Original Article

COMPARISON OF ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF TWO CULTIVATED *CISTUS* SPECIES FROM TUNISIA

COMPARAÇÃO DAS ATIVIDADES ANTIOXIDANTE E ANTIMICROBIANA DE DUAS ESPÉCIES CULTIVADA DE Cistus *DA TUNÍSIA*

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ABSTRACT: The secondary metabolite composition, antioxidant activities, and microbial inhibition properties of leaves of two *Cistus* species*; C. monspeliensis and C. salvifolius* were investigated using three solvent extracts (ethanol, hexane and distilled water). Ethanol extracts were most efficient at extracting phenolics, flavonoids and condensed tannins compared to hexane and distilled water for both *Cistus* species. A total antioxidant test (TAA) and two radical scavenging tests (DPPH and ABTS) indicated that the 70% ethanolic extract from *C. salvifolius* leaves had stronger antioxidant activity compared to the *C. monspeliensis* 70% ethanol extract, while the aqueous extract of *C. monspeliensis* was much stronger than the aqueous extract or the 70% ethanol extract of *C. salvifolius*. Overall, the polar extracts were more active in both species than the non-polar extracts. Thus aqueous ethanol extracts of the leaves of each Cistus species were tested for their ability to inhibit seven pathogenic microbial strains, including *Escherichia coli* ATCC 8739, *Salmonella typhimurium* NCTC 6017, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis*, *Aspergillus niger*, and *Candida albicans*. Leaf ethanol extracts from both species were active against each microbial species, but the *C. monspeliensis* leaf ethanolic extract was much more active against several microbial species than that of *C. salvifolius.* For example, the *C. salvifolius* 70% ethanol extract showed its highest antimicrobial activity against *P. aeruginosa* and *A. niger* (MIC 3.1 mg/ml and MBC 6.3 mg/ml for both), while the *C. monspeliensis* extract showed much higher overall activity against *E. coli*, *P. aeruginosa* and *C. albicana.* (MIC 6.25 mg/ml and MBC 12.5 mg/ml for all three). In conclusion, maceration with 70% ethanol was the most efficient method for extracting total polyphenols, flavonoids, and condensed tannins from the two *Cistus* species, and the aqueous-ethanol extracts displayed the highest antioxidant and antimicrobial activities. Hence, the aqueous-ethanolic extracts of both species may be considered as potential sources of natural antioxidants and antimicrobial agents.

KEYWORDS: *Cistus salvifolius*. *Cistus monspeliensis*. Antioxidant activity. Antifungal activity.

INTRODUCTION

The *Cistaceae* family (consisting of eight genera and 175 species) includes the genus *Cistus,* which comprises about 20 shrub species found broadly throughout the entire Mediterranean region (COMANDINI et al., 2006, TOMAS-MENOR et al., 2013) In Mediterranean folk medicine, *Cistus* species have been used as general remedies (BARRAJÓN-CATALÁN et al., 2010) and for their anti-inflammatory (DEMETZOS et al., 2001), anti-ulcerogenic, wound healing, anti-microbial (DEMETZOS et al.,1999), antifungal (BAYOUB et al., 2010), antiviral, anti-tumor (DIMAS et al., 2000), cytotoxic (BEN JEMIA et al., 2013) and anti-nociceptive (BARRAJÓN-CATALÁN et al., 2010) properties. For example, tea prepared from *C. salvifolius* L. herb (AL-KHALIL, 1995) has traditionally been used for the treatment of gout (AL-KHALIL, 1995) and ulcers (YESILADA et al., 1999). In Morocco, *C. salvifolius* also has been found to exhibit anti-bacterial activity against *Mycobacterium aurum* and *Mycobacterium smegmatis* (HAOUAT et al., 2013). *Cistus incanus spp. tauricus* contains a specific polyphenol-rich extract (called CYSTUS052) that has demonstrated antiviral activity against influenza A virus infections (EHRHARDT et al., 2007). The antimicrobial activity of many plant species is related to their phenolic compounds (RAUHA et al., 2000), and these types of antioxidant compounds are widely distributed in the plant kingdom and are considered as potential therapeutic

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agents against a wide range of diseases (MIDDLETON et al., 2000). The available literature reports chemical studies on the composition of extracts of *Cistus* species, obtained by different solvents. Catechin related compounds were identified in the aqueous extracts of *C. monspeliensis* (POMPONIO et al, 2003). In *C. ladanifer*, the most abundant group was ellagic acid derivatives with punicalagin gallate being the most abundant phenolic compounds (BARROS et al., 2012). These compounds (i.e., ellagitannins) could be related to the strong inhibition of *Candida albicans*, *C. glabrata* and *C. parapsilosis* growth (BARROS et al., 2012).

