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Ahlem Rebaya, Igueld Souad, Safa Hammrouni, Abderrazak Maaroufi, Malika Ayadi, Jamila Chérif

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Research Article

Antibacterial and Antifungal Activities of Ethanol Extracts of *Halimium halimifolium, Cistus salviifolius and Cistus monspeliensis*

Ahlem Rebaya[1], Souad Igueld Belghith[2*], Safa Hammrouni[3], Abderrazak Maaroufi[3], Malika Trabelsi Ayadi[1], Jamila Kalthoum Chérif[1,4]

[1]*Laboratory of Applications of Chemical Resources, Natural Substances and the Environment (LACReSNE), Faculty of Sciences of Bizerte, 7021 Zarzouna - Bizerte, Tunisia.*
[2]*Preparatory Institute for Engineering Studies of El-Manar B.P.244 El Manar II - 2092 Tunis, Tunisia.*
[3]*Laboratory of Epidemiology and Veterinary Microbiology, Group of Bacteriology and Biotechnology, Pasteur Institute of Tunisia (IPT), BP 74, 13 place Pasteur, Belvédère, 1002 Tunis, Tunisia.*
[4]*Preparatory Institute for Engineering Studies of Tunis, 2 rue Jawaharlal Nehru, Monfleury, 1008 Tunis, Tunisia.*

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ABSTRACT

The objective of this study was to evaluate antimicrobial and antifungal activities of crude extracts from leaves and flower of *Halimium halimifolium*, and compared with those of *Cistus salviifolius* and *Cistus monspeliensis.* The tested plants (leaves and flowers) were extracted with ethanol, the activities were screened against three Gram-positive (*Listeria monocytogenes, Bacillus subtilis and staphylococcus aureus*), three Gram-negative bacteria (*Salmonella enteric, Pseudomonas aeruginosa and Escherichia coli*), and two pathogenic fungi (*Candida albicans* and *Aspergillus niger*). The efficacy of these extracts was tested against those microorganisms through a disc-diffusion method employing 15 μL of each sample per paper discs (6 mm in diameter). Comparable results were carried out using Gentamicin and Amphotericin as standard antibiotics. Ethanol extracts of different parts of plant exhibited good activity against all microorganisms tested. The inhibition zone measured ranged from 10 to 26 mm against all the bacteria and 8 to 20 mm against fungal strains. The minimum inhibitory and bactericidal concentrations (MIC and MBC) values against *Staphylococcus aureus* ranged from 1.562 to 12.5 mg. mL^{-1} and 3.125 to 25 mg. mL^{-1} respectively. The *Cistus salviifolius* leaves had the most potent bactericidal activity. On the other hand, *Candida albicans* and *Aspergillus Niger* were the least susceptible microorganisms to all *Cistus* extracts. In the present work, the antimicrobial potential of ethanol extracts of leaves and flower of *Halimium halimifolium, Cistus salviifolius* and *Cistus monspeliensis* is demonstrated. The high levels of antibacterial activities of have been detected, indicating that this plant may serve as an excellent source of natural antibacterial for disease prevention.

Key words: antibacterial, antifungal, stokes disc diffusion, *Halimium halimifolium* and *Cistus salviifolius*

INTRODUCTION

The *Cistaceae* is a Mediterranean native family of almost 200 species of shrubs[1]. This family known for their beautiful shrubs is formed by different genera, including *Halimium* and Cistus[2]. The genus *Cistus* comprises a group of about 20 shrub species[3]. In the Mediterranean folk medicine, all the *Cistus* species are frequently used as herbal tea infusions for the treatment of various skin diseases, rheumatism, fever and diarrhea[4-6]. Recent pharmacological studies provided the beneficial effects of *Cistus* extracts in inflammatory or infectious diseases by demonstrating their antiproliferative, cytotoxic activity and strong gastric antiulcer activity, antibacterial, antiviral, antifungal, hypotensive, skin care and spasmolytic activities [4,6-8]. Studies on the effect of *Cistaceae* species against plant pathogens are limited. The previous investigations showed that aqueous extracts of *C. monspeliensis* growing in Morocco inhibited the bacterial growth of *Clavibacter michiganensis subsp*[9]. Other works have tested the effectiveness of ethanol extract of *Cistus salviifolius* growing in Spain and found to have an inhibitory effect against *E. coli and S. aureus*[10]. To our knowledge very few investigations were performed on the biological properties of the *H. halimifolium*. This encouraged us to study the antimicrobial properties of the extracts of rockrose species collected in Tunisia against eight microorganisms, including six bacteria reference pathogenic, one fungi and one yeast. The study was realized using ethanol extracts of *H. halimifolium* (leaf and flower) and compared with those of *C. salviifolius* and *C. monspeliensis.*

