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Phenolic profile and *in vitro* bioactive potential of Saharan *Juniperus phoenicea* L. and *Cotula cinerea* (Del) growing in Algeria

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The aim of this study was to characterize the individual phenolic profile and antioxidant, anti-inflammatory, cytotoxic, and antimicrobial activities of hydroethanolic and infusion extracts prepared from Algerian Saharan *Juniperus phoenicea* L. and *Cotula cinerea* (Del). The phenolic profile was determined using a liquid chromatograph coupled to a diode array detector and an electrospray ionization mass spectrometer (LC-DAD-ESI/MS). A total of thirteen and nine individual phenolic compounds were identified in *J. phoenicea* and *C. cinerea*, respectively. 3-*p*-Coumaroylquinic acid, quercetin- and myricetin-*O*-pentoside were the major compounds present in *J. phoenicea*; on the other hand, *C. cinerea* presented luteolin-7-*O*-glucoside, luteolin-*O*-malonylhexoside, and 5-*O*-caffeoylquinic acid as the main molecules. In general, all samples exhibited interesting antioxidant activity when compared to the standard Trolox, but *J. phoenicea* extracts presented the highest bioactivity. Likewise, all the samples exhibited anti-inflammatory activity; thus *J. phoenicea* hydroethanolic extracts showed the highest potential (88 ± 8 μg mL^{-1}). In addition, their cytotoxicity was evaluated towards a panel of four selected cell lines (HeLa, NCI-H460, MCF-7 and HepG2), and all the extracts showed cytotoxic effects, with *J. phoenicea* extracts being the most effective. The *in vitro* antimicrobial activity of the plant extracts was moderate, Gram-positive bacteria thus being more sensitive than the Gram-negative strains (MIC values between 5 and 20 mg mL^{-1}). The present work suggests that *J. phoenicea* and *C. cinerea* are sources of bioactive ingredients with potential use in the food and pharmaceutical industries.

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1. Introduction

Medicinal plants are recognized for their therapeutic effects and biological characteristics, due to the presence of several bioactive substances belonging to different molecule classes, such as terpenoids, alkaloids, and phenolic compounds, like flavonoids, tannins, coumarins and phenolic acids.[1] These biomolecules are widespread in different plant parts, such as fruits, leaves, and seeds.[2] Nowadays, there is an increasing interest in the consumption of phytochemicals, like phenolic compounds, due to their important health effects, which are caused by their protective properties against a variety of pathologies mainly related with oxidative stress.[3] Many authors have reported the beneficial effects of polyphenols and mentioned that they present numerous bioactive properties, such as antibacterial,[4,5] antiviral, antifungal,[6] cytotoxic,[7] and anti-inflammatory[8,9] properties. These properties depend on their specific chemical structures[10] and much attention has been paid to the extraction and isolation of polyphenols from herbs having the ability to protect food and living systems from peroxidative damage.[11]

In order to search for effective natural bioactive compounds, the current study selected two traditional medicinal plants based on their ethno-pharmacological importance, for

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being applied as pharmaceutical and dietary supplements. Among the medicinal plants, *Juniperus phoenicea* L. and *Cotula cinerea* (Del) are Algerian Saharan herbs, harvested from southwest of Algeria and known to possess biological properties. *J. phoenicea* (Cupressaceae) is a small shrub of the Mediterranean basin, mostly distributed in its western part and largely growing in the Algerian mountains.[12] *J. phoenicea* is considered an important medicinal plant that is used in folk medicine, where decoctions and infusions of its leaves are used to treat diarrhea, rheumatism, bronco-pulmonary, diuretic, diabetic and obesity diseases;[13,14] it is also used in the treatment of hepatotoxicity and nephrotoxicity.[15] A recent study on *J. phoenicea* growing in Egypt reported that the crude extracts (petroleum ether, chloroform, ethyl acetate, and methanol) showed antiproliferative activity against lung carcinoma (H460), liver tumor (HepG2), and breast carcinoma (MCF7) cell lines.[16] Moreover, the hydroethanolic extract prepared from Algerian species demonstrated antioxidant activity.[17] The main bioactive compounds described in *Juniperus* species are mostly phenolic compounds.[18] Boulanouar *et al.*[17] analyzed these molecules using spectrophotometric methods and concluded that hydroalcoholic extracts of the aerial parts of *J. phoenicea* mainly presented phenolic acids and flavonoids. In addition, the presence of the biflavone, agathisflavone, in hydroalcoholic extracts of *J. phoenicea* leaves growing in Egypt was also reported.[16] Four flavonoid derivatives (cupressuflavone, hinokiflavone, hypolaetin-7-*O*-β-xylopyranoside and catechin) present in petroleum ether, chloroform, and methanol fractions obtained from the crude ethanol extract of the aerial parts of *J. phoenicea* growing in Saudi Arabia were isolated and detected by Alqasoumi *et al.*[19]

