eppendorf tubes and stored at -20 °C until analysis.

Total phenolic content

Total phenolic content (TPC) in samples was determined using a spectrophotometric procedure originally described by Singleton and Rossi (1965). Briefly, 0.1 mL of extract (diluted in the extraction solvent) was mixed with 0.5 mL of Folin-Ciocalteu's reagent, 0.4 mL of a saturated sodium carbonate solution (7.5%) and incubated for 30 min in a dark room. Absorbance was read in a T70+ UV/Vis spectrometer (PG Instruments, Ltd, Lutterworth, UK) at 765 nm against a blank. Phenolic content was calculated using a gallic acid calibration curve and the results were expressed as mg GAE/g dw (gallic acid equivalents per gram of dry weight).

Total flavonoid content

Total flavonoid content (TFC) was determined according to the method of Lamaison and Carnat (1990). Briefly, 0.4 mL of diluted extract was mixed with 0.8 mL of a 2% methanolic AlCl₃.6H₂O solution. After standing for 10 min in the dark the absorbance was measured at 430 nm. Flavonoid content was calculated using a quercetin calibration curve, and the results were expressed as mg QE (quercetin equivalents)/g dw.

Total antioxidant activity

Total antioxidant activity (TAA) of extracts was determined using a spectrophotometer and the method proposed by Prieto et al. (1998). Briefly, 0.1 mL of diluted extract was mixed with 1.0 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). This mixture was then incubated in a water bath at 95 °C for 90 min. Absorbance was read at 695 nm against a blank (negative control of water, ethanol or 80% ethanol: water according to the solvent used) and results were calculated from an ascorbic acid (positive control) calibration curve. Results were expressed as mg AAE (ascorbic acid equivalents)/g dw.

Reducing power

Reducing power (RP) was determined using the method previously described by Oyaizu (1986). Briefly, 0.2 mL of diluted extract were mixed with 0.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 0.5 mL potassium ferricyanide (1%). The mixture was then incubated at 50 °C for 20 min. After incubation, 0.5 mL of trichloroacetic acid (10%) were added and the mixture was centrifuged at 650 g for 10 min. 0.5 mL of the supernatant were mixed with 0.5 mL of distilled water and 0.1 mL of ferric chloride (0.1%). Absorbance was measured at 700 nm. Trolox was used as positive control and the negative control was water, ethanol or 80% ethanol: water according to the solvent used in the extraction. Results were expressed as mg TE (Trolox equivalents)/g dw.

Ferric-reducing antioxidant power

Ferric-reducing antioxidant power (FRAP) was determined using the method previously described by Benzie and Strain (1996). First, three solutions were prepared, a 300 mM acetate buffer, pH = 3.6, a 10 mM TPTZ, 40 mM HCl solution, and a 20 mM FeCl₃·6H₂O solution. Mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution and 2.5 mL of FeCl₃·6H₂O solution and heating the mixture to 37 °C prepared the working solution. After cooling down, 0.9 mL of the working solution were mixed with 0.1 mL of diluted extract. After staying 30 min in the dark the absorbance was read at 593 nm. Trolox was used as positive control and water, ethanol or 80% ethanol:water were used as negative control, according to the solvent used. Results were calculated from a Trolox calibration curve and expressed as mg Trolox/g dw.

DPPH radical scavenging activity

Radical scavenging activity was determined using the DPPH radical (Blois 1958) method (DPPH) with slight modifications. Briefly, 1.0 mL of a 0.16 mM DPPH solution was added to a test tube, which contained 1.0 mL of extract at different concentrations (two with more than 50% scavenging activity and two with less than 50% scavenging activity). This mixture was then vortexed and kept in the dark for 30 min, after which absorbance was measured at 517 nm. The radical scavenging percentage (IC) was calculated using the following formula: $(IC) = ((A_0 - A_t) / A_0) \times 100$, where A_0 is absorbance of control at 30 min and A_t absorbance of sample at 30 min. Trolox was used as positive control and the negative control was water, ethanol or 80% ethanol: water according to the solvent used. Results were expressed as µg sample/mL in form of IC₅₀, determined by linear regression of IC and extract concentration at 50% inhibition.

RP-HPLC analysis

Phenolic acids and flavonoids in dry biomass were quantified in methanol extracts (80 °C, 2 h) and after hydrolysis with 2 M aqueous HCl, 100 °C, 1 h (Harborne 1998). RP-HPLC analysis was conducted according to Ellnain-Wojtaszek and Zgorzka (1999) but with modification on Merck–Hitachi liquid chromatograph (LaChrom Elite) (San Jose, CA) equipped with a DAD detector L-2455 and Purospher® RP-18e (250x4 mm/5 µm) column. Analysis was carried out at 25 °C, with a mobile phase consisting of A – methanol, B – methanol: 0.5% acetic acid 1: 4 (v/v). The gradient was as follows: 100% B for 0–20 min; 100–80% B for 20–35 min; 80–60% B for 35–55 min; 60–0% B for

55–70 min; 0% B for 70–75 min; 0–100% B for 75–80 min; 100% B for 80–90 min at a flow rate 1 ml/min, $\lambda = 254$ nm (phenolic acids), $\lambda = 370$ nm (flavonoids). Identification was done by comparing retention times of peaks with standards and by adding the standards to the tested samples and verifying the increase in the peak area of the compound being identified (standard addition). Quantification was done by measurement of peak area with reference to the standard curve derived from five concentrations (0.03125 to 0.5 mg/mL). Standards were caffeic, chlorogenic, cinnamic, gallic, gentizic, *o*-coumaric, protocatechuic, salicylic, sinapic, syringic acid, isorhamnetin, kaempferol, luteolin, quercetin, quercitrin, rutin, vitexin, *p*-coumaric, vanillic, ferulic and *p*-hydroxybenzoic acid diluted in HPLC grade methanol.