MATERIALS AND METHODS

Plant material

The aerial parts of *Halimium halimifolium*, *Cistus salviifolius* and *Cistus monspeliensis* were collected at the full flowering stage, during June 2011 from the north-western part of Tunisia (Sidi Mechreg). The plant material was botanically identified according to the morphological description presented in Tunisian flora[11].

**Author for Correspondence*

Table 1: *Antibacterial screening* of plant extract as shown by the inhibition zone diameter

	Bacterial strain	Inhibition zone (mm)						
		H. halimifolium		*C. salviifolius*		*C.monspeliensis*		Antibiotic
		L	F	L	F	L	F	Gentamycin
Gram-	*E. coli*	18	10	24	22	11	22	11
	P. aeruginosa	23	19	26	23	15	25	21
	S. typhimurium	24	21	25	21	15	24	21
Gram+	*S. aureus*	23	19	25	22	15	25	16
	B. subtilis	22	20	24	23	15	24	21
	L. monocytogenes	25	20	25	20	15	22	20

Values are mean inhibition zone (mm) ± S.D of three replicates; L: leaves, F: flowers

Table 2: *Antifungal screening of* plant extract as shown by the inhibition zone diameter.

Fungal strain	Inhibition zone (mm)						
	H. halimifolium		*C. salviifolius*		*C. monspeliensis*		Antibiotic
	L	F	L	F	L	F	Amphotéricine
Candida albicans	13	2	20	14	8	13	20
Aspergillus niger	9	8	15	13	8	13	15

Values are mean inhibition zone (mm) ± S.D of three replicates; L: leaves, F: flowers

Table 3: Minimum Inhibitory Concentration (MIC) for plants tested

Microorganism	MIC values (mg /mL)					
	H. halimifolium		*C. salviifolius*		*C. monspeliensis*	
	L	F	L	F	L	F
Staphylococcus aureus	1.562	3.125	1.562	3.125	1.562	1.562
Escherichia coli	12.5	12.5	12.5	12.5	12.5	12.5
Bacillus subtilis	12.5	12.5	6.25	12.5	12.5	12.5
Pseudomonas aeruginosa	12.5	12.5	12.5	12.5	12.5	12.5
Salmonella typhimurium	12.5	12.5	12.5	12.5	12.5	12.5
Listeria monocytogenes	6.25	3.125	1.562	6.25	3.125	1.562
Candida albicans	6.25	6.25	6.25	6.25	6.25	6.25
Aspergillus niger	-	-	-	-	-	-

L: leaves, F: flowers

The collected plant material was separated into leaves and flowers and dried in darkness at ambient temperature. After, each part was manually ground to a fine powder and stored in plastic bags until the use.

Preparation of the extracts

Plant material used in this study was ground to a fine powder before extraction. The rockrose powder (1 g of leaves and flowers) was extracted with ethanol (10 mL) for 24 h. The extracts obtained was filtered and stored in a refrigerator at 4 °C until further use.

