



Antimicrobial activities of proteinic extracts of *Allium roseum* L., a wild edible species in North Africa

Hanen Najjaa ^{1*}, Emna Ammar ² and Mohamed Neffati ¹

¹Laboratoire d'Ecologie Pastorale, Institut des Régions Arides, 4119 Médenine, Tunisie. ²UR Gestion des Environnements Côtiers et Urbains, Ecole Nationale d'Ingénieurs de Sfax, B.P. "W" - 3038 Sfax, Tunisie. *e-mail: hanen.najjaa@yahoo.fr

Received 12 June 2009, accepted 22 September 2009.

Abstract

This work was carried out on *Allium roseum* L., a North African endemic species, growing in the South-East of Tunisia and used as a vegetable, spice or herbal remedy in traditional medicine. Three protocols of extraction (Tris HCl buffer, phosphate buffer and sulphuric acid) were used in order to extract proteins from four different organs of this species (flowers, bulbs, leaves and seeds). Tris HCl buffer extract showed the highest content of *A. roseum* protein. Leaves had the highest protein content and seed had the highest diversified molecular weight varying from 10 to 50 kDa comparatively to the other organs. Antimicrobial activities of different protein extracts were tested by disc diffusion and well diffusion and evaluated by the determination of minimum inhibitory concentration (CMI) and minimum bactericidal concentration (MBC). Antimicrobial activities of the extracts against ten tested microbial strains differed depending on the extraction method and plant material used. The determination of CMI and CMB relieved a very significant antimicrobial activity to some strains such as *C. albicans* and *E. coli*. Extracts selected for highest antimicrobial activity were tested for sensitivity to heat and proteases. Tested extracts were thermolabile and their activities were sensitive to several proteases including α -chymotrypsine, pepsin and trypsin. Results suggest the potential use of the plant as condiment and preservative in the food industry.

Key words: Tunisia, antimicrobial activity, peptides, food pathogens.

Introduction

Food-borne illness resulting from consumption of food contaminated with pathogenic bacteria has been of vital concern to public health ¹. The over-use of antibiotics in agriculture and medicine has led to the emergence of highly resistant pathogenic microorganisms, which now represent a very serious public health problem ^{2,3}. In the food sector, the increasing prevalence of food pathogens in several food commodities is in large part due to a recent tendency to limit the use of traditional microbiological hurdles such as chemical additives and salt. To reduce health hazards and economic losses due to food-borne microorganisms, the use of natural products as antibacterial compounds seems to be an interesting way to control the presence of pathogenic bacteria and to extend the shelf life of processed food ². Indeed, natural crude extracts and biologically active compounds from plant species used in traditional medicine may represent valuable sources for such new preservatives ⁴. Antimicrobial proteins are currently receiving increased attention as defense compounds because of their dual action against phytopathogenic bacteria and fungi ^{5,6}. To date, several proteins with antimicrobial and/or antifungal properties have been isolated and characterized from different plant species and tissues ^{7,8}.

Allium, the largest genus of Alliaceae, includes about 700 species, widespread throughout the world ⁹. Several *Allium* species such as *A. sativum* (garlic) and *A. cepa* (onion) have shown in previous studies various activities. For example, a wide

array of therapeutic effects of garlic has attracted particular attention of modern medicine because of its widespread use as antiatherosclerotic, antidiabetic, antihypertensive, antimicrobial, anticancerous, antioxidant, antifungal and antiviral agent ^{10,11}.

Allium roseum, a North African endemic species, is known since old times as vegetable, spice or herbal remedy and treatment for headache and rheumatism ¹². In the food industry, the potential condiment and preservative use of the plant is also reported ¹³. In this work, we report the extraction and characterization of antimicrobial protein from flowers, bulbs, leaves and seeds of *Allium roseum* L. var. *odoratissimum*. The extracts of this species were tested against pathogenic food bacteria (*E. coli*, *E. fecalis*, *P. aeruginosa*, *S. typhimurium*, *S. aureus*, *S. epidermidis*, *B. cereus*, *B. subtilis*, *M. luteus* and *C. albicans*). Physicochemical properties of active compounds were also assessed.

Materials and Methods

Plant material: *Allium roseum* var. *odoratissimum* (Desf.) Coss (syn. *A. roseum*) samples were collected from the arid South-East of Tunisia (Bengardane) at three periods of the growing cycle in 2008: in January for the vegetative stage (leaves), in February for the flowering (flowers) and in April and May for bulbs and seeds, respectively. Botanical identification was made by Prof. Mohamed Neffati, Director of the Range Ecology Laboratory of the "Institut des Régions Arides", Tunisia (I.R.A.), according to the "Flora of

Tunisia¹⁴. Voucher specimens were deposited at the herbarium of the I.R.A.