Cotula cinerea (Del) is a synonym of *Brocchia cinerea* (Dil.) Vis. (Asteraceae); it is known as a xerophytic herb in Algeria by the name "Guertouffa" or Saharan camomile (English) and is commonly found in the Algerian desert.[12] It is traditionally used as infusions and decoctions to treat digestive troubles, rheumatoid arthritis, urinary and pulmonary infections, fever, headaches, migraines, coughs, and joint inflammation.[20] This species has been described to have antioxidant, analgesic, antiseptic, and antimicrobial properties.[21–24] The main compounds have been isolated from the methanol extracts of Egyptian and Moroccan *C. cinerea*, and identified as phenolic acids (neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, and 3,4-dicaffeoylquinic acid) and flavonoids (luteolin-4′-*O*-glucoside, luteolin-7-*O*-β-D-glucoside, luteolin-6-hydroxy-7-*O*-β-D-glucoside, apigenin-7-*O*-α-rhamnoside, apigenin-6-*C*-arabinosyl-8-*C*-glucoside, isoschaftoside, quercetin-3-*O*-β-D-glucoside, and 5,3′,4′-trihydroxy 3,6,7-trimethoxyflavone).[25,26]

In this study, the most common form of consumption (infusion) of *J. phoenicea* and *C. cinerea* (Del) collected in southwest of Algeria was compared with a crude hydroethanolic extract. The extracts of both species were characterized in terms of phenolic composition and bioactive properties, such as antioxidant, cytotoxic, anti-inflammatory, and antimicrobial activities.

2. Materials and methods

2.1. Plant material

The samples of *Juniperus phoenicea* L. leaves and *Cotula cinerea* (Del) aerial parts were collected in southwest of Algeria at EL Bayadh (33° 05′ 18″ North; 0° 34′ 52″ East) and Bechar (31° 01′ 00″ North; 2° 44′ 00″ West), respectively, during the flowering period (May 2015). The plants were identified by Okacha Hasnaoui, Professor at the University of Tlemcen, Algeria. The samples were dried and ground to a fine powder (~20 mesh) prior to analysis.

2.2. Extraction procedure

Hydroethanolic and infusion extracts were prepared from *J. phenica* leaves and *C. cinerea* aerial parts. The hydroethanolic extraction (80% ethanol, 30 mL) was performed by maceration (150 rpm), with 1 g of each sample at 25 °C for 1 h and then filtered; the residue was re-extracted using the same methodology. Afterwards, the extracts were evaporated in order to remove the ethanol, under reduced pressure (Büchi R-210 rotary evaporator, Flawil, Switzerland). For aqueous extracts, 2 g of plant material was infused with boiling distilled water (200 mL) for 15 min and then filtered. Both extracts were previously frozen before lyophilizing (FreeZone 4.5, Labconco, Kansas City, MO, USA), in order to obtain a dry extract.

The lyophilized hydroethanolic and infusion extracts were dissolved in ethanol/water (80 : 20, v/v) and water, respectively, to obtain a stock solution of 10 mg mL^{-1} for the antioxidant activity assays: 5 mg mL^{-1} for the phenolic compound characterization; 20 mg mL^{-1} in culture medium for the antimicrobial assays; finally, 8 mg mL^{-1} in water for anti-inflammatory and cytotoxicity tests. In the bioactivity evaluation assays, the stock solutions were further diluted and tested.

2.3. Phenolic compound characterization

LC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) was used for phenolic compound characterization (identification and quantification), as previously detailed by Bessada *et al.*[27] The detection was preformed using a diode array detector (DAD, 280, 330, and 370 nm as the preferred wavelengths) and an ESI mass spectrometer operated in the negative mode (Linear Ion Trap LTQ XL mass spectrometer, Thermo Finnigan, San Jose, CA, USA). Calibration curves were prepared with different available standards and the results are expressed as mg per g of extract.

2.4. Antioxidant activity assays

Four different *in vitro* methods were used to evaluate the antioxidant activity, DPPH radical-scavenging activity, reducing power, β-carotene bleaching inhibition assay and lipid peroxidation inhibition by TBARS using methodologies previously described by Sobral *et al.*[28] Results are expressed as EC_{50} values (μg mL^{-1}) and Trolox was used as a positive control.

2.5. Anti-inflammatory activity assay

LPS-induced NO (nitric oxide) production by the murine macrophage (RAW 264.7) cell line was determined using the

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nitrite concentration in the culture medium according to a method previously described by Sobral *et al.*[28] Results are expressed as EC_{50} values (μg mL^{-1}), and dexamethasone was used as a positive control.

2.6. Cytotoxicity assays

Cytotoxicity was evaluated against four human tumor cell lines, MCF-7 (breast carcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma), and non-tumor porcine liver cell line (PLP2), using the sulphorhodamine assay previously described by Sobral *et al.*[28] Results are expressed as GI_{50} (μg mL^{-1}) and Ellipticine was used as a positive control.

2.8. Antimicrobial activity assays

The antimicrobial activity of the samples was tested against a range of strains from different microorganism: four Gram-positive bacteria (MRSA – methicillin-resistant *Staphylococcus aureus*, MSSA – methicillin-susceptible *Staphylococcus aureus*, *Listeria monocytogenes* and *Enterococcus faecalis*) and five Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Morganella morganii* and *Pseudomonas aeruginosa*) and one yeast (*Candida albicans*). The minimal inhibitory concentrations (MIC) were performed by the microdilution method using the *p*-iodonitrotetrazolium chloride (INT) colorimetric assay according to Dias *et al.*[29] The minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC) were calculated by adding 10 μL of the MIC value to fresh culture medium to see whether the bacteria were able to grow. After 24 h of incubation at 37 °C, MBC and MFC were registered. The antibiotic susceptibility profile of microorganisms has previously been described.[29]

2.8. Statistical analysis

For each species, three samples were used and all the assays were carried out in triplicate. Statistical comparisons were performed using the SPSS Statistics v. 23.0 program (differences were significant at the level of $\alpha = 0.05$) by using one-way analysis of variance (ANOVA) followed by Tukey's HSD. When necessary, a Student's *t*-test was used to determine the significant differences between less than three different samples, with $p = 0.05$. All the data were expressed as the mean values with standard deviations (SD).

