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Radical scavenging, antioxidant and antimicrobial activities of halophytic species

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Abbreviations: ABTS: 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid); ATCC: American Type Culture Collection; BCC: Brittany Culture Collection; GAE: Gallic Acid Equivalents; TAC: Total Antioxidant Capacity.

Introduction

Food poisoning is still a concern for both consumers and the food industry despite the use of various preservation methods. Because of the resistance that pathogens build against antibiotics, there is a growing interest to use natural antibacterial products for food preservation, like extracts of herbs and spices (Smid and Gorris, 1999). Indeed, natural crude extracts and biologically active compounds from plant species used in traditional medicine may represent valuable sources for such new preservatives (Al-Fatimi et al., 2007).

Eryngium maritimum L. (sea holly, Apiaceae), *Crithmum maritimum* L. (sea fennel, Apiaceae) and *Cakile maritima* Scop. (sea rocket, Brassicaceae) are three halophytic species commonly found along Atlantic coast. They generally grow in sand hills, sea fennel being also found in rocky cliffs. These halophytes have found many applications in folk medicine. Thus, *C. maritimum* and *C. maritima* are thought to have diuretic, antiscorbutic, digestive and purgative properties (Guil Guerrero et al., 1996; Davy et al., 2006). Various properties were traditionally attributed to sea rocket. Indeed, this emetic plant was good to cleanse the lungs of tough viscid phlegm, could help jaundice and dropsy, and was prescribed in scrofulous affections and lymphatic disturbances (www.herbnet.com). Sellam et al. (2007) described antifungal activity of phytoalexins and glucosinolates, two natural compounds present in *Cakile maritima*. Antimicrobial activity was also proved in other Brassicaceae (Sekiyama et al., 1996 ; Brown and Morra, 1995). *E. maritimum* has numerous medicinal uses as a diaphoretic, a diuretic, a stimulant, a cystotonic, a urethritis remedy, a stone inhibitor, an aphrodisiac, an expectorant and as an anthelmintic (www.botanicals.com, www.crescentbloom.com). However, literature on possible activities of sea holly only highlights anti-inflammatory and antinociceptive properties (Küpeli et al., 2006). Studies on antioxydant or antibiotic properties of these halophytes mainly deal with sea fennel essential oil (Ozcan, 2000; Ruberto et al., 2000). Sea rocket was recently studied for its radical

scavenging activity (Ksouri et al., 2006), but no antimicrobial properties have been scientifically demonstrated.

In the present work, we report for the first time the results of the combined investigations on in vitro antibacterial, radical scavenging and antioxidant activities of the extracts of these three halophytic species, with the aim of demonstrating their biological activities and confirming their potential as natural preservatives.

2. Materials and methods

2.1 Plant material

Leaves of *Crithmum maritimum* L., *Eryngium maritimum* L. and *Cakile maritima* Scop. were collected along the shoreline at “Pointe du Toulinguet” (Brittany, France) in August 2006. The leaves were cleaned with deionized water, rapidly soaked, stored at - 25°C and then freeze dried. The dry material was ground to a fine powder.

2.2 Extraction of plant material

For the determination of total phenol contents and antioxidant activities, 200 mg of powder were homogenized with 5 ml water-methanol (1:1) under magnetic stirring at 4°C for 20 min. After centrifugation for 15 min (4°C, 4000 g), the resulting pellet was extracted twice following the same protocol. The supernatants were collected, pooled and filtrated over glass wool. The obtained extract was concentrated by rotary evaporation at 30°C. The residue was dissolved in deionized water.

For the antibacterial tests, 200 mg of powder were homogenized with 5 ml methanol-chloroform-water (12:5:3) under magnetic stirring for 20 min. As mentioned previously, three successive extractions were performed. After phase separation by adding deionized water, chloroformic and aqueous fractions were concentrated by rotary evaporation at 30°C. The

residues were weighed and dissolved in methanol to a concentration of 10 $mg.ml^{-1}$ and 2 $mg.ml^{-1}$ for the polar and apolar phases, respectively.