Microorganisms: Antimicrobial activity was tested against a panel of microorganisms, mainly food-borne pathogens as well as clinical isolates (Table 1): Gram-positive bacteria, Gram-negative bacteria and one yeast strain. Strains were grown in liquid nutritive broth at 37°C overnight before being used for antibacterial activity test.

Table 1. Bacterial and yeast strains used to study antibacterial activities.

Gram staining	Species	Culture collection, strain reference
Gram-positive	<i>Staphylococcus aureus</i>	ATCC 25923
	<i>Staphylococcus epidermidis</i>	CIP 106510
	<i>Micrococcus luteus</i>	NCIMB 8166
	<i>Bacillus cereus</i>	ATCC 11778
	<i>Bacillus subtilis</i>	ATCC 168
Gram-negative	<i>Enterococcus faecalis</i>	ATCC 29212
	<i>Salmonella typhimurium</i>	NRRLB 4420
	<i>Escherichia coli</i>	ATCC 25922
	<i>Pseudomonas aeruginosa</i>	ATCC 27853
Yeast	<i>Candida albicans</i>	ATCC 1405

Preparation of plant extracts: For each sample (bulbs, leaves, flowers and seeds), 1 g was extracted separately by the following three methods. Method 1¹⁵ comprised powdering of each *A. roseum* sample with a mortar and pestle, stirring the powder in 0.05 M sulfuric acid (3 ml g⁻¹) for 3 h, neutralizing the suspension with NaOH and removing the insoluble material by centrifugation at 10,000 x g and subsequent microfiltration through a 0.22 µm membrane. For Method 2¹⁶ the plant material was homogenized for 5 min with 10 ml of 1 M Tris HCl buffer (pH 8.8) in a Waring blender for 2 h 30 min and filtered through gauze, and the filtrate was centrifuged at 12,000 x g for 15 min. For Method 3¹⁷, each sample was homogenized in 0.02 M phosphate buffer (pH 7.2) containing 0.1 M NaCl, stirred overnight, filtered through gauze, adjusted to pH 4.0 with acetic acid (50%, v/v), stirred for 4 h and centrifuged at 10,000 x g for 40 min. All extraction steps were done at 4°C. The plant extracts were stored at -20°C until testing.

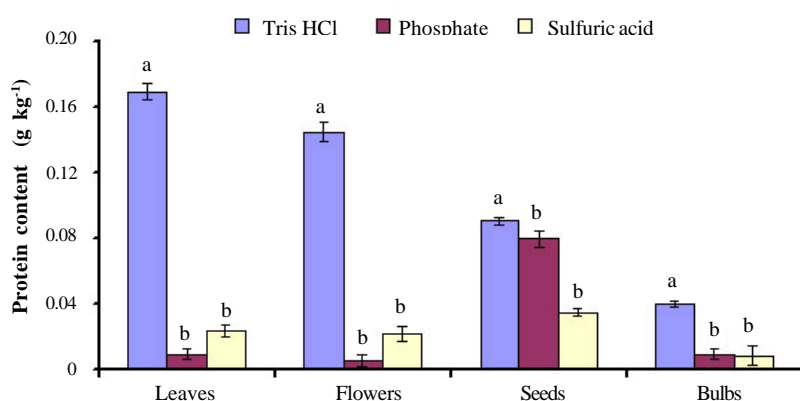


Figure 1. Total protein content of *Allium roseum* bulbs, leaves, seeds and flowers expressed as g kg⁻¹ dry matter. For the extraction three different buffers were used. Means with different letters (a or b) are significantly different at $P < 0.05$.

Determination of protein content: Protein concentrations were determined in triplicate by the Bradford method¹⁸ using bovine serum albumin (Sigma, Mo, USA) as a standard.

Molecular weight determination of total protein: Size separation of the protein samples were solubilized in Tris-HCl buffer (pH 8) using polyacrylamide gel electrophoresis (PAGE), carried out according to the method of Laemmli¹⁹ in a vertical slab gel system (16 cm×18 cm×1 mm) under denaturing conditions. Polyacrylamide gels (12%) running under a constant voltage of 60 V for stacking and of 180 V for separating were used. Low and high molecular weight proteins (Bio-Rad) were used as standards and the gels were stained with Commassie Brilliant Blue R-250.

Antimicrobial screening: Two different methods, agar well diffusion and agar disc diffusion methods, were used for the determination of antimicrobial activities for the proteinic extracts²⁰. MICs and MBCs of these extracts against the test organisms were determined by broth microdilution method²¹.