3. Results and discussion

3.1. Phenolic compound characterization

As shown in Tables 1 and 2, a total of thirteen and nine individual phenolic compounds were identified in *J. phoenicea* and *C. cinerea*, respectively. Nine flavonols, one biflavone, flavan-3-ol, flavone, and a phenolic acid were identified in *J. phoenicea*; on the other hand, four flavones, two flavonols, and three phenolic acids were identified in *C. cinerea*.

Regarding *J. phoenicea*, peaks 2^{JP}, 6^{JP} and 9^{JP} were positively identified according to their retention time, mass and UV-vis

Table 1 Retention time (R_t), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, and identification and quantification of phenolic compounds in *Juniperus phoenicae* L. extracts (mean ± SD)

Peak	R_t (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg per g of extract)		
						Hydroethanolic extract	Infusion extract	Student's *t*-test
1^{JP}	6.2	310	337	191(100), 173(15), 163(22), 155(7), 137(5), 119(3)	3-*p*-Coumaroylquinic acid[1]	4.1 ± 0.1	3.09 ± 0.03	<0.001
2^{JP}	7.7	278	289	245(100), 231(22), 205(13), 179(20), 137(10)	Catechin[2]	0.230 ± 0.001	0.230 ± 0.001	<0.001
3^{JP}	13.3	356	611	479(100), 317(66)	Myricetin-*O*-pentoside-*O*-hexoside[3]	0.514 ± 0.005	0.305 ± 0.003	<0.001
4^{JP}	14.1	354	641	479(100), 317(28)	Myricetin-*O*-dihexoside[3]	0.50 ± 0.01	0.230 ± 0.001	<0.001
5^{JP}	14.5	356	479	317(100)	Myricetin-*O*-hexoside[3]	0.40 ± 0.01	0.337 ± 0.001	<0.001
6^{JP}	16.5	346	431	341(20), 311(100)	Apigenin-6-*C*-glucoside[4]	0.023 ± 0.001	tr	–
7^{JP}	17.6	357	463	317(100)	Myricetin-3-*O*-rhamnoside[3]	0.337 ± 0.001	0.27 ± 0.01	<0.001
8^{JP}	18.0	356	449	317(100)	Myricetin-*O*-pentoside[3]	1.00 ± 0.02	0.33 ± 0.01	<0.001
9^{JP}	18.8	350	463	301(100)	Quercetin-3-*O*-glucoside[3]	0.38 ± 0.01	0.230 ± 0.01	<0.001
10^{JP}	20.5	354	463	301(100)	Quercetin-*O*-hexoside[3]	0.362 ± 0.001	0.29 ± 0.01	<0.001
11^{JP}	22.4	355	433	301(100)	Quercetin-*O*-pentoside[3]	1.033 ± 0.003	0.48 ± 0.01	<0.001
12^{JP}	23.4	270, 330	551	537(100)	Methyl-biflavone[5]	0.31 ± 0.01	0.243 ± 0.004	<0.001
13^{JP}	26.9	350	447	315(100)	Isorhamnetin-*O*-pentoside[3]	0.42 ± 0.01	0.29 ± 0.01	<0.001
					Total phenolic acids	4.1 ± 0.1	3.1 ± 0.03	<0.001
					Total flavonoids	5.50 ± 0.03	3.2 ± 0.04	<0.001
					Total phenolic compounds	9.6 ± 0.1	6.3 ± 0.1	<0.001

tr – traces. Standard calibration curves: (1) *p*-coumaric acid ($y = 301950x + 6966.7$; $R^2 = 0.999$); (2) catechin ($y = 84950x - 23200$; $R^2 = 0.999$); (3) quercetin-3-*O*-glucoside ($y = 34843x - 160173$; $R^2 = 0.999$); (4) apigenin-6-*C*-glucoside ($y = 107025x + 61531$; $R^2 = 0.999$); (5) apigenin-7-*O*-glucoside ($y = 10683x - 45794$; $R^2 = 0.996$). When only two samples were present, a Student's *t*-test was used to determine the significant differences between two different samples, with $\alpha = 0.05$.