2.3 Chemicals

Folin-Ciocalteu reagent, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, sodium phosphate and ammonium molybdate were purchased from Sigma-Aldrich. Methanol and chloroform were from Carlo Erba Reagenti. All other reagents were of analytical grade.

2.4 Microorganisms

Antimicrobial activity was tested against a panel of microorganisms, mainly food-borne pathogens as well as clinical isolates (Table 1): gram-positive bacteria (cocci and bacilli), gram-negative bacteria and one yeast. For each type of bacteria, at least one strain was obtained from American Type Culture Collection, all the others were from Brittany Culture Collection. Strains were grown in liquid nutritive broth at 37°C overnight before being used for antibacterial activity test.

2.5 Total phenol content

The concentration of total phenols in each plant extract was determined with Folin–Ciocalteu reagent following the colorimetric method adapted by Sanoner et al. (1999). Measurements were carried out in triplicate and calculations were based on a calibration curve obtained with gallic acid. The levels of total phenols were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg $GAE.g^{-1}$ DW).

2.6 Scavenging activity of ABTS radical cation

ABTS radical cation ($ABTS^{\bullet+}$) scavenging activity was measured according to the method described by Re et al. (1999). ABTS was dissolved in water to a 7 mM concentration and the ABTS radical cation was produced by adding potassium persulfate to a final concentration of 2.45 mM. The completion of radical generation was obtained in the dark at room temperature for 12–16 h. This solution was then diluted with ethanol to adjust its absorbance at 734 nm to 0.70 ± 0.02. To determine the scavenging activity, 1 ml of diluted $ABTS^{\bullet+}$ solution was added to 10 µl of plant extract (or water for the control), and the absorbance at 734 nm was measured 6 min after the initial mixing, using ethanol as the blank. The percentage of inhibition was calculated by the equation:

$$\text{Inhibition percentage (\% IP)} = [(A_c - A_s)/ A_c] \times 100$$

where A_c and A_s are the absorbances of the control and of the test sample, respectively.

From a plot of concentration against %IP, a linear regression analysis was performed to determine the IC_{50} value for each plant extract.

2.7 Total antioxidant activity

The total antioxidant capacity (TAC) of the plant extracts was evaluated by the phosphomolybdenum method of Prieto et al. (1999). A 0.1 ml aliquot of the plant extract was mixed with 1 ml of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The absorbance of the mixture was measured at 695 nm and standard curve was performed with ascorbic acid solution. The antioxidant activity of the samples was expressed as milligrams of ascorbic acid equivalents per gram of dry weight.

2.8 Antimicrobial activity

The microplate bioassay (microdilution) was used to study the antimicrobial activities of *C. maritimum*, *E. maritimum* and *C. maritima*. Plant extracts (at the concentration of 1, 2, 10 and 100 µg.ml^{-1}) were dropped in sterile 96-well plates (NUNC microplate, Fisher Bioblock). Twelve repetitions were made for each concentration. After evaporation of the solvent, 100 µl of microorganism suspensions (10^2 cells.ml^{-1}) were added in wells. Antibiotic solution (mixture of streptomycin and penicillin G at 5 and 10 mg.ml^{-1}, respectively) was used in the presence of microorganisms for negative control. The microplate was aseptically sealed and incubated at 30°C for 24 h. After agitation, microorganism growth was estimated by reading the absorbance in microplate wells at 405 nm with a microplate spectrophotometer (Multiskan MCC/340, Titertek). The MIC was defined as the lowest concentration of plant extracts able to inhibit microorganism growth.