Due to antibiotic side effects and the resistance that pathogenic microorganisms have developed against our current spectrum of antibiotics, much attention has recently been given to extracts and biologically active compounds isolated from plant species used in herbal medicine. However, most studies have focused only on the use of one solvent to extract bioactive compounds and have paid little attention to the efficiency and costs of solvent extraction and environmental disposal, characteristics that should be considered for the transition from small laboratory scale to commercial processes on a large-scale. In the current study, we report on the effectiveness of three different solvents (ethanol, hexane and distilled water) in the extraction of antioxidant activity, secondary metabolite levels, and antimicrobial activities from leaves of two *Cistus* species.

MATERIAL AND METHODS

Plant material

Samples of *Cistus monspeliensis* and *Cistus salvifolius* leaves were collected from October 2013 to March 2014 from the locality of Ghardimaou, Jendouba (Northwestern Tunisia). The geographic position of the collection site was determined with a Majellan Pro handheld global positioning system (GPS) and is as follows: latitude (36° 36' 55" (N) and longitude 8° 25' 7" (E). The climate at the collection site is temperate with cold, rainy winters, dry, hot summers, and a mean annual rainfall higher than 1000 mm. The most frequent annual wind direction is from the west. Plant material was botanically identified according to the morphological description presented in Tunisian flora (POTTIER-AlAPETITE, 1981) , and by the botanist Prof. Mouhiba Ben Nasri (Département de Biologie, Faculté des Sciences de Tunis, Tunisia) where voucher specimens (CMG1013 and CS0313) were deposited.

Phenolic extraction

Dried leaf samples were independently ground into a fine powder by hand with a mortar and pestle; then independent triplicate samples of 1 g dry powder (from each sample) were separately extracted by stirring with 10 ml 70% ethanol, distilled water, or 100% hexane for 30 min. at room temperature. The extracts were then kept in the dark for 24 h at 4◦ C, each one then filtered once through Whatman No. 4 filter paper and then stored at 4◦ C until analysis.

Total polyphenol quantification

Folin-Ciocalteu (F-C) reagent was used for the quantification of total phenolics on each of the three extract types as described by Mau et al. (2001). An aliquot of 125 µl diluted extract (20%, v/v in ethanol diluted to fit within the calibration range below) was added to 500 µl deionized water and 125 µl F-C reagent. After shaking, the mixture was incubated for 3 min at room temperature (RT). Then, 1.25 ml of 7% Na_2CO_3 solution was added, the volume adjusted to 3 ml using distilled water, then the extract was mixed vigorously and held for 90 min at RT before measuring the optical density at 765 nm. The sample was analyzed in triplicate against a blank compared with a gallic acid commercial standard (Sigma-Aldrich Inc. Steinheim, Germany), and the total phenolic content was expressed as mg gallic acid equivalents (GAE) per g dry weight (DW) using a calibration curve ranging from 50–400 $\mu g.mL^{-1}$.

Flavonoid quantification

The flavonoid content was measured on triplicate independent leaf extracts (for each of the three extract types) according to Dewanto et al. (2002). One ml of diluted leaf sample (5% (v/v in diluted to fit within the calibration range below) was added to a 10 ml volumetric flask containing 4 ml distilled water. This was followed immediately by the addition of 0.3 ml of 5% (w/v) $NaNO_2$, then by 0.6 ml of 10% (w/v) $AlCl_3$ after 5 min and by 2 ml of 1 mol l^{-1} NaOH after another 6 min. Contents of each reaction flask were then diluted with 2.4 ml distilled water and mixed immediately. Absorbance of the resulting pink-colored solution was measured in triplicate at 510 nm against distilled water compared with a gallic acid commercial standard. Flavonoid content was expressed as mg gallic acid equivalents

(GAE) per g DW using a calibration curve ranging from 50–500 mg ml^{-1}.

Tannin quantification

Ethanolic (70%) , aqueous or hexane extracts of ground leaves (500 µl) were added to 3 ml of 4% vanillin (prepared in 100% methanol) with 1.5 ml concentrated HCl according to Sun et al. (1998). The mixture was incubated for 15 min at RT, and the absorbance of the resulting pink colored solution was measured in triplicate at 500 nm against 100% methanol as a blank. Tannin monomer and polymer content was expressed as mg catechin equivalents (CE) per g DW using a calibration curve of commercial catechin (Sigma-Aldrich Inc. Steinheim, Germany).