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Table 2 Retention time (R_t), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, and identification and quantification of phenolic compounds in *Cotula cinerea* (Del) extracts (mean ± SD)

Peak	R_t (min)	λ_{max} (nm)	$[M-H]^-$ (m/z)	MS^2 (m/z)	Tentative identification	Quantification (mg per g extract)		Student's *t*-test
						Hydroethanolic extract	Infusion extract	
1CC	7.2	324	353	191(100), 179(10), 161(5), 135(3)	5-*O*-Caffeoylquinic acid[1]	5.9 ± 0.1	5.9 ± 0.1	0.999
2CC	15.5	342	609	447(20), 285(100)	Luteolin-dihexoside[2]	2.38 ± 0.05	3.1 ± 0.1	<0.001
3CC	15.9	352	463	301(100)	Quercetin-*O*-hexoside[3]	1.70 ± 0.04	1.17 ± 0.04	<0.001
4CC	17.9	343	579	285(100)	Luteolin-*O*-pentosyl-hexoside[2]	3.3 ± 0.1	1.9 ± 0.1	<0.001
5CC	18.9	345	447	285(100)	Luteolin-7-*O*-glucoside[2]	36 ± 1	5.8 ± 0.1	<0.001
6CC	20.4	325	515	353(80), 191(100), 179(47), 173(5), 135(7)	3,5-*O*-Dicaffeoylquinic acid[1]	4.0 ± 0.1	1.27 ± 0.03	<0.001
7CC	22.4	350	549	505(80), 463(5), 301(100)	Quercetin-*O*-malonylhexoside[3]	1.39 ± 0.02	1.66 ± 0.03	<0.001
8CC	22.9	327	515	353(78), 191(28), 179(65), 173(100), 135(5)	4,5-*O*-Dicaffeoylquinic acid[1]	4.1 ± 0.2	0.915 ± 0.005	<0.001
9CC	23.8	345	533	489(80), 447(10), 285(100)	Luteolin-*O*-malonylhexoside[2]	6.6 ± 0.3	6.9 ± 0.2	0.168
					Total phenolic acids	14.1 ± 0.1	8.12 ± 0.04	<0.001
					Total flavonoids	51 ± 2	20 ± 1	<0.001
					Total phenolic compounds	65 ± 1	29 ± 1	<0.001

Standard calibration curves: (1) chlorogenic acid ($y = 168823x - 161172$; $R^2 = 0.999$); (2) apigenin-7-*O*-glucoside ($y = 10683x - 45794$; $R^2 = 0.996$); (3) quercetin-3-*O*-glucoside ($y = 34843x - 160173$; $R^2 = 0.999$). When only two samples were present a Student's *t*-test was used to determine the significant differences between two different samples, with $\alpha = 0.05$.

characteristics by a comparison with commercial standards. The remaining compounds were tentatively identified based on their pseudomolecular ions and fragmentation pattern. Flavonols were the most abundant flavonoids present in this species, representing 51–57% of the phenolic composition, with peaks 3^{JP}, 4^{JP}, 5^{JP}, 7^{JP}, and 8^{JP} being identified as myricetin glycosides based on their UV spectra (λ_{max} around 356 nm) and the production of a MS^2 fragment ion at m/z 317. Similarly, peaks 9^{JP}, 10^{JP} and 11^{JP} were identified as quercetin (λ_{max} around 353 nm, MS^2 fragment at m/z 301) glycosides, and peak 13 as isorhamnetin (λ_{max} around 350 nm, MS^2 fragment at m/z 315) glycoside. Peak 3^{JP} ($[M-H]^-$ at m/z 611) was identified as myricetin-*O*-pentosyl-*O*-hexoside, in which the MS^2 fragments revealed the alternative loss of pentosyl (m/z at 479; −132 u) and hexosyl (m/z at 317; −162 u) residues, indicating the location of each residue at different positions of the aglycone. The remaining compounds presented MS^2 fragments corresponding to distinct losses of dihexosyl (−324 mu), hexosyl (−162 mu), rhamnosyl (−146 mu), and pentosyl (−132 mu) moieties. An elution order coherent with the type of sugar substituent and according to their expected polarity was presented, although the position and nature of the sugar moieties could not be identified, because their retention times did not correspond to any of the standards available. Peak 7 was identified as myricetin-3-*O*-rhamnoside, which was previously identified in berries and leaves of *J. phoenicea* by Ali *et al.*[15] Peak 12 ($[M-H]^-$ at m/z 551) was identified as a methyl-biflavonoid taking into account the findings of Innocenti *et al.*[30] and Miceli *et al.*,[31] identifying this compound in fruits of *Juniperus communis*. Peak 1 ($[M-H]^-$ at m/z 337) was identified as 3-*p*-coumaroylquinic acid taking into account the hierarchical keys previously reported by Clifford *et al.*[32] This was the only phenolic acid present, and accounted for 43–49% of the total phenolic compounds.

In general, 3-*p*-coumaroylquinic acid was the main phenolic compound present in *J. phoenicea*, followed by quercetin-*O*-pentoside and myricetin-*O*-pentoside. Moreover, the hydroethanolic extract presented a higher concentration in these than in the infusion extract.

The obtained individual profile was different from those reported for the Egyptian *J. phoenicea* berries and leaves' extracts, which revealed the presence of two major biflavonoid compounds; cupressuflavone and amentoflavone in the ethyl acetate fraction, four flavonoids (myricitrin, quercetin, cosmosin, quercitrin), and two phenolic acids (*p*-coumaric acid and caffeic acid) in the methanol fraction.[15] The only compound that was similar to the one identified in the present work was myricitrin (peak 7). Moreover, Alqasoumi *et al.*[19] studied *J. phoenicea* petroleum ether, chloroform, and methanol fractions from Saudi Arabia, resulting in the identification of five known diterpenoids (13-epicupressic acid, imbricatolic acid, 7-α-hydroxysandaracopimaric acid, 3-β-hydroxysandaracopimaric acid and isopimaric acid) and four flavonoid derivatives (cupressuflavone, hinokiflavone, hypolaetin-7-*O*-β-xylopyranoside, and (+)-catechin). Maamoun *et al.*[16] identified 4 flavonoids (isoetin-7-*O*-β-glucoside, isoscutellarein, amentoflavone, and