Zinc

A multipurpose electrochemical analyzer M161 with the M164 electrode stand (both MTM-ANKO, Poland) was used for all voltammetric measurements. The standard three-electrode cell consisted of a controlled growth mercury drop electrode (CGMDE) as a working electrode, Ag/AgCl in 3 M KCl with a double junction filled with 3 M KCl (Mineral, Krakow, Poland) as reference and platinum wire as an auxiliary electrode. Voltammograms were recorded, interpreted and stored by EAGRAPH (MTM-ANKO, Krakow, Poland) software. A standard stock solution of Zn(NO₃)₂ was prepared by proper dilution of solution both with concentration of 1 g/L (OUM, Łódź, Poland). The electrolyte used as ionic medium was prepared by dissolving KNO₃ (Merck, Suprapur®). For digestion procedures HNO₃ (Merck, Suprapur®) was used. All the solutions were prepared with double-distilled water from quartz distiller (SZ-97A, Chemland, Stargard, Poland) and all reagents were of analytical grade. Samples were powdered in agate mortar and then dried at over 70 °C for 4 h. Approximately 250–500 mg of the sample material was weighed and inserted to high-pressure acid digestion vessel 4748 (Parr Instruments, Moline, IL) and treated with 5 mL of nitric acid. Next, the acid digestion bomb was closed and kept in the oven at 170 °C for 24 h. The digested sample was placed at the heated plate to let it evaporate and to remove the nitrate. The sampled solutions were cooled to room temperature, transferred quantitatively into volumetric flasks (10 mL) and filled up to the mark with double distilled water. All the procedures were repeated three times for each sample.

The zinc content of the selected samples was determined using a differential pulse anodic stripping voltammetry (DP ASV) with a controlled growth mercury drop electrode (CGMDE) under following parameters: pulse amplitude –20 mV; step height 2 mV; pulse width (waiting time – 20 ms; sampling time – 20 ms); resting time 5 s (Szlósarczyk et al. 2011). The voltammograms were recorded in the potential range from –1200 mV to 75 mV in diluted sample solution. Before each measurement, the solution in the voltammetric cell was de-aerated by high purity argon for 5 min. Voltammograms corresponding to individual additions were taken three times according to the standard addition method. All the experiments were performed at room temperature.

Cell lines culture

Human cell lines were obtained from the American Type Cell Culture collection, ATCC (LGC Standards-ATCC, Teddington, Great Britain). ATCC designations were as follows: BJ, normal adherent human skin fibroblasts, CRL-2522; HEP G2,

Table 1. Antioxidant and radical scavenging activities of the five tested plants.

Extract		TPC (mg GAE/g dw)	TFC (mg QE/g dw)	TAA (mg AAE/g dw)	RP (mg TE/g dw)	FRAP (mg TE/g dw)	DPPH IC ₅₀ (µg sample/mL)
<i>Crataegus monogyna</i>	Water	55.02 ± 2.68	6.05 ± 1.32	173.13 ± 8.86	52.57 ± 2.51	74.54 ± 4.64	61.56 ± 4.00
	80% Ethanol: water	478.12 ± 8.45	10.70 ± 1.66	243.31 ± 9.61	177.86 ± 7.54	225.52 ± 10.91	37.64 ± 2.20
	Absolute ethanol	241.87 ± 10.77	40.08 ± 1.74	137.79 ± 6.82	26.88 ± 3.69	168.56 ± 5.37	43.14 ± 2.02
<i>Equisetum telmateia</i>	Water	66.38 ± 3.05	8.68 ± 1.05	128.20 ± 7.27	8.67 ± 1.27	17.64 ± 1.96	79.53 ± 2.38
	80% Ethanol: water	371.82 ± 14.62	60.37 ± 1.28	370.05 ± 17.23	242.50 ± 2.66	353.42 ± 11.75	32.52 ± 2.32
	Absolute Ethanol	585.76 ± 8.83	16.50 ± 1.23	252.92 ± 9.94	9.17 ± 2.21	46.43 ± 5.47	22.60 ± 1.41
<i>Geranium purpureum</i>	Water	219.52 ± 9.35	24.73 ± 3.01	333.30 ± 15.01	169.07 ± 3.21	467.24 ± 7.85	211.57 ± 5.82
	80% Ethanol: water	293.22 ± 14.28	36.79 ± 1.19	472.04 ± 22.99	295.51 ± 9.53	705.91 ± 15.21	211.44 ± 10.33
	Absolute ethanol	326.90 ± 7.82	59.82 ± 2.84	536.90 ± 21.67	681.58 ± 20.18	783.48 ± 20.50	197.16 ± 7.38
<i>Lavandula stoechas</i> spp. <i>luisieri</i>	Water	276.67 ± 12.77	13.26 ± 1.31	343.50 ± 7.61	35.81 ± 1.85	80.35 ± 2.80	26.00 ± 1.59
	80% Ethanol: water	99.21 ± 5.56	9.80 ± 1.94	173.84 ± 7.55	313.57 ± 6.61	447.40 ± 21.26	38.32 ± 1.54
	Absolute ethanol	482.59 ± 10.67	7.18 ± 1.81	164.49 ± 8.56	51.98 ± 5.23	52.42 ± 8.12	123.02 ± 3.25
<i>Mentha suaveolens</i>	Water	172.97 ± 7.01	17.47 ± 1.23	289.00 ± 14.72	298.58 ± 14.34	396.60 ± 17.54	169.45 ± 5.54
	80% Ethanol: water	205.23 ± 5.40	39.74 ± 1.79	340.32 ± 12.74	333.67 ± 6.06	534.34 ± 21.12	150.11 ± 4.26
	Absolute ethanol	101.66 ± 4.83	56.98 ± 2.73	229.88 ± 7.17	90.80 ± 5.81	218.03 ± 11.92	219.40 ± 6.00