Chlorophyll, carotenoid, and lycopene extractions

Total chlorophyll and carotenoid concentrations were determined on 80% acetone (v/v) leaf extracts at 470, 645 and 663 nm as described by Lichtenthaler (1988). The individual carotenoid, lycopene, was determined on 100% hexane/100% acetone/100% ethanol (50/50/1, v/v/v) leaf extracts at 472 nm as described by Benakmoum et al. (2008).

Total Antioxidant Activity (TAA) Assay

The quantification of total antioxidant activity was determined by a colorimetric assay using a method described by Prieto et al. (1999). A 0.2 ml of a solution containing sulfuric acid (H_2SO_4; 0.6M) sodium phosphate (NaH_2PO_4 H_2O; 28mm) and ammonium heptamolybdate ($(NH4)_6 MO_7 O_{24}$, $4H_2O$; 4 mM) at acidic pH was added to the 70 % ethanol, distilled water, or 100% hexane extracts (0,1 ml) . The mixture was then placed in a water - bath at 95° C for 90 min. After cooling to room temperature, the absorbance was measured at 695 nm, and total antioxidant activity was expressed mg gallic acid equivalents (GAE) per g DW.

DPPH Radical-Scavenging Activity

The electron donation ability of 70 % ethanol, distilled water, or 100% hexane extracts was measured by bleaching of the purple-colored 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical according to Sun et al. (1998). The 70 % ethanol, distilled water, or 100% hexane extracts (2 ml, 10–1000 µg.ml^{-1}) were added to 0.5 ml of 0.2 mmol.l^{-1} DPPH, incubated for 30 min at RT, then the absorbance was measured in triplicate against a blank at 517 nm using a Shimadzu 160-UV spectrophotometer (Tokyo, Japan). The antiradical activity (% DPPH scavenging effect) was determined using the equation: [(*A*blank − *A*sample)/*A*blank]× 100 (1), where *A* blank is the absorbance of the control reaction and *A* sample is the absorbance in the presence of plant extract. The effective concentration having 50% radical inhibition activity EC50, expressed as µg extract per millilitre (µg.ml^{-1}), were determined plotting inhibition percentage *versus* extract concentrations.

Free radical scavenging using the ABTS radical cation

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free radical assay was also conducted since occasionally some compounds showing ABTS radical scavenging activity do not show DPPH inhibition (WANG et al., 1998). The radical cation (ABTS+) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at RT for 24 h before use (RE et al., 1999). Afterwards, the ABTS+ solution was diluted with ethanol to an absorbance of 0.7 at 734 nm, and 990 ml of diluted ABTS+ solution was added to 10 ml of sample. The absorbance measured in triplicate at 734 nm (at 30° C) against a blank (negative control) sample (containing the same proportion of ethanol and ABTS+ solution) exactly 6 min after initial mixing using the spectrophotomer above. The percentage inhibition of the ABTS cation radical (by the samples) was calculated according to the following formula: Scavenging (%) = [(A0-A1)/A0] × 100, where A0 is the absorbance of the blank sample and A1 is the absorbance of the sample. The EC50 (µg.ml^{-1}) was determined as above mentioned.

Growth of microbial strains

Seven pathogenic microbial strains were grown for inclusion in the antimicrobial assays detailed below. These included three gram-negative bacteria (*Escherichia coli* ATCC 8739, *Salmonella typhimurium* NCTC 6017, and *Pseudomonas aeruginosa* ATCC 27853), two gram-positive bacteria (*Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis),* and two fungal (yeast) species (*Aspergillus niger* and *Candida albicans*). Bacterial strains were manually streaked onto trypto-caseine soy agar (TCS) and incubated at 30° C or 37° C for 24 h (depending on the strain optimum) to develop fresh inoculum. Fungal species were grown on potato

dextrose agar (PDA) at 28°C for 48 h or 72 h, depending on the strain optimum.

Microbial disc diffusion assay

A disc diffusion assay was used for evaluating the antimicrobial activity of *C. salvifolius* and *C. menspeliensis* ethanolic extracts based on the ranking of all three extracts in the antioxidant activity assessment. Briefly, 100 µL of fresh microbial suspension containing 10^8 colony forming units (CFU)/mL of bacteria cells or 10^6 CFU/mL of fungi were spread onto petri plates containing TSA or PDA solid (agar) medium, respectively. Paper discs (6 mm in diameter) were fully impregnated each with 15 µl of the tested extract. Discs were placed on the inoculated agar, and then the plates were maintained at 4° C for 1 h. Gentamicin (10 µg/disc) and amphotricin B (20 µg/disc) were used as a positive (inhibitory) reference for bacteria and fungi, respectively, whereas discs without plant extract were used as negative controls. The inoculated plates were incubated for 24 h at 37° C for pathogenic bacterial strains and 48 h at 30° C for yeast species. Antimicrobial activity was assessed by measuring the diameter of the growth-inhibition zone in millimetres (including disc diameter of 6 mm) for the tested microorganisms compared to the negative controls. The measurements of inhibition zones were carried out three times with independently generated extract samples.