Characterization of the active compounds: The stability of the putative antimicrobial compounds under exposure to heat and proteases was evaluated for extract selected for high antimicrobial activity. Extracts were boiled (100°C) for 10 or 20 min. Protease treatment was for 2 h with trypsin (99, 3 U/mg), α-chymotrypsin (63, 6 U/mg) and pepsin (31 U/mg) from Sigma (St. Louis, MO, USA). The protease concentration in the reaction solutions was 1 mg/ml. Enzymatic digestion was performed at 37°C for pepsin and 25°C for trypsin and α-chymotrypsin for 2 h. The residual activities of the extracts thus treated were measured by the agar well diffusion method as described.

Statistical analyses: All analytical determinations were performed in triplicate. Results were expressed as mean values ± standard deviations of three separate determinations. A multiple comparison test (LSD) was used to test the significant differences between the treatment means ($P < 0.05$).

Results and Discussion

Content and molecular weight of total proteins: The protein contents of *A. roseum* bulb, leaf, flower and seed extracts using three methods are presented in Fig. 1. Protein content varied significantly ($P < 0.001$) as a function of plant material (bulbs, leaves, flowers and seeds) as well as the extraction buffer. The protein content in flowers and leaves were significantly higher than in bulbs and seeds reaching values in Tris HCl extracts up to 0.17 and 0.14 g kg⁻¹ DW, respectively. Tris HCl extracts showed clearly higher protein contents than phosphate and sulfuric acid extracts. Thus, Tris HCl seems to be the most effective buffer for total protein content from this species. However, these data show the highest content of protein extracted by phosphate buffer in seed. This last result could be explained by the presence of another type of protein, reserve protein, in seed. The protein contents in the organs of *A. roseum* can be ordered as follows: leaves, flowers, seeds and bulbs. *A. roseum* represents an important source of protein as compared to other *Allium* species such as garlic bulbs (1-2% based on fresh weight)²² and *A. sativum* (9.3% based

on dry matter)²³. SDS-PAGE analysis of the 4 extracts was performed to estimate their molecular masses. After treatment with disulfide-reducing agents, all extracts show a major band of 50 and 17 kDa and some minor traces of 10-45 kDa (Fig. 2). Indeed, in all *Allium* species Hanelt *et al.*²⁴ revealed three strong protein bands by electrophoresis under non-reducing conditions. Flowers and leaves proteins have apparent six and seven bands, respectively, with molecular masses between 12 and 72 kDa, but bulb extracts migrate as nine bands of about 100, 72, 50, 43, 35, 30, 26, 17 and 13 kDa. Seed extract shows a mixture of bands of 50, 45, 40, 28, 20, 17 and 10 kDa. According to Oomah²⁵, proteins in seeds are specially globulins with molecular weight <30 kDa and rich in arginin and histidin. The storage proteins might play an important role in plant defense mechanisms^{26,27}. It seems that these proteins function as storage molecules when not required for plant defense as antimicrobial components²⁸.

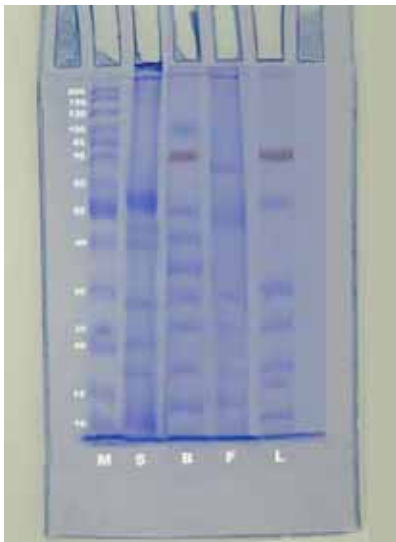


Figure 2. SDS-polyacrylamide gel electrophoretic profile of *Allium roseum* bulbs, leaves, seeds and flowers proteins. M molecular weight marker (Sigma); S: Seeds; B: Bulbs; F: Flowers; L: Leaves.

Antimicrobial activity: The *in vitro* antibacterial effects of *A. roseum* extracts, obtained by the three extraction methods, against the tested bacteria and their activity potentials were qualitatively and quantitatively assessed by the presence or absence of inhibition zones. Zone diameters, MIC and MBC values are presented in Tables 2 and 3. Table 2 shows that extracts had great potential as antimicrobial agent against the tested bacteria having caused serious food poisoning, and preservatives to eliminate their growth are needed.