The results are represented as mean ± standard deviation from three replicates.

hepatocellular carcinoma. Cells were cultured according to ATCC's catalogue instructions. Briefly, cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ to confluence in Eagle's Minimum Essential Medium (EMEM, ATCC), supplemented with 10% v/v FBS (ATCC) and with antibiotic solution (100 IU/mL penicillin, 0.1 mg/mL streptomycin, Gibco Laboratories, Grand Island, NY). For experiments, the cells were at an exponential phase of growth.

Bacterial strains culture

Prior to the assay a culture of each microorganism was initiated in 2 mL Luria Broth and incubated at 37 °C for 24 h. After the 24 h the microorganism was cultured on solid Plate Count Agar medium using the streak method, and grown at 37 °C for 24 h.

MTT assay

Cell Viability Assay Microculture tetrazolium assay (MTT) was used as an indicator of cell viability. MTT assay determined by measuring the mitochondrial-dependent reduction of MTT to formazan was conducted as described previously (Tyszka-Czochara et al. 2014). Briefly, cells were seeded in 96-well microtiter plates (Sarstedt, Numbrecht, Germany). After optimum confluence, cells were treated with the extracts and incubated for 24 h at 37 °C. Five different concentrations (0.01, 0.1, 0.5 1.0 and 5.0 mg/mL) for each extract (diluted in DMSO) were used. Cells treated with equivalents of vesicle were considered as controls. After incubation, cells were washed before adding medium containing MTT (5 mg/mL) and kept for 1 h at 37 °C. Medium was then discarded and the formazan salt that had formed in cells was dissolved in DMSO. The optical density was measured at 570 nm (the reference wavelength was 630 nm) using a microplate reader (Tecan Austria GmbH, Grodig, Austria). Results were reported using the following formula: (average OD value of three measurements for each experimental group/average OD value of control group) × 100% (Nunes et al. 2014).

Disc diffusion assay

For the determination of minimum concentration of diffusion inhibition (MCDI) a method similar to that used in antibiotic susceptibility testing was used (Bauer et al. 1966), but with plant extracts instead (Gaudreau & Gilbert 1997). Briefly, a colony was

collected from the agar plate and suspended in a flask containing Ringer solution. An aliquot of 50 µL was spread on a petri dish containing solid plate count agar. After dried, discs were put in the Petri dish and 10 µL of extract diluted in distilled water (10 mg/mL) were added to each disk. Ampicillin (10 mg/mL) and streptomycin (10 mg/mL), diluted in distilled water, were used as positive controls and distilled water was used as negative control. After 24 (not shown) and 48 h of incubation, the inhibition diameter was checked. After assessing which strains were inhibited, several extract concentrations with 1 mg/mL of difference between them were tested to find the MCDI. All bioassays were repeated thrice in separated plates, and each plate had three discs per concentration. All the results are means ± SD.

Statistical methods

A one way analysis of variance (ANOVA) and the Student's *t* test were used for the evaluation of MTT test. *p* < 0.05 was considered as the level of significance.

Results

Antioxidant activity, phenolic compounds and trace elements

Out of the five plants screened, those with highest content in phenolic compounds (Table 1) were *E. telmateia* (585.76 ± 8.83) and *L. luisieri* (482.59 ± 10.67) in absolute ethanol, followed by *C. monogyna* (475.12 ± 10.31 mg GAE/g dw) in 80% ethanol:water. Regarding flavonoids content *E. telmateia* provided the highest results (60.37 ± 1.28) when a mixture of both solvents was used, while *G. purpureum* was the best on water (24.73 ± 3.01) and absolute ethanol (59.82 ± 2.84 mg QE/g dw). Regarding radical scavenging activity, the plants with most potential (lower IC₅₀), were *L. stoechas* spp. *luisieri* for water (26.00 ± 1.59) and *E. telmateia* for absolute ethanol (22.60 ± 1.41) and 80% ethanol: water (32.52 ± 2.32 µg/mL).