Determination of MIC and MBC

The minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) values were determined using a broth dilution method as described by Aouadhi et al. (2013). All antibacterial tests were performed in TCS and sabouraud dextrose (SD) broth. Overnight broth cultures were diluted in 0.1% peptone water (v/v) to obtain working microbial cultures of 10^5 CFU ml^{-1}. Serial dilutions of the studied extract (ranging from 0.07 to 50 mg/ml) were incubated with each working microbial culture in tubes. Additionally, one extract-minus growth control (TCS or SD broth) and one sterility control (appropriate medium + tested extract without microbial culture) were included in the study. Tubes were incubated for 24 h at 30 or 37° C depending on the optimum temperature for each strain. Microbial growth was indicated by turbidity in the liquid and a visible 'pellet' on the tube bottom. MICs were determined presumptively as the first serial dilution (in ascending order), which did not produce a tube pellet. To confirm MICs and to establish MBCs, 10 µl of broth was removed from each tube, inoculated on TCS or PDA plates, and after incubation, the number of surviving organisms (colonies) was determined. The MIC was the lowest extract concentration, which resulted in a substantial decrease in inoculum viability (> 90%), while the MBC was the concentration at which 99.9% or more of the initial inoculum was killed. Each experiment was repeated at least three times and the modal MIC and MBC values were selected.

Statistical analysis

All measurements were carried out in triplicate and the results were presented as mean values ± SD (standard deviation). Statistical analyzes were performed using two-way analysis of variance (ANOVA) and differences between means were determined by Duncan's multiple range test. Differences at $P<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Total phenolic, flavonoid, and condensed tannin contents

Polyphenol extraction is usually a long process that typically includes maceration and expensive organic solvents (GARCIA SALAS et al., 2010) hazardous to the environment, but it is usually the only method used when extracting a set of fragile molecules. Thus, ethanol and water would be economically better choices with respect to environmental and health protection than other solvents used to extract antioxidant phenolic compounds for use in the food industry if their extraction efficiencies were also high compared with more expensive typical solvents.

Leaf 70% ethanol extracts of *C. salvifolius* and *C. monspeliensis* had similar levels of total phenols (ranging from 50 to 56 mg GAE/g DW), and were richer in these compounds than leaf hexane and leaf aqueous extracts (Table 1). The values we obtained for total leaf phenol contents in these two *Cistus* species were in the concentration range found for *C. ladanifer* (40-57 mg GAE/g DW) and *C. libanotis* (40-44 mg EGA/g DW) collected in eastern Morocco in May (ZIDANE et al., 2013). In contrast, a 2nd Moroccan study reported a much lower content of total phenols (18 and 12 mg GAE/g DW) in the latter

two species, respectively (AMENSOUR et al., 2010). In Turkish, *C. laurifolius*, leaves collected from the town of Dörtdivan, Bolu province in June, were found to contain very high phenols contents (312-354 mg/g DW) in the butanol and ethanol extracts, respectively (AKKOL et al., 2012). In a more recent phytochemical investigation on *C. salviifolius* leaves and flower buds collected in March 2013 from Nahli mount located in Ariana governorship in northern Tunisia, El Euch et al. (2015) reported phenolic levels similar to those found in *C. laurifolius* and 6-fold higher than those found in our study. As with phenolics, the total flavonoid content was also similar in leaves of both species (in the order of 7 mg EC/g DW). Again, flavonoids detected in these leaf ethanolic extracts were higher in content than those detected in hexane and aequous extracts (Table 1). These values were much lower than those observed for *C. ladaniferus* (AMENSOUR et al., 2010), *C. salviifolius* (EL EUCH et al., 2015), *C. laurifolius* (AKKOL et al., 2012) and *C. libanotis* (ZIDANE et al., 2013). For example, Zidane et al. (2013) reported much higher total flavonoids in methanolic and ethanolic extracts of the Moroccan *C. ladaniferus* (61 and 51 mg GAE/g DW), and *C. libanotis* (16 and 25 mg GAE/g DW), respectively. At this point, it seems that total phenolic and flavonoid contents are somewhat variable in *Cistus* and depend on the species, plant tissue evaluated, season, growth conditions, sample preparation, and analytical methods. However, our results demonstrate (at least with *C. salviifolius* and *C. monspeliensis*) that 70% ethanol is a more efficient solvent for extracting total phenols and flavonoids when compared with distilled water and hexane.