3. Results

3.1 Total phenol contents and antioxidant activity

Sea holly (*E. maritimum*) exhibited the lowest phenolic content (Table 2), with 30% and 50% less phenols than in sea rocket and sea fennel, respectively. Similar observations could be made with the radical scavenging activity. Thus, *E. maritimum* presented a high ABTS IC_{50} while *C. maritimum* showed the best radical scavenging activity. Sea rocket, which accumulated a medium level of phenolics, presented quite a strong antiradical activity (ABTS IC_{50} = 0.144 $mg.ml^{-1}$) and a good antioxidant capacity.

3.2 Antimicrobial activity

Table 3 shows MIC of plant extracts against several bacteria and one yeast. Apolar fractions were generally more active compared to polar fraction. Thus, sea holly apolar fraction inhibited 9 microorganisms, whereas its polar fraction only inhibited 2 microorganisms. *Listeria monocytogenes* was the only bacteria not affected by any of the plant extracts. Conversely, *Pseudomonas aeruginosa* and *P. fluorescens* were the most sensitive bacteria to all plants extracts. Indeed, only 1 $\mu g.ml^{-1}$ of the three polar fractions was necessary to inhibit *P. aeruginosa* growth. *Candida albicans* was particularly sensitive to the two fractions of sea rocket (1 $\mu g.ml^{-1}$) as well as *Salmonella arizonae* with sea fennel extracts (MIC of 1 $\mu g.ml^{-1}$). The average MIC was 100 $\mu g.ml^{-1}$ for most of bacteria.

Discussion

The halophytic plants studied here differed in their capacity to accumulate phenolic compounds. Even if they grew in the same environment, experiencing the same stressful conditions, they produced different patterns of total phenols. Thus, *Eryngium maritimum* exhibited the lowest phenolic level as well as the lowest radical scavenging activity. Conversely, *Crithmum maritimum* exhibited the highest total phenol content and ABTS radical scavenging activity. *Cakile maritima* plants from Brittany coast presented here a lower phenolic level than that measured recently by Ksouri et al. (2006) in several accessions from Tunisia. Although the three halophytic plants differed in their radical scavenging activity and total phenol contents, they presented close antioxidant capacities (32 to 48 $mg.g^{-1}$ DW). Moreover, there was no relationship between total antioxidant and anti-ABTS radical activities. These results can be explained since the global antioxidant property of a plant extract is generally considered as the result of the combined activity of a wide range of compounds including, beside phenolics, peptides, organic acids and other components (Gallardo et al., 2006).

Concerning antimicrobial properties, we report here for the first time antimicrobial activity in both polar and apolar fractions of halophyte extracts. The three halophytes presented a good antimicrobial activity, particularly in their organic (apolar) fraction. Most of the extracts showed a MIC of 100 $\mu g.ml^{-1}$, some others even being active at 1 $\mu g.ml^{-1}$. However, they did not show antilisterial activity. That result could be related, in the case of *Crithmum maritimum*, to the predominance of chlorogenic acid in sea fennel (Meot and Magné, unpublished results) and the absence of antilisterial activity of plant phenolics shown recently for phenolics such as chlorogenic acid (Wen et al., 2003).

Apolar fractions were more active than polar ones. Thus, sea holly polar fraction inhibited the growth of only 2 bacteria, whereas its apolar phase was active against nine

pathogens. Interestingly, some bacteria were sensitive to both polar and apolar extracts of a same plant (*e.g. Salmonella arizonae* vs sea fennel, or *Pseudomonas fluorescens* vs sea holly), though these two fractions greatly differed in chemical composition. *Eryngium maritimum* presented a strong antibacterial activity against two of the three *Pseudomonas* species tested (*P. aeruginosa* and *P. fluorescens*). Indeed, its 2 fractions had low MIC : 1 and 2 $\mu g.ml^{-1}$ for polar and apolar fractions, respectively. *Cakile maritima* was very active against *Salmonella arizonae* (MIC of 1 $\mu g.ml^{-1}$ for its 2 fractions). *Crithmum maritimum* had a good antimicrobial activity against *Pseudomonas aeruginosa* and *Candida albicans* (MIC of 1 $\mu g.ml^{-1}$).