From the examined plants *C. monogyna* was the richest in phenolic compounds, with nine detected (Table 2) and had the highest zinc content (37.21 ± 4.03 mg/kg) (Table 3). The plant with the least phenolic diversity was *E. telmateia* in which only protocatechuic acid was detected (2.85 ± 0.41 mg/100 g dw). This compound was present on most analyzed samples, (was only absent in *M. suaveolens*) but always on small amount. On the other hand

Table 2. Individual compounds of the five tested plants identified by RP-HPLC.

	<i>Crataegus monogyna</i>	<i>Equisetum telmateia</i>	<i>Geranium purpureum</i>	<i>Lavandula stoechas</i> spp. <i>luisieri</i>	<i>Mentha suaveolens</i>
Caffeic acid	3.58 ± 0.92	n.d.	n.d.	3.94 ± 0.71	n.d.
Chlorogenic acid	37.05 ± 2.33	n.d.	n.d.	12.64 ± 1.32	n.d.
Coumaric acid ^a	4.26 ± 0.52	n.d.	n.d.	n.d.	n.d.
Ferulic acid	n.d.	n.d.	n.d.	17.30 ± 2.10	n.d.
Gallic acid ^a	n.d.	n.d.	162.38 ± 9.20	n.d.	n.d.
Kaempferol	n.d.	n.d.	24.14 ± 0.05	n.d.	n.d.
Neochlorogenic acid	92.91 ± 5.33	n.d.	115.35 ± 7.51	n.d.	n.d.
p-Hydroxy benzoic acid ^a	1.79 ± 0.07	n.d.	n.d.	1.70 ± 0.04	0.19 ± 0.02
Protocatechuic acid ^a	17.46 ± 1.35	2.85 ± 0.41	2.55 ± 0.21	1.29 ± 0.29	n.d.
Quercetin	23.12 ± 2.15	n.d.	27.13 ± 2.76	n.d.	n.d.
Rosmarinic acid	n.d.	n.d.	n.d.	301.71 ± 10.15	161.40 ± 5.32
Rutin	n.d.	n.d.	53.87 ± 1.20	n.d.	n.d.
Syringic acid	14.60 ± 2.00	n.d.	n.d.	n.d.	n.d.
Vanillic acid ^a	n.d.	n.d.	n.d.	1.07 ± 0.07	n.d.
Vitexin	20.10 ± 0.07	n.d.	n.d.	n.d.	n.d.

Results are expressed as mg/100 g of dry weight.

^aAfter HCl 2M digestion.

Table 3. Zinc content of the five tested plants.

Sample	Content of Zn(II) mg/kg ± SD
<i>Crataegus monogyna</i>	37.21 ± 4.03
<i>Equisetum telmateia</i>	28.30 ± 0.69
<i>Geranium purpureum</i>	11.63 ± 0.96
<i>Lavandula stoechas</i> spp. <i>luisieri</i>	23.55 ± 2.71
<i>Mentha suaveolens</i>	16.36 ± 1.58

The results are represented as mean ± standard deviation from three replicates.

rosmarinic acid, was only present in *L. stoechas* spp. *luisieri* and *M. suaveolens*, but was the phenolic present in greatest amount (301.71 ± 10.15 and 161.40 ± 5.32 mg/100 g, respectively).

Antibacterial activity

Equisetum telmateia and *C. monogyna* were the plants that needed the lowest concentration to inhibit the growth of a microorganism, with a MIC around 6000 µg/mL for *P. aeruginosa* (*E. telmateia*) and *L. monocytogenes* and *S. aureus* (*C. monogyna*). On the other hand *G. purpureum* and *L. stoechas* spp. *luisieri* inhibited the highest number of microorganisms. *G. purpureum* inhibited *B. cereus*, *E. coli*, *S. aureus* and *E. faecalis*, while *L. stoechas* spp. *luisieri* inhibited the first three. All *L. stoechas* spp. *luisieri* extracts demonstrated some inhibitory activity, while only the ethanolic extract of *G. purpureum* had activity (Table 4). *Crataegus monogyna* only inhibited two microorganisms, *L. monocytogenes* and *S. aureus*, but this was the only plant with the ability to inhibit the growth of *L. monocytogenes* and had the highest activity towards *S. aureus*. All microorganisms were inhibited by at least one plant. The most and least resistant microorganisms were *L. monocytogenes* and *S. aureus*, respectively. The positive control, ampicillin, inhibited all the microorganisms tested, with the exception of *P. aeruginosa*, for which streptomycin was used instead, on the other hand the negative control, water, inhibited none.

Antiproliferative activity

The anticancer potential of herbal extracts was analyzed according to proliferation inhibition of cells using MTT assay. Cell lines were exposed to plant extracts for 24 h, at concentrations of 0.01, 0.1, 0.5 1.0 and 5.0 mg/mL. Studies were conducted on cancer and normal cell lines of human origin, hepatocellular carcinoma (HEP G2) and normal skin fibroblast (BJ) to address the specific anti-tumour activity of the herbal extracts toward cell types of

different lineage. Dose response studies of the tested extracts are summarized in Figure 1.