Aqueous leaf extracts of *C. salvifolius* were richer in soluble condensed tannins compared to 70% ethanol and hexane extracts, whereas both 70% ethanol and aqueous extracts of *C. monspeliensis* leaves were richer in tannins compared to hexane extracts (Table 1). Condensed tannins are extracted more efficiently in 70% ethanol and water than in hexane owing to the higher polarity and stronger solubility of these polymeric hydroxylated compounds (WIELAND et al., 2006). Studies with grapes (KATALINIC et al., 2010), artichokes (MULINACCI et al., 2004), and Ivorian plants (KOFFI et al., 2010) confirm our results that ethanol in combination with water enables a more efficient extraction of total polyphenols.

Table 1. Total phenolic, flavonoid, and condensed tannin contents in *Cistus salvifolius and Cistus monspeliensis leaves.* Values are given as mean ± SD (n = 3). Means within each row followed by different superscript letters are significantly different by a Duncan test ($P \leq 0.05$).

	Cistus salviifolius			***Cistus monspeliensis***		
	100% Ethanol	Distilled water	Hexane	100% Ethanol	Distilled water	Hexane
Total phenol content (mg GAE/g DW)	49,98^{a}± 3,39	45,61 ^{a}± 0,47	9,02^{b}± 0,39	56,40^{a}± 1,64	37,39^{b}± 3,43	5,26^{c}± 1,89
Total flavonoid content (mg CE/g DW)	7,00 ^{a}± 1,80	5,27^{a}± 0,85	1,97^{b}± 0,01	7,00^{a}± 0,72	5,27^{b}± 0,45	2,00^{c}± 0,04
Condensed tannin content (mg CE/g DW)	10,47 ^{b}± 2,95	17,88^{a}± 2,95	2,10^{c}± 0,66	16,77^{b}± 1,97	22,23^{a}± 1,10	1,45^{c}± 0,01

Chlorophylls, carotenoids and lycopene contents

C. salvifolius leaves were two-fold richer in carotenoids (59.7 mg/100 g FW) compared to *C. monspeliensis* (29.5 g/100 mg FW) (Table 2), but much lower in carotenoids than in phenolics, flavonoids, and tannins. The leaf carotenoid content for *C. salvifolius* was similar to that reported for *C. albidus* (52 mg/100 g FW) (BEGONA et al., 2010). Total chlorophyll content was also two-fold higher in *C. salvifolius* (200 mg/100g FW) than in *C. monspeliensis* (103 mg/100 g FW) and even higher proportionately for chlorophyll b (141 mg/100 g FW vs. 29 mg/100 g FW, respectively) (Table 2). However, lycopene which is a major carotenoid found in tomatoes and other plant species (KAMILOGLU et al., 2014) formed only 1/4 to 1/2 of the carotenoid component in our two *Cistus* species (13 g/100 g FW) (Table s) and was four-fold lower than that reported for *C. albidus* leaves (40 mg/100 g FW) (BEGONA et al., 2010). As with phenolics, flavonoids, and condensed tannins, growing conditions likely play a major role in levels of these metabolites.

Table 2. Levels of foliar pigments (chlorophylls, carotenoids and lycopene) in leaves of *C. salviifolius* and *C. monspeliensis*. Concentration of chorophylls (Chl), carotenoids and lycopene expressed as mg/100 g fresh weight (FW). Values are given as mean ± SD (n = 4).

Plants	Chl a	Chl b	Tot Chl	Carotenoids	Lycopene
C. salviifolius	$59,5^{a} \pm 6,2$	$140,9^{a} \pm 46,9$	$200,5^{a} \pm 40,3$	$59,7^{a} \pm 4,39$	$13,1^{a} \pm 2,7$
C. monospeliensis	$73,3^{b} \pm 1,6$	$29,2^{b} \pm 7,7$	$102,5^{b} \pm 7,8$	$29,5^{b} \pm 3,98$	$13,2^{a} \pm 1,0$_

Means within each column followed by different superscript letters are significantly different ($P \leq 0.05$)

Antioxidant activities

Phenolics and carotenoids often form the basis of antioxidant capacity (MULLEN et al., 2007). Hence, we examined our *Cistus* extracts using three types of anti-oxidant assays. Ethanol (70%) extracts of *C. salvifolius* leaves exhibited enhanced antioxidant activity (in the order of 35 mg EAG/g DW) compared with the two other leaf extracts (aqueous and hexane) using a total antioxidant activity assay (Table 3). However, aqueous extracts of *C. monspeliensis* leaves displayed the highest total antioxidant capacity (65 mg EAG/g DW, respectively) using this assay compared with 70% ethanol and hexane extracts.