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Table 3 Antioxidant activity and NO production inhibition capacity of *J. phoenicae* and *C. cinerea* extracts (mean ± SD)

	J. phoenicea		*C. cinerea*		
	Hydroethanolic extract	Infusion extract	Hydroethanolic extract	Infusion extract	Positive control[a]
Antioxidant activity (EC_{50} values, μg mL^{-1})					
DPPH scavenging activity	12 ± 1d	22.4 ± 0.6c	26.0 ± 0.1a	24.8 ± 0.2b	42 ± 1
Reducing power	12.0 ± 0.4d	18.2 ± 0.3c	31.9 ± 0.2b	38 ± 1a	41 ± 1
β-Carotene bleaching inhibition	11.57 ± 0.08c	20 ± 1a	14.7 ± 0.2b	20.2 ± 0.8a	18 ± 1
TBARS inhibition	7.6 ± 0.3a	6.7 ± 0.2b	7.4 ± 0.3a	7.5 ± 0.2a	23 ± 1
Anti-inflammatory activity (EC_{50} values, μg mL^{-1})					
Nitric oxide (NO) production	51 ± 4d	70 ± 5c	105 ± 9b	122 ± 6a	16 ± 1

[a] Trolox and dexamethasone for antioxidant and anti-inflammatory activities, respectively. The antioxidant activity was expressed as EC_{50} values (mean ± SD), which means that higher values correspond to lower reducing power or antioxidant potential. EC_{50}: extract concentration corresponding to 50% of antioxidant activity or 0.5 absorbance in the reducing power assay. Results of anti-inflammatory activity are expressed as EC_{50} values: the sample concentration providing 50% of inhibition of nitric oxide (NO) production. In each row different letters mean significant differences between extracts ($p < 0.05$).

agathisflavone) from ethanol, petroleum ether, chloroform, ethyl acetate, and methanol extracts of *J. phoenicea* growing in Egypt. These differences could be attributed to several factors such as geographical location, the part of the plant used and especially the use of different extraction methodologies and solvents.

Concerning *C. cinerea*, phenolic acids were the minor group of compounds present (22–28%), while flavonoids represented 72–78% of the phenolic composition. Peaks 1^{cc}, 6^{cc} and 8^{cc} were identified as 5-*O*-caffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, and 4,5-*O*-caffeoylquinic acid, respectively, according to the hierarchical keys previously developed by Clifford *et al.*[32] These compounds were also found in the areal parts of Morocco *C. cinerea* methanol extracts.[25] The remaining compounds correspond to flavonoid derivatives, mainly luteolin (λ_{max} around 345 nm, MS^2 fragment at *m*/*z* 285) and quercetin (λ_{max} around 350 nm, MS^2 fragment at *m*/*z* 301) glycosides. Peaks 2^{cc} ($[M-H]^-$ at *m*/*z* 609), 3^{cc} ($[M-H]^-$ at *m*/*z* 463), 4^{cc} ($[M-H]^-$ at *m*/*z* 579), and 5^{cc} ($[M-H]^-$ at *m*/*z* 447) were identified as luteolin-dihexoside, quercetin-*O*-hexoside, luteolin-*O*-pentosyl-hexoside, and luteolin-7-*O*-glucoside (identified in comparison with a commercial standard), using a similar reasoning as for the compounds described in *J. phoenicea*. Furthermore, peaks 7 ($[M-H]^-$ at *m*/*z* 549) and 9 ($[M-H]^-$ at *m*/*z* 533) released a MS^2 fragment at *m*/*z* 301 and 285 ($[M-H-162-86]^-$, loss of a malonylhexoside moiety), being tentatively assigned quercetin and luteolin-*O*-malonylhexoside, respectively.

Overall, luteolin-7-*O*-glucoside, luteolin-*O*-malonylhexoside, and 5-*O*-caffeoylquinic acid were the main molecules present in both infusion and hydroethanolic extracts obtained from *C. cinerea*; thus the latter extracts revealed the highest concentration in all the detected compounds.

Dendougui *et al.*[33] studied the ethanolic extract of *C. cinerea* from Algeria, revealing the presence of germacranolide, tatridin A, and seventeen flavonoid derivatives, such as luteolin, apigenin, and quercetin glycosides. Khallouki *et al.*[25] studied Morocco *C. cinerea* methanol extracts, describing the presence of phenolic acids (chlorogenic acid and dicaffeoylquinic acid derivatives) and one flavonoid (luteolin-4′-*O*-glucoside), thus being very similar to the herein described phenolic acid composition. Ahmed *et al.*[26] reported different flavonoids in this species, namely 7-*O*-β-D-diglucoside and 7-*O*-β-D-glucoside of luteolin, and luteolin itself, as well as apigenin-7-*O*-α-L-rhamnoside. All of these described profiles present some similarities to the ones presented herein. Thus, the variances in the chemical composition could also be due to the same factors mentioned above for the precedent species, including different types of extraction methodologies, the nature of the solvents used, the bioclimatic zone divergence and the plant parts used to obtain the extracts.

