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Assessment of Antimicrobial Effect of the *Artemisia herba-alba* Aqueous Extract as a Preservative in Algerian Traditional Fresh Cheese

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Abstract

The present work was carried out in order to study the antimicrobial activity of *A. herba-alba* extracts and their application as food preservative. A crude extract was prepared by steeping of the dry leaves of *A. herba-alba* in phosphate buffer. In order to obtain an enriched fraction in active molecules, a series of ammonium sulfate precipitation (ASP) was carried out. Extracts showed antibacterial activity against *E. feacalis*, *M. leteus* and *L. monocytogenes* strains. The best activity is noted for the precipitate obtained at ammonium sulfate precipitation of 60% (ASP60), with inhibition zones of 23.67 ± 0.44 mm for the *E. feacalis* and *M. leteus* strains and 18.00 ± 0.67 mm for *L. monocytogenes*. This extract shows a MIC (minimum inhibitory concentration) of 0.23 and 0.9 mg/ml for *E. feacalis* and *L. monocytogenes*, respectively. The application of ASP60 on "Takammérite", traditional fresh cheese as preservative, cause a significant slowdown in microbial growth under refrigeration during 15 days of storage.

Keywords: *Artemisia herba-alba;* Antimicrobial activity; Purification; Fresh cheese; Bio preservation

Introduction

In food industry, the major challenge is to oppose food alterations. Industrial have always resorted to the use of synthetic additives developed by the chemical industry. These synthetic compounds are widely used to protect food, reducing lipid oxidation and microbial growths during food storage. However, some of them have shown a number of disadvantages and limits of use like butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT), which are suspected to have pathological and toxic effects as chronic toxicity (carcinogenic and allergenic effects) [1-3]. Hence, the new antimicrobial agents should be from natural wholesome sources. Thus, essential oils, proteins and peptides represent the new generation of antimicrobial agents [4].

The efficiency of medicinal plants has driven researchers to conduct thorough studies of defense systems to identify active molecules. These latter synthesized by the plant, as being secondary metabolites are classified in to different families according to their chemical structure. The active molecules might be: phenolic and proteinaceous compounds, and peptides with antimicrobial activities [5-7].

The discovery of the first antimicrobial peptide in plants dates back to 1942 when BALLS et al. have purified α-purothionine from wheat. Since, several studies describing new antimicrobial peptides from plant tissues have been reported by several authors [8,9]. These peptides were isolated from leaves, seeds, tubers, fruits, and roots [6,10,11].

The PhytAMP database (http://phytamp.pfba-lab-tun.org) lists almost 300 peptides of plants considered as antimicrobials, including Defensins, Lipid transfer proteins, Knottins, Hevein and Vicilin-like peptides, Snakins and Cyclotides [10].

In this context, our work focuses on the study of the antimicrobial activity of peptide extracts from *Artemisia herba-alba* and the application of these extracts as food preservative.

A. herba-alba known as "desert wormwood" or "Chih", as it is commonly named in North Africa is part of the genus Artemisia which includes more than 450 species [12,13]. It is a plant that grows spontaneously on the high steppe plains, the highlands and the Sahara [14]. In Algeria the steppe with "desert wormwood" covers 3 million hectares in potential area [15].

It is a popular medicinal herb, infusions of this species are used in traditional medicine to calm abdominal pain, cure diabetes, bronchitis, abscess, diarrhea and as, analgesic, antispasmodic and as a diuretic agent [16-19].

In Algeria, especially in the south, various plants are traditionally used to flavor and preserve many foods such as meats and locally produced cheeses [20,21].

Several extracts and essential oils of *A. herba-alba* have shown biological activities, such as anti-malarial, anti-viral, anti-tumor, anti-hemorrhagic, anti-coagulant, anti-oxidant, antidiabetic activities and strong antibacterial activities against several human pathogens [22-24]. *A. herba-alba* essential oil contain mainly aromatic substances such as terpenoid, flavonoid, coumarin, acetylenes, caffeoylquinic acids and sterols [25].

In addition to the previously described components, *A. herba-alba* contains antimicrobial peptides. The first identification of which was made by [26]. These PAMs inhibited the growth of *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and the new approved species *Bacillus cytotoxicus*.

The aim of the present work was to highlight antibacterial activity of *A. herba-alba* aqueous extract after partial purification of active molecules by ammonium sulfate precipitation and the study of application of the extract obtained as food preservative by assessment of its antimicrobial effect on the storage time of "Takammérite" traditional fresh cheese.

Materials and Methods

Plant material and bacterial cultures

The aerial part of the plant *A. herba-alba (chih),* was collected from Khenchla city (north-south of Algeria) during August-September 2016.