Microbial strains

Antimicrobial activities of ethanol extracts were tested against 8 microorganisms, including 6 strains of bacteria: *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enterica* NCTC 6017 (Gram-negative bacteria) and *Listeria monocytogenes ATCC* 7644, *Bacillus subtilis* ATCC 6051, *Staphylococcus aureus* ATCC 25923 (Gram-positive bacteria) one fungi: *Aspergillus niger* and one yeast *Candida albicans* ATCC 2091 The cultures were sub cultured regularly and stored at 4°C for further uses. All the bacteria used were obtained from international culture collections ATCC and the local culture collection of Pasteur Institute of Tunisia. Bacterial strains were cultured on trypto-caséine agar soja (TCS) and fungal strains were cultured on Sabouraud Dextrose agar (SDA).

Inoculums preparation

The microbial strains were suspended in a normal saline solution to a turbidity of 0.5 Mac Farland standards (10^8 CFU (Colony Forming Units) / mL of bacterial cells and 10^6 CFU / mL for fungal cell[12]. The inoculums were stored at 4°C until use.

Disc diffusion method

Antimicrobial activity was determined by agar disc diffusion method[13]. Filter paper discs (6 mm in diameter) were impregnated with15 µL of each extract and placed on the agar surface These plates, after staying at 4°C for 1 hours, were incubated at 37° C for 24 h for bacteria , 48 h for yeast and 72 h for fungi. Gentamicin (10 µg/disc) was used as a positive control in antibacterial activity assays, while Amphotericin B (10 µg/disc) was used in antifungal tests. The diameters of the zones of inhibition were measured and reported in millimeters. Each test was performed in triplicate. Determinations of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The minimum inhibitory concentration and the minimum bactericidal concentration were performed according to Cosentino et al [14] with

Table 4: Minimum bactericidal concentration (MBC) for plants tested

Microorganism	MIB values (mg/mL)					
	H. halimifolium		*C. salviifolius*		*C. monspeliensis*	
	L	F	L	F	L	F
Staphylococcus aureus	3.125	6.25	3.125	6.25	3.125	3.125
Escherichia coli	25	12.5	12.5	25	25	25
Bacillus subtilis	25	25	12.5	25	25	25
Pseudomonas aeruginosa	12.5	12.5	12.5	12.5	12.5	12.5
Salmonella typhimurium	12.5	12.5	25	12.5	25	12.5
Listeria monocytogenes	12.5	6.25	3.125	12.5	6.25	3.125
Candida albicans	6.25	6.25	6.25	6.25	6.25	6.25
Aspergillus niger	-	-	-	-	-	-

Table 5: Total phenolic, flavonoid, and Proanthocyanidin content in *Cistus salviifolius* and *H. halimifolium*

	Inhibition zone (mm)			
	H. halimifolium[20]		*C. salviifolius*[21]	
	L	F	L	F
TP	55.98±1.30	40.55±0.30	53.62±0.38	38.2±0.17
TF	20.11±1.02	15.50±0.47	27.84±0.02	26.40±0.35
TCT	2.20±0.20	1.99±0.02	1.35±0.00	0.75±0.13

TP: Total phenolics (g gallic acid eq.100g-1 DM); TF: Total flavonoid (g catechin eq. 100g-1 DM); Total condensed tannins (g catechin eq. 100g-1 DM); Values are presented as means ± S.E.M (n = 3) .

modifications. Details of both methods are described elsewhere. All antibacterial and antifungal tests were performed in TCS and Sabouraud broth, respectively. Overnight broth cultures were diluted in peptone water (0.1% (v/v)) to obtain a working culture (10^5 CFU/mL). The initial test concentration of samples was serially diluted (1/2, 1/4, 1/8, 1/16, 1/32, 1/46). For pathogenic bacterial strains, the tubes were incubated for 24 h, whereas for fungi, the tubes were incubated for 48 to 72 h at 37°C. Gentamycin and amphotericin were used as positive controls, and ethanol as a negative control in parallel experiments. The MIC is defined as the lowest concentration without sign of visible bacterial growth on the culture plates, whereas, the minimum bactericidal concentration was identified by determining the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculums by ≥99.9%. It can be determined from broth dilution minimum inhibitory concentration (MIC) tests by subculturing to agar plates that do not contain the test agent**.** MIC of all plants tested was estimated for each of the test organisms in triplicates.