The statistical analysis showed a highly significant antibacterial effects ($P < 0.001$) of plant (bulb, leaf, seed and flower) extracts on tested strains. *S. aureus*, *B. subtilis*, *B. cereus*, *E. faecalis*, *E. coli*, *P. aeruginosa*, *S. typhimurium* and *C. albicans* were sensitive to the different extracts with the mean growth inhibition zones of 11-21, 12-22, 9-17, 11-23, 10-20, 9-17, 12-21 and 15-18 mm, respectively. *A. roseum* extracts did not have any selective antimicrobial activity on the basis of the tested bacterial groups (Gram+ or Gram-). Previous studies showed that Gram-negative

bacteria are most sensitive to *Allium sativum* extracts²⁹. In standard disc and well diffusion tests flower and seed extracts exhibited more antimicrobial potential than bulb and leaf extracts, reflecting the qualitative and quantitative biochemical differences among these plant parts. The three extraction methods showed variable effectiveness in extracting antimicrobial compounds, with Method 1 being most effective and yielding extract with a broad spectrum of activity. This method, which uses Tris HCl buffer, resulted in significant antibacterial activity against both Gram-positive and Gram-negative species, while the other two methods yielded weak antimicrobial compounds active only against Gram-positive species.

The electrophoretic pattern of seed protein showed the best effectiveness of antimicrobial agent against all the strains. Furthermore, supplement bands in electrophoretic pattern of seed extract, particularly those of molecular weight between 47 and 10 kDa, are presumably responsible for the observed antimicrobial activity. Antimicrobial activity of specific plant peptides was first reported for two isoforms isolated from radish seeds³⁰. Our work may be the first to investigate the prevalence of antimicrobial peptides in wild *Allium* species.

The broth microdilution method used to determine the MIC and MBC of the most active protein solutions against the most sensible microorganisms tested showed both bacteriostatic and bactericidal activities of the extracts with MICs and MBCs ranging from 1 to 4.49 $\mu\text{g protein}/\mu\text{l}$ (Table 3). Seeds, bulbs and flowers had the lowest MIC values (1, 1.05 and 1.45 $\mu\text{g}/\mu\text{l}$) against *S. aureus* and *C. albicans*. MIC values of these organs reflect high sensitivity of *C. albicans* and *S. aureus* of this extract.

Characteristics of antimicrobial compounds: Extracts obtained by Method 1 were selected for a further characterization. Extracts were partially or totally inactivated by tested enzymes, which converted the bioactive compounds to inactive molecules, suggesting that the active compounds are peptide in nature. In contrast, the antimicrobial properties of *Allium* have usually been attributed to a variety of sulphur containing compounds, their precursors and phenolic compounds^{3,31,32}. Our results showed that the active compounds in the plant extracts were quite stable to heating, remaining at least partially active even after 20 minutes at 100°C. This result seems to be important, especially in the case of antimicrobial peptides to be used as food cold preservatives.

Conclusions

Tris HCl buffer extracted relative high protein content of *A. roseum*. Leaves and flowers have the highest protein content and seeds have the highest diversified molecular weight compared to the other parts organs. The work clearly demonstrated the potential of *A. roseum* recovered from arid regions of Tunisia as a source of antimicrobial protein. Some of peptides, extracted by Tris HCl buffer from *A. roseum*, have a broad spectrum of antimicrobial activity against both Gram-positive and Gram-negative bacteria. *A. roseum* may offer a promising alternative for purposes used as a condiment with appreciable flavour and taste, and therefore, this species would be a valuable vegetable (fresh or dried) and an important food ingredient.

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Table 2. Antibacterial activity of *Allium roseum* leaves, flowers, bulbs and seeds protein extracts obtained by three methods.