3.2. Antioxidant activity

The results on the antioxidant activity of both *J. phoenicea* and *C. cinerea* hydroethanolic and infusion extracts are shown in Table 3. For a broader evaluation of the antioxidant capacity, four assays were carried out: DPPH radical scavenging activity, reducing power, β-carotene bleaching inhibition and lipid peroxidation inhibition in brain cell homogenates (TBARS). The results were compared with the standard Trolox and expressed as EC_{50} values. Based on these results, *J. phoenicea* and *C. cinerea* extracts showed interesting antioxidant properties, particularly for the TBARS assays, in which all samples were demonstrated to be two to three times more effective than Trolox, with EC_{50} values of 7.6 ± 0.3 and 7.4 ± 0.3 μg mL^{-1} for hydroethanolic extracts and EC_{50} of 6.7 ± 0.2 and 7.5 ± 0.2 μg mL^{-1} for infusion extracts of *J. phoenicea* and *C. cinerea*, respectively. These extracts showed anti-lipid peroxidation proprieties, which are of great interest in food preservation.[11] The scavenging activity evaluated by the DPPH assay showed that *J. phoenicea* presented very low EC_{50} values for both extracts (12 ± 1 and 22.4 ± 0.6 μg mL^{-1} for hydroethanolic and infusion extracts, respectively), indicating a strong ability to donate hydrogen and scavenge the free DPPH radical, probably due to

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its phenolic composition. The results obtained were higher than the ones reported by El Jemli *et al.*[34] and Keskes *et al.*,[14] who evaluated the ability of a Morrocan *J. phoenicea* water extract for the scavenging activity of DPPH (EC_{50} = 30.7 ± 0.1 μg mL^{-1}), and also Tunisien *J. phoenicea* ethyl acetate extract (EC_{50} = 220 μg mL^{-1}) and hexane extract, which did not show activity using this method. Nevertheless, Keskes *et al.*[14] reported the methanol extract to show a stronger ability to scavenge DPPH radicals (EC_{50} value = 2 μg mL^{-1}). Taviano *et al.*[35] studied the antioxidant activity of methanol and water extracts of five different Turkish *Juniperus* species, which showed a weaker activity in comparison with the herein studied extracts. On the other hand, the *C. cinerea* infusion extract demonstrated a higher DPPH radical scavenging activity (EC_{50} = 24.8 ± 0.2 μg mL^{-1}) in comparison with its hydroethanolic extract (EC_{50} = 26.0 ± 0.1 μg mL^{-1}), contrary to what happened for all the remaining methods for both species. Moreover, the activities of all the samples were higher than the ones displayed by the commercial standard Trolox (EC_{50} = 42 ± 1 μg mL^{-1}). These data are in accordance with the results obtained by Khallouki *et al.*,[25] who also reported a strong antioxidant capacity (DPPH and FRAP assays) of the methanol extract from Moroccan *C. cinerea*, and correlated this activity with its significant content in echinoids and flavonoids. In fact, both extracts from the two plant species revealed a relevant reducing power, which increased with the increase of the extract concentration, ranging from 12.04 ± 0.4 to 38.1 ± 1 μg mL^{-1}, being more effective than Trolox (41 ± 1 μg mL^{-1}). Once more, *J. phoenicea* hydroethanolic and infusion extracts exhibited the best ability to reduce Fe^{3+} to Fe^{2+} (Table 3). The susceptibility of an antioxidant capable of neutralizing free radicals and inhibiting lipid peroxidation can be evaluated through a β-carotene bleaching inhibition assay. For this methodology, the hydroethanolic extracts of *J. phoenicea* and *C. cinerea* demonstrated the highest activity (EC_{50} values of 11.57 ± 0.08 and 14.7 ± 0.2 μg mL^{-1}, respectively), revealing a higher activity than Trolox (EC_{50} values = 18.0 ± 1 μg mL^{-1}), while the infusion extract of both plants showed lower or closer activity in comparison with the commercial standard. The effectiveness of the hydroethanolic extracts can be explained by their higher concentration in phenolic compounds, which could influence their capacity to scavenge free radicals and prevent lipid peroxidation.[36]

3.3. Anti-inflammatory activity

The anti-inflammatory effect was evaluated using murine macrophage-like RAW 264.7 cells and quantified through the nitric oxide (NO) production, and the results are summarized in Table 3. All the samples revealed inhibition of NO production with EC_{50} values ranging between 51 ± 4 and 122 ± 6 μg mL^{-1}. It can be noticed that the highest activity was shown by both extracts of *J. phoenicea* (hydroethanolic and infusion, with EC_{50} values of 51 ± 4 and 70 ± 5 μg mL^{-1}, respectively). This activity could be attributed to the high content of 3-*p*-coumaroylquinic acid and the flavonol derivatives found in this species. The obtained results are also in agreement with those reported by Jeong *et al.*,[37] who found a powerful anti-inflammatory activity in a Korean *Juniper rigida* methanolic extract and its fractions (*n*-hexane, chloroform, ethyl acetate and *n*-butanol). This ability was attributed to the phenolic composition present in the extract, especially the presence of phenylpropanoid glycosides, with *p*-hydroxy groups, massoniaside A, (+)-catechin, and amentoflavone, which effectively inhibited LPS-induced NO production in RAW264.7 cells.[37] Otherwise, *C. cinerea* hydroethanolic and infusion extracts revealed lower inhibition of NO production, with EC_{50} values of 105 ± 9 and 122 ± 6 μg mL^{-1}, respectively. The activity observed for both extracts of *C. cinerea* could be attributed to the presence of phenolic compounds, such as luteolin-7-*O*-glucoside, luteolin-*O*-malonylhexoside, and 5-*O*-caffeoylquinic acid. These findings could be associated with the important usage of this species in traditional African medicine.[25,33] To the best of our knowledge, this is the first report on the anti-inflammatory potential of *J. phoenicea* leaves and *C. cinerea* areal parts.