The *in vitro* radical scavenging activity of aqueous, 70% ethanolic, and hexane extracts of *C. salvifolius* and *C. monspeliensis* were also evaluated by two specific tests using DPPH and ABTS. DPPH• is a stable free radical and accepts an electron or hydrogen radical to become a stable molecule (GULCIN et al., 2004). The ABTS assay was also conducted, since occasionally some compounds showing ABTS radical scavenging activity do not show DPPH inhibition (WANG et al., 1998). For both tests, their radical scavenging activities depended on the nature of the solvent and on the test plant species. Hexane extracts showed the highest EC50 radical scavenging value for C. *salvifolius* and *C. monspeliensis* (DPPH 7 µg/ml, ABTS 34 µg/ml and DPPH 9 µg/ml, ABTS 35 µg/ml, respectively); thus the hexane extracts had the lowest antioxidant activity. In contrast, 70% ethanol extracts of *C. salvifolius* and *C. monspeliensis* leaves showed the lowest EC50 (i.e., highest antioxidant activity with DPPH 0.1 µg/ml, ABTS 16.8 µg/ml and DPPH 0.2 µg/ml, ABTS 18.9 µg/ml, respectively). In addition, the EC50 radical scavenging activity of aqueous leaf extracts was also substantially low for *C. salvifolius* leaves (DPPH 1.3 µg/ml**,** ABTS: 29.8 µg/ml) and *C. monspeliensis* leaves (DPPH 1.2 µg/ml, ABTS 24.1 µg/ml), indicating high antioxidant activity. These latter results suggest that the high total antioxidant capacity of the 70% ethanol and aqueous extracts compared to hexane extracts may be due (at least in part) to the phenolic and flavonoid contents found in these polar extracts.

Aqueous-ethanol extracts were more efficient ABTS radical scavengers compared to the water extracts in both of our *Cistus* species evaluated, whereas DPPH scavenging activity was lower. Wang et al. (1998) found that some compounds possessing ABTS•+ scavenging activity did not show DPPH-scavenging activity. Our ABTS+ scavenging data suggest that components within the extracts are capable of scavenging free radicals *via* a mechanism of electron/hydrogen donation and should be able to protect susceptible matrices from free radical-mediated oxidative degradation.

Antimicrobial activity

Because of undesirable side effects and the resistance that pathogenic microorganisms eventually develop against antibiotics, much attention has been paid recently to extracts and biologically active compounds isolated from plant species used in herbal medicine.

Since our 70% ethanol extracts from both *C. salvifolius* and *C. monspeliensis* had moderately high levels of antioxidant-type secondary metabolites and strong free radical inhibiting activity (above), these specific extracts were next evaluated for antimicrobial activity using inhibition zone diameter (IZ), minimum inhibitory concentration (MIC), and minimum bacteriocidal concentration (MBC) tests against common gram-positive and gram-negative bacteria and two fungal (yeast) species. Results indicate that 70% ethanol extracts showed antibacterial and antifungal activity with specific differences according to the evaluated plant species and the test microbial strain (Table 4).

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Table 3. Antioxidant and radical scavenging activities of the ethanol extracts (EE), aqueous extract (AE), and hexane (HE) in the leaves of plants of *C.salvifolius* and *C. monspeliensis*. Values are given as mean ± SD (n = 3). Means for all data within each assay followed by different superscript letters are significantly different ($P \leq 0.05$).

	TAA activity (mg GAE/ µg DW)			**DPPH activity (EC50/ µg/ ml)**			**ABTS activity (EC50 µg/ml)**		
	EE	AE	HE	EE	AE	HE	EE	AE	HE
C.salvif.	$35,36^{a}$± 4, 93	$9,58^{b}$± 1,83	$4,48^{c}$± 3,8	$0,13^{c}$± 0,04	$1,29^{b}$± 0,02	$7,13^{a}$± 0,02	$16,75^{c}$± 0,76	$29,75^{b}$± 1,06	$34,68^{a}$± 1,5
C. monsp.	$25,46^{b}$± 1,73	$65,32^{a}$± 1,67	$3,19^{c}$± 0,23	$0,24^{c}$± 0,02	$1,22^{b}$± 0,03	$9,48^{a}$± 1,08	$18,92^{c}$± 0,26	$24,12^{b}$± 0,86	$35,33^{a}$± 2,8