Herbal extracts proliferation inhibition and cytotoxicity against cancer and normal cells varied in a dose-dependent manner. At a concentration of 0.01 mg/mL, only *L. stoechas* spp. *luisieri* had effect on the viability of Hep G2 cells. The incubation of both cell lines, tumour and normal ones, with addition of 5 mg/mL of any tested herbal extract, caused over 90% cell death. The highest anti-proliferative activity against Hep G2 cells was measured on *L. stoechas* spp. *luisieri* followed by *C. monogyna*. As shown in Figure 1, *G. purpureum* and *E. telmateia* revealed mild anti-proliferative potential while the inhibitory effect of *M. suaveolens* extract was the lowest.

Discussion

Antioxidant activity, phenolic profile and trace elements

For the majority of the tested plants, the ethanol–water mixture was better at extracting phenolics while pure ethanol was better at extracting flavonoids. Exceptions were *L. stoechas* spp. *luisieri* and *E. telmateia*. On these two plants ethanol was much better than the mixture at extracting phenolics and the ethanol–water mixture was better at extracting flavonoids. Regarding antioxidant activity, the ethanol–water mixture was the overall best solvent for all the plants except *G. purpureum* (for this plant pure ethanol was better). This solvent dependence is in accordance with the literature and shows the importance of using more than one solvent when studying the antioxidant activity of plants (Mimica-Dukic et al. 2008; Radojevic et al. 2012; Keser et al. 2014).

The individual phenolic compounds distribution was dependent on the plant being analyzed. The most common phenolics were protocatechuic and p-hydroxybenzoic acids, present in four and three of the five samples, respectively. Despite being the most common, they were only found in low amounts. Both are strong antioxidants and protocatechuic acid has mild activity against leukemia cells, which is an early indicator that some of the plants may have antiproliferative activity (Tseng et al. 2000). Those present in highest amounts were rosmarinic, neochlorogenic and gallic acids, rutin, quercetin and chlorogenic acid in decreasing order. Rosmarinic acid is a phenolic compound with antioxidant activity both in lipid protection and radical scavenging. It also has antimicrobial activity against several microorganisms including two tested in this work, *E. faecalis* and *P. aeruginosa* (Abedini et al. 2013). Chlorogenic acid and its isomer

Table 4. Antibacterial activity of the five tested plants.

Plant	Extraction solvent	MIC ($\mu\text{g/mL}$) after 48h					
		<i>Bacillus cereus</i>	<i>Escherichia coli</i>	<i>Enterococcus faecalis</i>	<i>Listeria monocytogenes</i>	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>
<i>Crataegus monogyna</i>	Water	x	x	x	x	x	9830 \pm 410
	80% ethanol:water	x	x	x	5830 \pm 410	x	5830 \pm 410
	Ethanol	x	x	x	x	x	7830 \pm 410
<i>Equisetum telmateia</i>	Water	x	x	x	x	6000 \pm 0	x
	80% ethanol:water	x	x	x	x	x	x
	Ethanol	x	8000 \pm 0	9670 \pm 520	x	x	x
<i>Geranium purpureum</i>	Water	x	x	x	x	x	x
	80% ethanol:water	x	x	x	x	x	x
	Ethanol	10000 \pm 0	9670 \pm 520	9830 \pm 410	x	x	8000 \pm 0
<i>Lavandula stoechas</i> spp. <i>luisieri</i>	Water	9830 \pm 410	x	x	x	x	9830 \pm 410
	80% ethanol:water	x	7000 \pm 0	x	x	x	x
	Ethanol	7670 \pm 520	x	x	x	x	x
<i>Mentha suaveolens</i>	Water	x	x	x	x	x	x
	80% ethanol:water	x	8000 \pm 630	x	x	9670 \pm 520	8330 \pm 520
	Ethanol	x	x	x	x	x	x

The results are represented as mean \pm standard deviation from nine replicates. x – No inhibition at maximum tested concentration (10,000 $\mu\text{g/mL}$).

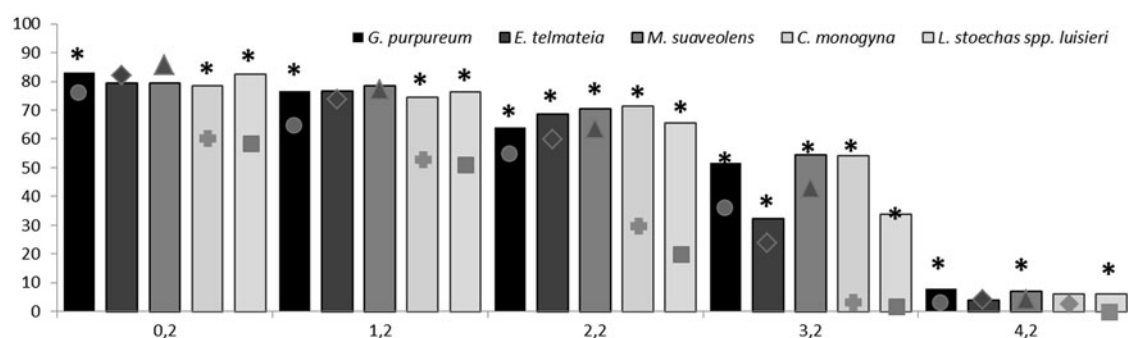


Figure 1. Effect of *Lavandula stoechas* L. spp. *luisieri*, *Crataegus monogyna* L., *Geranium purpureum* Vil., *Equisetum arvense* Ehrh. and *Mentha suaveolens* Ehrh. on the proliferation of human tumour (Hep G2) and non-tumour (fibroblasts) cell lines as determined by the MTT assay. The bars represent the fibroblasts while the marks represent the Hep G2 cells. The mean values with asterisks were significantly different ($p < 0.05$, $n = 3$).