3.4. Cytotoxic effects

The results on the antiproliferative effects of *J. phoenicea* and *C. cinerea* hydroethanolic and infusion extracts are summarized in Table 4. All the tested samples presented significant

Table 4 Cytotoxic properties of *J. phoenicae* and *C. cinerea* extracts in human tumor cell lines and non-tumor liver primary cells (mean ± SD)

	J. phoenicae		*C. cinerea*		
	Hydroethanolic extract	Infusion extract	Hydroethanolic extract	Infusion extract	Ellipticine
Human tumor cell lines (GI_{50} values, μg mL^{-1})					
MCF-7 (breast carcinoma)	11 ± 1d	19 ± 1c	53 ± 4b	77 ± 6a	0.91 ± 0.04
NCI-H460 (non-small cell lung cancer)	30 ± 1c	51 ± 3b	50 ± 3b	101 ± 10a	1.03 ± 0.09
HeLa (cervical carcinoma)	9 ± 1d	17 ± 2c	47 ± 5b	51 ± 4a	1.91 ± 0.06
HepG2 (hepatocellular carcinoma)	15 ± 1d	22.4 ± 0.7c	31 ± 2b	42 ± 4a	1.1 ± 0.2
Non-tumor cells (GI_{50} values, μg mL^{-1})					
PLP2 (porcine liver primary cells)	88 ± 8d	137 ± 12b	120 ± 8c	198 ± 5a	3.2 ± 0.7

GI_{50} values (mean ± SD) correspond to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. In each row different letters mean significant differences between extracts (p < 0.05).

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cytotoxic properties for the four human tumor cell lines tested (MCF-7, NCI-H460, HeLa and HepG2), with GI_{50} values ranging from 9 ± 1 to 101 ± 10 μg mL^{-1}. Furthermore, the samples also presented cytotoxic effects against non-tumor cells (PLP2 – porcine liver primary cells); thus the GI_{50} values were much higher than those needed to exert antiproliferative activity in tumor cells and also much higher than those exhibited by the standard Ellipticine. *J. phoenicea* hydroethanolic and infusion extracts demonstrated a higher cytotoxic potential in comparison with *C. cinerea*. The obtained results are also in agreement with those reported by Maamoun *et al.*,[16] who found higher activity against lung carcinoma (H460), liver tumor (HepG2), and breast carcinoma (MCF7) cell lines with the crude extract of Egyptian *J. phoenicea* leaves. Consecutively, these authors screened the flavonoid agathisflavone, which recorded higher cytotoxicity in the lung carcinoma (H460).[16] Additionally, a significant *in vivo* hepatoprotective effect was reported for *J. phoenicea* leaves.[15,38] Tavares *et al.*[39] also proved the neuroprotective potential of the phenolic fractions obtained from Portuguese *J. phoenicea* and other species of *Juniperus*, attributing this capacity to the biflavones detected, especially to amentoflavone. Furthermore, *J. phoenicea* is well known to contain lignans, which are recognized for their interesting antiproliferative and antiviral activities.[40] Many reports supposed that *Juniperus* species could be a source of bioactive compounds with potential anti-cancer effects.[41]

On the other hand, *C. cinerea* hydroethanolic and infusion extracts also presented antiproliferative activity, being more active in the following order: HepG2 (31 ± 2 and 42 ± 4 μg mL^{-1}), HeLa (47 ± 5 and 51 ± 4 μg mL^{-1}), NCI-H460 (50 ± 3 and 101 ± 10 μg mL^{-1}) and MCF-7 (53 ± 4 and 77 ± 6 μg mL^{-1}). In fact, the cytotoxicity of *C. cinerea* extracts was lower than the one observed for *J. phoenicea* hydroethanolic and infusion extracts; these differences could be due to the distinct individual phenolic profiles of both extracts. It has been previously highlighted that some phenolic compounds can contribute to protection against cancer disease, for example, *p*-coumaroylquinic acid, quercetin, and myricetin derivatives, which were the major phenolic compounds found in *J. phoenica*. These compounds have exhibited a protective role for *in vivo* hepatotoxicity and nephrotoxicity.[15] To the best of our knowledge there are no previous reports on the cytotoxic activity of *C. cinerea*.

3.5. Antimicrobial activity

The antimicrobial activity results of both *J. phoenicea* and *C. cinerea* extracts (hydroethanolic and infusion) tested against ten pathogenic strains, and expressed as the minimal inhibitory concentrations (MIC), minimal bactericidal concentrations (MBC) and minimal fungicidal concentrations (MFC), are presented in Table 5. Overall, the extracts obtained from both species were found to have moderate to weak activity, with a higher effectiveness towards Gram-positive bacteria, in comparison with Gram-negative. MIC values ranged between 5 and 20 mg mL^{-1}, and the inhibitory effect tested against all the bacterial strains was more bacteriostatic than bactericidal.