Table 4. Antimicrobial activity expressed of *C. salvifolius* and *C. monspeliensis* ethanolic extracts as diameter of inhibition zone (mm), minimum inhibitory concentration MIC (mg/ml) and minimum bactericidal concentration MBC (mg/ml). [1]Gentamicin. [2]Amphotricin

	Diameter of inhibition zone (mm)			**MIC (mg/ml)**		**MBC (mg/ml)**	
	Antibiotics	***C. salvifolius***	***C. monspeliensis***	***C. salvifolius***	***C. monspeliensis***	***C. salvifolius***	***C. monspeliensis***
Gram + Bacteria							
Escherchia coli	24[1]	$18,0^{b}$ ± 0,5	$14,0^{b}$ ± 1,0	$12,5^{b}$ ± 0,0	$6,3^{b}$ ± 0,0	$25,0^{b}$ ± 0,0	$12,5^{b}$ ± 0,0
Salmonella typhimurium	23[1]	$20,0^{a}$ ± 1,0_	$17,0^{a}$ ± 0.5	$25,0^{a}$± 0,0	$12,5^{a}$ ± 0,0	$50,0^{a}$ ± 0,0	$25,0^{a}$ ± 0,0
Enterococcus faecalis	20[1]	$13,0^{c}$ ± 0,5	$16,0^{a}$ ± 0.5	6.3^{c} ± 0,0	$12,5^{a}$ ± 0,0	$12,5^{c}$ ± 0,0	$25,0^{a}$ ± 0,0
Gram - Bacteria							
Staphylococcus aureus	23[1]	$17,5^{b}$ ± 0,5	$17,0^{a}$ ± 1,0	$12,5^{b}$ ± 0,0	$12,5^{a}$ ± 0,0	$25,0^{b}$ ± 0,0	$25,0^{a}$ ± 0,0
Pseudomonas aeriginosa	21[1]	$10,0^{d}$ ± 0,5	$10,0^{c}$ ± 1,5	$3,1^{d}$ ± 0,0	$6,3^{b}$ ± 0,0	$6,3^{d}$ ± 0,0	$12,5^{b}$ ± 0,0
Fungi							
Candida albicans	17[2]	$17,0^{b}$ ± 0,5	$12,0^{c}$ ± 0,5	$12,5^{b}$ ± 0,0	$6,3^{b}$ ± 0,0	$25,0^{b}$ ± 0,0	$12,5^{b}$ ± 0,0
Aspergillus niger	12[2]	$9,0^{d}$ ± 0,5	$15,0^{a}$ ± 1,5	$3,1^{d}$ ± 0,0	$12,5^{a}$ ± 0,0	$6,3^{d}$ ± 0,0	$25,0^{a}$ ± 0,0

In fact, the IZ and MIC tests ranged from 9-20 mm and 3.1-25 mg/ml, respectively for the *C. salvifolius* extract and 10-17 mm and 6.3-12.5 mg/ml, respectively for the *C. monspeliensis* extract. In more detail, the *C. salvifolius* 70% ethanol extract showed highest antimicrobial activity essentially against *P. aeruginosa* and *A. niger* (MIC 3.1 mg/ml and MBC 6.3 mg/ml), while the *C. monspeliensis* extract showed highest activity (MIC 6.25 mg/ml and MBC 12.5 mg/ml) against *E. coli*, *P. aeruginosa* and *C. albicana.*

Previous studies reported that gram-positive bacteria are more sensitive to plant oils and extracts than gram-negative bacteria (MAHBOUBI; HAGHI, 2008; BOUKHEBTI et al., 2011), due to the presence of hydrophobic lipopolysaccharide in the outer gram-negative membrane (which provides protection against several agents) (NIKAIDO; VAARA, 1985). However, neither of our *Cistus* 70% ethanolic extracts showed selective antimicrobial activity based on bacterial cell wall differences. This finding is distinct from that of extracts of *C. ladaniferus* and *C. monspeliensis* which were markedly more active against gram-positive than gram-negative bacteria (BENAYAD et al., 2013).