neochlorogenic acid are antioxidant and antibacterial compounds. Their antibacterial activity is due to the disruption of the bacterial membrane, which changes the intracellular potential and leads to the bacteria death (Lou et al. 2011). These are known to have activity against four of the six tested bacteria, *B. cereus*, *E. coli*, *E. faecalis* and *S. aureus* (Fiamegos et al. 2011; Lou et al. 2011). Gallic acid is a trihydroxybenzoic acid with high antioxidant potential and anti-mutagenic potential that potentiates the expression of antioxidant-related and DNA repairing enzymes (Abdelwahed et al. 2007). Its effect on protecting diabetic-induced rats was also demonstrated (Punithavathi et al. 2011). According to Borges et al. (2013) gallic acid inhibits the growth of *E. coli*, *E. faecalis*, *L. monocytogenes*, *S. aureus* and *P. aeruginosa*, with higher activity towards the latter. Rutin is a quercetin derivate with antibacterial activity against *S. aureus*. Quercetin is a flavonoid with high antioxidant and antibacterial activities against *S. aureus* and *E. coli* (Rauha et al. 2000). The fact that gallic acid was present in only one of the five samples used in this study was somewhat surprising since it is one of the most common phenolic compounds in terrestrial plants (Fernandes & Salgado 2016). Kaempferol was only present in *G. purpureum*, ferulic acid was only present in *L. stoechas* spp. *luisieri*, and vitexin, coumaric acid and syringic acid were only present in *C. monogyna*.

According to the phenolic compound results, the high antioxidant activity detected in *G. purpureum* is due to gallic acid and quercetin, both are strong antioxidants and best extracted with water and ethanol, respectively. The antioxidant activity of *L. stoechas* spp. *luisieri* can be attributed to rosmarinic acid,

commonly found in *Rosmarinus* and *Lavandula* genus (Erkan et al. 2008; Slobodnikova et al. 2013). The higher ethanolic extract TPC results can be explained by its higher solubility in ethanol than in water (Wüst et al. 2016). Surprisingly, these results are not reflected in the antioxidant assays, since for RP and FRAP the best results were obtained with the mixture of both solvents. Pearson's correlation (data not shown) indicates that for *G. purpureum* and *L. stoechas* spp. *luisieri*, phenolics and flavonoids probably have a shared role on the antioxidant activity. However, while for *G. purpureum* both phenolics and flavonoids contribute positively for the antioxidant activity, in the case of *L. stoechas* spp. *luisieri*, and confirming the observation made before when comparing our results with other authors, TPC is negatively correlated with RP and FRAP and positively correlated with DPPH, meaning a higher TPC will lead to worse results. On the other hand, the antioxidant activity of *C. monogyna* and *M. suaveolens* is strongly correlated with phenolics, while *E. telmateia* antioxidant activity is probably mostly due to flavonoids. Regarding the trace elements analysis, only zinc was detected out of the tested elements. Zinc is an important mineral in human health, especially for pregnant woman and children on the developing stage (Favier & Hininger-Favier 2005). It is also important to test for the presence of heavy metals such as (Cd, Pb, Cu) because they can interfere on the living cells (Chen et al. 2002; Hwang et al. 2013) leading to false positive results. Since none was detected it is safe to assume that the results obtained are in fact due to the compounds present in the tested samples.

Similarly to our work, Keser et al. (2014) found the FRAP and DPPH radical scavenging activity of *C. monogyna* to be