Sea fennel essential oil was largely studied, both for its antioxidant (Ozcan, 2000) and antibacterial properties (Ruberto et al., 2000). In particular, the latter work reported no antibacterial activity against *Pseudomonas aeruginosa* and a light activity against *Micrococcus luteus*, results which were obtained in our chloroform extracts too. However, Ruberto et al. (2000) did not find any activity against *Erwinia carotovora* and *Escherichia coli*, suggesting that our positive results were due to molecules not present in essential oil. In another study, Glowniak et al. (2006) presented the activity of sea fennel against Gram-positive bacteria. Accordingly, we confirmed that *C. maritimum* apolar fraction inhibited the growth of *B. cereus* and *M. luteus*. However, unlike the results of Glowniak et al. (2006), we found activity against *E. coli*, and apolar fraction was inactive against *S. aureus*.

The results of these investigations confirm the capacity of halophytic plants to produce bioactive compounds and the great potential for using them as natural food (or cosmetic) preservatives, as the extracts possess antibacterial and antioxidant activities. The phytochemical characterization of the extracts, as well as the identification of responsible bioactive compounds, is under progress in our laboratory to reinforce the notion of a potent valorization of such plant extracts.

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Table 1: Bacterial strains and yeast used in this study.

Organism or gram morphology and strain	Species	Culture Collection , identification number
Cocci		
gram positive	*Staphylococcus aureus subsp. aureus*	ATCC, 33862
	Micrococcus luteus	ATCC, 10240
Bacilli		
gram positive	*Listeria monocytogenes*	ATCC, 19112
	Bacillus cereus	BCC
gram negative	*Salmonella enterica subsp. arizonae*	ATCC, 13314
	Salmonella enterica subsp. montevideo	BCC
	Pseudomonas aeruginosa	BCC
	Pseudomonas fluorescens	BCC
	Pseudomonas marginalis	BCC
	Escherichia coli	BCC
	Erwinia carotovora subsp. carotovora	BCC
Yeast	*Candida albicans*	BCC

Table 2

Phenolic contents, free radical scavenging activity (ABTS) and total antioxidant capacity (TAC) of polar fractions of plant leaf extracts.

	Total phenol content ($mg.g^{-1}$ DW)	ABTS IC_{50}.($mg.ml^{-1}$)	TAC ($mg.g^{-1}$ DW)
Eryngium maritimum	16.44 ± 0.30	0.28 ± 0.020	32.74 ± 1.11
Crithmum maritimum	31.93 ± 2.03	0.11 ± 0.004	39.46 ± 1.78
Cakile maritima	22.24 ± 0.84	0.14 ± 0.004	48.64 ± 2.27

Table 3.

Antimicrobial activity of plant extracts expressed as minimum inhibitory concentrations (MICs) in µg.ml^{-1}

Bacteria strains	MIC (µg.ml^{-1})					
	Eryngium maritimum		*Crithmum maritimum*		*Cakile maritima*	
	Polar	Apolar	Polar	Apolar	Polar	Apolar
Staphylococcus aureus ATCC 33862	-	10	-	-	-	100
Listeria monocytogenes ATCC 19112	-	-	-	-	-	-
Micrococcus luteus ATCC 10240	-	100	-	1	-	100
Salmonella arizonae ATCC 13314	-	100	1	1	-	10
Salmonella montevideo	-	100	-	-	-	100
Erwinia carotovora	-	100	-	100	-	100
Pseudomonas marginalis	-	-	100	100	2	100
Pseudomonas aeruginosa	1	2	1	-	1	1
Pseudomonas fluorescens	1	2	1	100	-	-
Escherichia coli	-	-	-	100	-	-
Bacillus cereus	-	1	-	10	-	100
Candida albicans	-	100	-	10	1	1

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