The antimicrobial activities of the two *Cistus* 70% ethanol leaf extracts were comparable to a range of extracts developed from other species in this genus. Bouamama et al. (1999; 2006) reported that organic and aqueous extracts of *C. incanus* and *C. monspeliensis* differed clearly in their antimicrobial activities. Here, *C. villosus* extracts exhibited stronger activity from *C. monspeliensis* when used on *Staphylococcus aureus* (MIC 0.8 mg/ml) and *Candida glabrata* (MIC 0.2 mg/ml). Güvenç et al (2005) demonstrated that water, methanol, chloroform, ethyl acetate, and butanol extracts of five *Cistus* species:
C. creticus. L., *C. laurifolius*. L., *C. monspeliensis*. L., *C. parviflorus*. Lam., and *C. salviifolius*. L. each showed at least some activity against *B. subtilis* and *B. cereus*. Here, lyophylized extracts showed highest activity against *S. aureus* and butanol extracts of *C. creticus* leaves and fruits showed highest activity against *S. subtilis*, *S. faecalis*., *B. subtilis*, *B. cereus*., *E. coli*., whereas all extracts were not active against *P. aeruginosa* or *C. albicans*.

One of the valuable outcomes of this study is that microbial species demonstrating the largest inhibition zones (using the disk diffusion method) do not necessarily present the lowest MIC and MBC values. Hence, more than one antimicrobial test should always be conducted when evaluating plant extracts. For example, the *C. salvifolius* 70% ethanol leaf extract tested against *P. aeruginosa* showed a low IZ (10 mm) and a low MIC (3.1 mg/ml), whereas, a high IZ (20 mm) and a high MIC (25mg/ml) were obtained for *S. typhimurium.* The size of the inhibition zone did not necessarily reflects the antibacterial effectiveness of a compound, since inhibition zones can be affected by the solubility of the extract, its diffusion range within agar, and its evaporation rate which can affect effective dosage, among others (KIM et al., 1995; CIMANGA et al., 2002; HERNANDEZ et al., 2005)

CONCLUSION

The leaf hydroalcoholic extracts (70%ethanol) of *Cistus salvifolius* and *C. monspeliensis* possess potential antioxidant and antimicrobial activities. Consequently, both species could be exploited effectively to be used as natural preservative in food, cosmeceutical and pharmaceutical applications. However, beside the identification of putative bioactive molecules which are responsible for the observed antioxidant and antimicrobial activities, further detailed *in vitro* and *in vivo* studies are needed to evaluate the safety and efficacy of these species.

RESUMO: A composição de metabólitos secundários, atividade antioxidante e propriedades de inibição microbiana de extratos das folhas de duas espécies de *Cistus*; *C. monspeliensis* e *C. salvifolius* foram investigados utilizando-se três solventes de extração (etanol, hexano e água destilada). A extração com etanol foi a mais eficiente na extração de compostos fenólicos, flavonóides e taninos condensados em comparação com hexano e água destilada para ambas as espécies de *Cistus*. Um teste antioxidante total (TAA) e dois testes de eliminação de radicais (DPPH e ABTS) indicaram que o extrato em etanol 70% das folhas de *C. salvifolius* teve maior atividade antioxidante em comparação com o extrato etanólico de C. *monspeliensis*, enquanto o extrato aquoso de *C. monspeliensis* teve maior atividade antioxidante que o respectivo extrato aquoso ou o extrato etanólico de *C. salvifolius*. Em geral, os extratos polares foram mais ativos em ambas as espécies do que os extratos não-polares. Assim, extratos etanólico e aquoso das folhas de cada uma das espécies de *Cistus* foram testados quanto à sua capacidade de inibir sete cepas microbianas patogênicas, incluindo *Escherichia coli* ATCC 8739, *Salmonella typhimurium* NCTC 6017, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis*, *Aspergillus niger*, e *Candida albicans*. Extratos etanólicos da folha de ambas as

espécies foram ativos contra cada uma das espécies microbianas, mas o extrato de *C. monspeliensis* foi muito mais ativo contra várias espécies microbianas do que o de *C. salvifolius*. Por exemplo, o extrato de C. salvifolius mostrou maior atividade antimicrobiana contra *P. aeruginosa* e *A. niger* (MIC 3,1 mg/ml e MBC 6,3 mg/ml para ambos), ao passo que o extrato de *C. monspeliensis* mostrou em geral maior atividade contra E. coli, *P. aeruginosa* e *C. albicana* (MIC 6,25 mg/ml e MBC 12,5 mg/ml para todos os três). Em conclusão, o método de extração com etanol 70% foi mais eficiente para a extração de polifenóis total, flavonóides e taninos condensados a partir de duas espécies de *Cistu*s, sendo os extratos aquoso-etanol com maiores atividades antioxidante e antimicrobianas. Assim, os extratos aquosos-alcoólicos de ambas as espécies podem ser consideradas como potenciais fontes de antioxidantes naturais e agentes antimicrobianos.

PALAVRAS-CHAVE: *Cistus salvifolius*. *Cistus monspeliensis*. Atividade antioxidante. Atividade antifúngica.

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