Table 5 Antibacterial and antifungal activities of *J. phoenicea* and *C. cinerea* extracts (MIC, MBC and MFC values, mg mL^{-1})

Antibacterial activity																					
		Gram-negative bacteria								Gram-positive bacteria										Yeast	
		Escherichia coli		*Pseudomonas aeruginosa*		*Klebsiella pneumoniae*		*Proteus mirabilis*		*Morganella morganii*		*Enterococcus faecalis*		*Listeria monocytogenes*		*MRSA*		*MSSA*		*Candida albicans*	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC
J. phoenicea	Hydroethanolic extract	**10**	>20	>20	>20	>20	>20	>20	>20	**10**	**20**	**10**	>20	**20**	>20	**5**	>20	**10**	>20	>20	>20
	Infusion extract	**10**	>20	>20	>20	>20	>20	>20	>20	**10**	**20**	**10**	>20	**20**	>20	**5**	>20	**10**	>20	>20	>20
C. cinerea	Hydroethanolic extract	>20	>20	>20	>20	**20**	**20**	>20	>20	>20	>20	**20**	>20	**20**	>20	**10**	>20	**5**	>20	>20	>20
	Infusion extract	>20	>20	>20	>20	**20**	**20**	>20	>20	>20	>20	>20	>20	**20**	>20	**10**	>20	**5**	**20**	>20	>20

MRSA – Methicillin resistant *Staphylococcus aureus*; MSSA – methicillin susceptible *S. aureus*; MIC – minimal inhibitory concentration; MBC – minimal bactericidal concentration.

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The strains MRSA and MSSA were demonstrated to be more susceptible to both *J. phoenicea* (MIC = 5 and 10 mg mL^{-1}, respectively) and *C. cinerea* (MIC = 10 and 5 mg mL^{-1}, respectively) extracts. On the other hand, *Morganella morganii* also showed susceptibility towards *J. phoenicea* extracts revealing MICs and MBCs values of 10 and 20 mg mL^{-1}, respectively. Regarding Gram-negative bacteria, *J. phoenicea* and *C. cinerea* only presented inhibitory activity for *Escherichia coli* (MIC = 10 mg mL^{-1}) and *Klebsiella pneumonia* (MIC and MBC = 20 mg mL^{-1}). It is important to highlight that both *J. phoenicea* extracts had the same behavior on all the bacteria tested, while *C. cinerea* presented variation in the used extracts, especially for the strains *E. faecalis* and MSSA. Regarding the yeast *Candida albicans*, the extracts did not express any activity up to the maximal tested concentration.

The antimicrobial activity of the *J. phoenicea* extract is supported by studies performed by Hammami *et al.*,[42] who demonstrated moderate to good activity of a Tunisian *J. phoenicea* aqueous extract, obtained from seeds, and its fractions (methanol and acetone), against Gram-positive (*Listeria monocytogenes*, *Listeria innocua*, and *Listeria ivanovii*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria. In a similar order, Alzand *et al.*[43] proved significant growth inhibition of *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella pneumonia* using alcoholic extracts of *J. phoenicea* barks from India. In contrast, Miceli *et al.*[31] described the antibacterial activity of different species of Turkish *Juniperus* (*J. communis* L. var. *communis* and *J. communis* L. var. *saxatilis*. Pall) methanol extracts, which showed moderate capacity only against Gram-positive bacteria. It should also be highlighted that the part of the plant used and the phenolic compound concentration will also have a great influence on the antibacterial activity obtained for each extract. Thus, the antimicrobial activity obtained for both extracts of *J. phoenicea* could also be related to the presence of phenolic compounds, such as quercetin derivatives and *p*-coumaroylquinic acid, which are recognized for their antimicrobial actions,[4,44] supporting the antibacterial effectiveness of the studied species.

Concerning *C. cinerea* antibacterial activity, it is in agreement with the results described by Bensizerara *et al.*,[23] who evaluated the antibacterial properties of extracts obtained from *C. cinerea* aerial parts using different polarity solvents (ethanol, *n*-butanol, ethyl acetate and petroleum ether). These authors revealed weak activity for all the mentioned extracts; thus the most active fraction was *n*-butanol. Additionally, Markouk *et al.*[22] reported that the *n*-butanol extract obtained from the Moroccan *C. cinerea* was highly effective against germs in the tested concentrations (ranging from 12 to 200 μg mL^{-1}). Nevertheless, the inhibition ability of the bacterial growth shown by *C. cinerea* could also be associated with important levels of flavonoids, such as quercetin and luteolin derivatives, as was reported by Dendougui *et al.*[33] and Ahmed *et al.*[26]

Overall, the wide range of bioactive properties (antioxidant, anti-inflammatory, cytotoxic and antibacterial) shown by the hydroethanolic and infusion extracts of the studied Saharan plants supports their traditional use as a popular remedy in the treatment of cancer, infectious and inflammatory diseases. These properties may be related to the presence of different phenolic compounds at variable contents. Thus, further studies are needed to establish the mechanisms of action, supporting the use of these plants in the pharmaceutical and food fields.

Conflicts of interest

No conflict of interest.

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