Technique	<i>S. aureus</i>			<i>S. epidermidis</i>			<i>B. subtilis</i>			<i>B. cereus</i>			<i>M. luteus</i>			<i>E. fecalis</i>			<i>E. coli</i>			<i>P. aeruginosa</i>			<i>S. typhimurium</i>			<i>C. albicans</i>		
	Organ	Disk	Well	Disk	Well	Disk	Well	Disk	Well	Disk	Well	Disk	Well	Disk	Well	Disk	Well	Disk	Well	Disk	Well	Disk	Well	Disk	Well	Disk	Well	Disk	Well	
Tris HCl buffer	Leaves	n	11±1.5	n	10±2.2	18±0.5	17±0.2	17±0.6	11±0.6	14±0.8	10±0.6	11±1.4	15±0.6	19±0.2	15±0.6	13±0.8	23±0.7	12±1.4	15±2.2	9±0.3	13±0.8	10±0.2	12±0.6	12±0.2	10±0.2	10±0.8	14±0.8	12±0.6	15±2.6	
	Flowers	9±1.4	19±0.5	9±0.7	10±0.2	16	22±2.0	11±0.3	11±0.6	12±1.4	15±0.8	13±0.8	23±0.7	12±1.4	15±2.2	9±0.3	13±0.8	10±0.2	15±2.2	9±0.3	13±0.8	13±0.3	19±0.7	11±2.2	11±2.2	13±0.3	19±0.7	11±2.2	15±0.5	
	Bulbs	n	9±0.6	10±1.8	13±2.2	9±2.0	12±0.2	15±0.3	15±1.8	n	n	13±2.0	23±2.6	14±1.4	17±1.1	10±1.8	17±0.4	12±0.7	14±0.5	10±1.8	17±0.4	12±0.7	14±0.5	14±2.6	20±2.6	14±0.5	14±2.6	20±2.6		
Phosphate buffer	Leaves	n	21±0.7	15±0.4	15±0.2	13±0.5	21±1.4	9±1.8	9±0.7	8±2.6	n	n	9±0.7	8±2.6	n	n	15±1.5	15±0.4	18	20±0.5	9±0.8	9±0.6	21±0.3	12±0.6	18±2.2	9±0.6	12±1.1	n	n	
	Flowers	n	9±0.6	9±0.4	12±0.7	n	9±0.2	18±0.5	17±1.4	n	n	9±0.2	18±0.5	17±1.4	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	
	Bulbs	10±1.5	12±0.6	9±0.4	13±0.8	12±1.5	14±0.6	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5	10±0.5
Sulfuric acid	Leaves	n	9±0.7	n	n	n	n	9±2.6	9±0.3	n	n	9±2.6	9±0.3	n	n	9±0.8	10±0.4	n	n	n	n	n	n	n	n	n	n	n	n	
	Flowers	10±0.6	11±0.4	n	n	n	n	12±0.8	16±0.7	8±0.7	8±0.7	8±0.7	8±0.7	8±0.7	8±0.7	8±0.7	8±0.7	8±0.7	8±0.7	8±0.7	8±0.7	8±0.7	8±0.7	8±0.7	8±0.7	8±0.7	8±0.7	8±0.7	8±0.7	8±0.7
	Bulbs	n	10±0.5	n	10±1.1	12±0.6	11±1.5	10±2.2	11±1.5	n	n	10±2.2	11±1.5	n	n	9±0.4	12±2.2	10±0.2	12±1.5	n	n	n	n	n	n	n	n	n	n	n
Seeds	n	10	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n

¹ Diameter including disc diameter (6 mm). Values are means ± SD of three separate experiments done in triplicate. n = no antimicrobial activity.

Table 3. Minimal inhibitory concentration (MIC) (µg/µl) and minimal bactericidal concentration (MBC) (µg/µl) of proteomic extracts of *Allium roseum*.

Microorganism	Flowers		Leaves		Bulb		Seeds	
	MIC ¹	MBC ²	MIC ¹	MBC ²	MIC ¹	MBC ²	MIC ¹	MBC ²
<i>S. aureus</i>	1.45±0.5	1.60±0.7	4.44±0.7	4.44±0.7	n	n	1.05±0.7	1.05±0.7
<i>B. subtilis</i>	1.40±0.7	1.40±0.2	2.45	2.70	2.5	3.01	2.49±0.1	2.60±0.7
<i>B. cereus</i>	4.05±0.7	4.11±0.5	3.01±0.7	3.01±0.7	4.50±0.7	4.50±0.7	4.25±0.7	4.30±0.7
<i>E. fecalis</i>	n	n	n	n	2.00±0.4	4.03±0.5	2.24±0.5	2.24±0.2
<i>P. aeruginosa</i>	2.44±0.5	3.23±0.2	3.44±0.1	3.44±0.5	2.00±0.6	2.00±0.6	2.24±0.5	2.24±0.4
<i>S. typhimurium</i>	3.07±0.7	3.49±0.7	3.49±0.74	2.49±0.7	2.45±0.7	2.49±0.7	n	n
<i>C. albicans</i>	2.55±0.7	3.11±0.7	3.50±0.7	3.70±0.7	4.01±0.7	4.10±0.7	1.45±0.7	1.45±0.7
	1.44±0.6	1.44±0.5	3.44±0.4	3.44±0.2	1.00±0.3	2.00±0.8	1.24±0.3	2.24±0.5

Values are means ± SD of three separate experiments done in triplicate. n = no antimicrobial activity. ¹ Minimal Inhibition Concentration (µg protein /µl) ² Minimal Bactericide Concentration (µg protein /µl)