RESULTS AND DISCUSSION

Antimicrobial activity

The in vitro antifungal and antibacterial activities of different extracts is a first step towards the development of new potential drugs. Antimicrobial activity of the extracts obtained from *H. halimifolium*, *C. salviifolius*, and *C. monspeliensis* (leaves and flowers) against the employed micro-organisms is summarized in Table 1 and 2. The obtained results revealed that all extracts showed antibacterial activity with varying magnitudes. The patterns of inhibition varied with the plant extract and the organisms tested. A high activity of rockrose species against all bacterial and fungal test strains compared with reference agents Gentamycin (for bacteria) and amphotericin (for fungi) are shown, the inhibition zone measured ranged from 10 to 26 mm for all the sensitive bacteria. Table 1 shows that *H. halimifolium* and *C. salviifolius* leaves extracts possess strong antibacterial activity more than the corresponding flowers, whereas in the *C. monspeliensis* the flowers were the most active. *C. salviifolius* leaves extracts showed the highest inhibitory effects with a measured value of zone of inhibition ranged from 24 to 26. This activity is significantly greater than that of the antibiotic reference. Ethanol extracts of *H. halimifolium* flowers were not effective against *Candida albicans* with only 2 mm zone of inhibition. The ethanol extracts of *Cistus salviifolius*, specially leaves extract showed the best potential activity against *Candida albicans*, higher than those the standard antifungal amphotericin.

The minimal inhibitory concentrations (MIC) and minimal bactericidal concentration (MBC) of the three plant extracts against six bacterial species and two fungal species were calculated by using a broth micro dilution technique (Tab.3 and Tab.4). MIC values range between 1.562 to 12.5 mg/ mL. The three plants tested showed the strongest activity against *Staphylococcus aureus* (gram positive bacteria) (MIC =1.562 mg/ mL and MBC = 3.125 mg/ mL), with the exception of the flowers of H. *halimifolium* and *C. salviifolius* with (MIC =3.125 mg/mL and MBC = 6.25 mg/mL. The MIC concentrations against *B. subtilis*, *E. coli*, *P. aeruginosa* and *S. typhimurium* were relatively high and ranged from 6, 25 et 12, 5 mg/mL. The treated extracts showed excellent antimicrobial activity against both gram positive, gram negative bacteria. These results are in conformity with described by Luis Miguel Bedoya et al [15] who indicated that plants of *Cistaceae* family had a potential antimicrobial activity against bacteria, fungi, viruses and parasites. Scientific studies

show that Gram positive bacteria were more resistant to extract and plant oil than the Gram negative bacteria due to the presence of lipopolysaccharide (LPS) which provides protection against different agents[16]. However, in this study, the plant extracts were effective against both types of microorganisms. This activity may be indicative of the presence of a broad spectrum of antibiotic compounds[17]. The antibacterial activity of *H. halimifolium* extract could be attributed to the presence of phenolic compound. It has been reported that phenols are one of the most efficient antimicrobial agents of various plants[18, 19]. These results could be explained by previous studies conducted by our group who reported that *H. halimifolium and C. salviifolius* are potentials sources of novel bioactive compounds (phenolic acids, flavonoids and tannins) (Tab. 5)[20,21.] One of the valuable outcomes of this study is that the bioactive compounds of plants are connected with antimicrobial properties.

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CONCLUSION
The present study was conducted to obtain preliminary information on the antibacterial activity of ethanol extracts of *Halimium halimifolium*, *Cistus salviifolius* and *Cistus monspeliensis*. The extracts obtained from these plants show potent antibacterial and antifungal activity, and so may be advantageously used as a therapeutic agent for bacterial infections of humans or animals. These results confirmed the medicinal potential of the leaves and flowers of *H. halimifolium* and are in agreement with the medicinal potential of *Cistaceae* family showed by several authors. This work provides scientific insight to further determine the antimicrobial principles and investigate other pharmacological properties of these plants.

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