better on ethanolic than on aqueous extracts. Also in agreement is the TPC (114.38 mg/GAE) of the aerial parts methanolic extract studied by Simirgiotis (2013) and the absence of gallic acid and kaempferol. That extract, however, had a higher TFC and DPPH radical scavenging activity (64.9 mg QE/g dw and IC of 3.34 µg/mL, respectively) and no chlorogenic acid (Simirgiotis 2013). In addition, when Urbonavičiūtė et al. (2006) studied this plant, they found the ethanolic % influences rutin and chlorogenic acid concentrations, with 60 to 80% ethanol providing the best results. Radojević et al. (2012) studied the methanol, acetone and ethyl acetate *E. telmateia* extracts while Mimica-Dukic et al. (2008) studied the aqueous and ethanolic extracts of *E. arvense* (a plant very similar to *E. telmateia*). Similarly to our work, they both found the TPC and TFC values to be solvent dependent while the RP value remained unaltered when extracted with water or ethanol. The radical scavenging activity obtained by Mimica-Dukic et al. (2008) was however higher than what we obtained, ranging from 2.37 to 37.20 µg/mL (versus 22.60 and 79.53 µg/mL). Despite the difference in values, which could be explained by the different plant and method of extraction used, in both cases ethanolic extracts were more powerful scavengers than aqueous extracts. When Milovanovic et al. (2007) studied *E. telmateia* they did not detect quercetin, similarly to us, but identified kaempferol which we did not. Ben Jemia et al. (2013), Radulović et al. (2012) and Proestos et al. (2013) studied plants from the *Geranium* genus and obtained much lower TPC values than ours, 32.24 mg GAE/g dw for *G. robertianum* methanolic extract, 109.5 mg GAE/g dw for *G. macrorrhizum* ethanolic extract and 4.0 mg GAE/g dw for *G. purpureum* methanolic extract respectively. While our plant TPC was higher, its scavenging activity of the DPPH radical was lower, with an average of 205 µg/mL versus 19.98 µg/mL for *G. robertianum* methanolic extract and approximately 70 µg/mL for *G. purpureum* aqueous extract (Sohretoglu et al. 2011). Proestos et al. (2006) also studied the individual phenolics of *G. purpureum*, and found gallic acid to be present at a much lower concentration (14 mg/100 g). In addition, they identified caffeic, coumaric, vanillic, syringic and *p*-hydroxybenzoic acids, which we did not. The TPC and DPPH IC₅₀ values of *L. stoechas* spp. *luisieri* studied by Baptista et al. (2015) and *L. stoechas* studied by Fouad and Mohamed (2014) are directly proportional while a higher TFC led to a lower DPPH IC₅₀, which is in agreement with our correlations. These results indicate that phenolics are not responsible for the DPPH scavenging activity and seem to in fact hamper it, since lower phenolic content extracts have a lower DPPH IC₅₀. Finally, the *L. stoechas* studied by Ceylan et al. (2015) had a higher caffeic and rosmarinic acids and lower ferulic acid concentrations (87.5 versus 3.94 mg/100 g; 834.7 versus 301.71 mg/100 g and 3.3 versus 17.30 mg/100 g, respectively). In addition they also identified quercetin, rutin and coumaric acid. Regarding the zinc content, *C. monogyna*, the plant with highest zinc content of our work was on par or above 19 out of 35 medicinal plants tested by Ansari et al. (2004).

Antibacterial properties

Strains from the microorganisms used, when detected from clinical samples, can cause serious health problems and some can even lead to death if treatment is not administered. Due to the increasing antibiotic and synthetic drug resistances of microorganisms, it is increasingly important to search for sources of natural compounds capable of inhibiting their growth. Considering the antioxidant activity and individual phenolic results, the plant

expected to exhibit the highest antibacterial activity was *G. purpureum* due to gallic acid and quercetin. Following *G. purpureum* would be *L. stoechas* spp. *luisieri* due to rosmarinic acid. This was in fact observed, with *G. purpureum* inhibiting the growth of *B. cereus*, *E. coli*, *E. faecalis* and *S. aureus* while *L. stoechas* spp. *luisieri* inhibited the same with the exception of *E. faecalis*. On the other hand, *C. monogyna* only inhibited *L. monocytogenes* and *S. aureus* but was highly active against the latter and was the only plant capable of inhibiting *L. monocytogenes*, while *E. telmateia* was the most effective plant against *P. aeruginosa*.

The solvent used in the extraction influenced the antibacterial activities displayed by the plants, which is in accordance with the literature. When Şöhretoğlu et al. (2011) tested aqueous and ethyl acetate *G. purpureum* extracts, their antibacterial activity was also dependent on the solvent, with the latter being much stronger. In that work the aqueous extract showed no inhibition towards *S. aureus* or *E. faecalis*, although that could be explained by the lower concentrations tested (1024 µg/mL), but *S. aureus* was, similarly to our work, more sensitive than *E. faecalis*. When Ceyhan et al. (2012) studied the antibacterial activity of *L. stoechas* using four different solvents (water, ethanol, ethyl acetate and hexane), they found that only aqueous and ethanolic extracts had antibacterial activity, and the latter was more powerful against all microorganisms, including *S. aureus* and *B. cereus*. This is not in accordance with our results and could be due to the different variety of the plant, our plant belongs to the *luisieri* subspecies, or due to the extraction process. These variations could also explain why the MIC of their water extract for *S. aureus* is 20 times higher than ours and why there was no inhibition towards *B. cereus* detected by them. Regarding *C. monogyna*, Ignat et al. (2013) found no inhibition towards *P. aeruginosa* and *E. coli*, but found, like us, this plant capable of inhibiting *S. aureus*.

Out of the tested microorganisms the least sensitive to the tested plant extracts was *L. monocytogenes* which was only inhibited by *C. monogyna*. On the other hand *S. aureus* was the most sensitive microorganism. It was inhibited by at least one extract from each plant. In the case of *C. monogyna*, all extracts showed inhibition. This sensitivity of *S. aureus* and resilience of *L. monocytogenes* can be explained by the compounds present on the plants. *S. aureus* can be inhibited by quercetin, rutin, kaempferol, gallic, coumaric, caffeic, ferulic, neochlorogenic, chlorogenic, protocatechuic and *p*-hydroxybenzoic acids, a total of 11 different compounds. Among them the most powerful are kaempferol, chlorogenic, neochlorogenic and protocatechuic acids (Pretto et al. 2004; Teffo et al. 2010; Fiamegos et al. 2011). From the tested plants *C. monogyna* was the plant with the highest number of them, 7, followed by *G. purpureum* with 6. Considering the compounds that were previously mentioned to be the most powerful against *S. aureus*, *C. monogyna* has the highest concentration of protocatechuic and chlorogenic acids, and a high concentration of neochlorogenic acid. *G. purpureum* also has some of the most powerful compounds, namely, the highest concentration of neochlorogenic acid and was the only plant with kaempferol. It also has protocatechuic acid but at a much lower concentration. On the other hand, *L. monocytogenes* can only be inhibited by three of the detected compounds: gallic, ferulic and protocatechuic acids (Borges et al. 2013; Stojkovic et al. 2013; Takahashi et al. 2015), with the latter being the strongest. Although *G. purpureum* and *L. stoechas* spp. *luisieri* had gallic acid and ferulic acid, respectively, the concentration present was not enough since none of these plants inhibited its

growth. *C. monogyna* did inhibit its growth, which can be explained by its high concentration of protocatechuic acid.

These results show that conducting a screening of the plants regarding their phenolic and flavonoid total contents along with antioxidant activity or their individual compounds is a good approach to identify those with the highest potential to be explored for their antibacterial properties.

Antiproliferative properties

The identification of Portuguese local herbs with anticancer potential is important. This allows the establishment of their role as potential natural anticancer medicine and increases the attention given by the natural populace of the area and scientific community, perhaps helping even in their conservation. The plants screened in this study (or plants of the same genus) have been previously reported to cause cytotoxic effects towards tumour cells and constitute therefore interesting candidates in a plant bioprospecting program for anticancer activity. The extract of *L. dentata* possess cytotoxic and pro-apoptotic properties assessed against human breast adenocarcinoma (MCF-7) cell lines using the MTT assay (Ali et al. 2014). Ku et al. (2014) evaluated antioxidant, anti-hyperglycemic, anti-cancer, anti-inflammatory, and anti-coagulant activities of hyperoside, an active compound from the genera of *Crataegus* and *Hypericum*. Venskutonis et al. (2010) reported that the extract of *G. macrorrhizum* possesses strong antioxidant properties. These are combined with cytotoxic and genotoxic activities on the bovine leukemia virus-transformed lamb kidney fibroblasts. *Mentha* leaf extracts were studied for pro-apoptotic properties and cell cycle arrest of tumor cells (HeLa, MCF-7, Jurkat, T24, HT-29, MIAPaCa-2) (Jain et al. 2011; Elansary & Mahmoud, 2015).

By screening wild Portuguese plant extracts for their antiproliferative properties against human hepatocellular carcinoma (HEP G2) and normal skin fibroblast cell lines, we found that most of them exhibited promising cytotoxic activity towards tumour cell lines. This cytotoxic activity was dependent on the herbal extract. The obtained results also indicate this cytotoxic activity is selective as the anti-proliferative influence on tumour cells was more pronounced compared to the effects measured in normal human fibroblasts (Figure 1).

Identifying novel bioactive compounds with anticancer properties from natural products is of great importance.

The selectivity in growth inhibition makes all tested herbal extracts interesting tools for further investigation of anticancer properties, although the most interesting for further studies regarding the identification of the mechanisms by which the bioactive compounds exerted the anticancer properties would be *L. stoechas* spp. *luisieri*.

The obtained results indicate that genus *Crataegus*, *Lavandula* and *Equisetum* contain not only polyphenolic compounds but also trace elements such as zinc. Zinc has been shown to exert beneficial effect on growth, proliferation and metabolism of normal human skin fibroblasts (Tyszka-Czochara et al. 2014), protecting non-tumour human cells from damage, when used simultaneously with high content of polyphenols. It was found that the anti-proliferative effects of *E. telmateia*, *C. monogyna* and *L. stoechas* spp. *luisieri* towards tumour cells were more pronounced than towards normal fibroblasts, at the same extract concentration (Figure 1). This was especially true for the latter two. These data indicate the protecting properties of the herb compounds, specifically regarding non-tumour cells, and suggests the pleiotropic action of traditionally local used Portugal herbs.

Conclusion

There are significant differences between the results when different solvents are used. Water was generally the least effective solvent at extracting antioxidant compounds. Out of all the tested plants *G. purpureum* had average the one the highest antioxidant activity while *C. monogyna* had the biggest polyphenolic compound variety, with nine different compounds identified. The compound present in highest amount was rosmarinic acid present in *L. stoechas* spp. *luisieri* and *M. suaveolens*. *G. purpureum* and *L. stoechas* spp. *luisieri* were the most promising in their composition with quercetin, caffeic and chlorogenic acids, all with known anti-cancer activity. The plants with the highest antibacterial activity were *C. monogyna* (lowest MIC) and *G. purpureum* followed by *L. stoechas* spp. *luisieri* (most microorganisms inhibited) which is attributed to the detected phenolic compounds. The highest concentrations of zinc, which has protective effect on normal fibroblasts, were detected in *C. monogyna*, *E. telmateia* and *L. stoechas* spp. *luisieri*. Overall the most promising plants for further studies were *C. monogyna* and *L. stoechas* spp. *luisieri* followed by *G. purpureum*.


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The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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