Cultures of *Bacillus subtilis* ATCC 6633 was from the American Type Culture Collection (ATCC) (Rockville, MD, USA), *Listeria innocua* LMG 11387, *Escherichia coli* JM 109, *Staphylococcus aureus* CIP 4.83, *E. coli* CIP 54127, *L. monocytogenes* ATCC 3512. *Enterococcus feacalis*, (CM/NCTC, UK), *Micrococcus leteus* A270 and *Staphyloccus aureus* CIP 4,83.

Preparation of crude extract and ammonium sulfate precipitates

Twenty gram of *A. herba-alba* dry leaves were ground in a mortar, and the resulting powder was steeped one night in 200 ml of phosphate buffer (sodium-phosphate 0.04 M, pH 7) [22]. Then, the extract was centrifuged at 6000Xg for 30 min, the supernatant recovered is designated crude extract.

In order to obtain an enriched fraction in active molecules from the crude extract, an ammonium phosphate precipitation was carried out as follows: Solid ammonium sulfate was slowly added to 100 mL of the crude, under continuous stirring, up to 20% saturation in an ice bath for 1 h. After centrifugation at 10000Xg for 15 min at 4°C, the formed precipitate was preserved, and the supernatant was added with an amount of ammonium phosphate corresponding to 40% saturation and treated as indicated above. This operation was repeated using amount of salt corresponding to 60% and 80% saturation. The precipitates obtained are recovered and dissolved in phosphate buffer. These were designed ASP20, ASP40, ASP60, ASP80 corresponding to the salt saturation used, 20%, 40%, 60% and 80%, respectively. The protein content of the precipitates was determined by the method of Lowry (1951). These samples were sterilized by filtration through 0.44 µm filter (Millipore, MA, USA).

Antibacterial activity assays

Antibacterial activities of the different precipitates were determinated by a plate diffusion assay [23,24]. *Bacillus subtilis, Listeria innocua, Listeria monocytogenes, Enterococcus faecalis, Staphyloccus aureus, Micrococcus leteus* grown in Brain Heart Broth were used as the indicator organism.

Műller-Hinton agar (containing 5 g glucose per litre) was seeded with strain (approximately 1×10^6 CFU/ml). Wells were punched in the agar plate using the wide end of a sterile Pasteur pipette (5 mm diameter). 50 µl of the different samples were dispensed into each well. plates were incubated at 37°C for 18-20 h and examined for zones of growth inhibition on the resultant bacterial lawn. Positive and negative controls consisted on Nisine A, 5.2×10^7 UI/g (Danisco, Beaminster Dorset, UK) and phosphate buffer, respectively.

The Minimum inhibitory concentration (MIC) assays were performed in sterile 96-well microplates (Costar 3799, Corning Incorporated, United Kingdom) [25-27]. In each well containing 190 µl of Műller-Hinton medium were added of 50 µl with various concentrations of AS-P (resulting from twofold serial dilutions of an initial 28.92 mg/ml protein set) and 10 µl of the strain tested prediluted in Muller-Hinton medium for a final bacterial load at 2-8 × 10^4 CFU/ml. Absorbance of wells containing serial dilutions of AS-P is compared to that of the wells of a negative control consisting of Muller-Hinton medium and a control culture. The MIC was defined as the lowest concentration of peptide that resulted in no increase of absorbance at 630 nm after incubation at 37°C for 18 h without shaking.

Diameters inhibition (mm)					
Test strain	**Ammonium sulfate precipitates**				**Nisine**
	ASP20	**ASP40**	**ASP60**	**ASP80**	
S. aureus CIP 4,83	–	–	–	–	15.00 ± 0.67
E. feacalis	15.00 ± 0.67	21.00 ± 0.67	23.67 ± 0.44	16.33 ± 0.89	16.33 ± 0.89
B. subtilis ATCC6633	–	–	–	–	14.33 ± 0.89
L. monocytogenes ATCC 3512	18.33 ± 0.44	19.00 ± 0.67	18.00 ± 0.67	10.33 ± 0.44	14.00 ± 0.67
M. leteus A270	14.00 ± 0.67	21.33 ± 0.89	23.67 ± 0.44	19.33 ± 0.44	13.00 ± 0.67
L. innocua LMG1138	–	–	–	–	13.33 ± 0.44
E. coli JM 109	–	–	–	–	–

Table 1: Antibacterial activity against several strains of ammonium sulfate precipitates of crude extract of *A. herba-alba.*

Cheese preparation and application of actif extract

The "Takammèrite" is traditionally prepared in south of Algeria regions from raw milk coagulated by chicken pepsin. In this study the preparation of fresh cheese was made with 10 L of raw milk. After filtration, the milk was heated to 37°C then added by 8 g of salt and 16 ml of chicken pepsin crude extract. After incubation for 30 min at 37°C, the formed coagulum was sliced and allowed to stand for about 10 min to exude the maximum of whey. After 1 h of draining, the cheese obtained was cut into small cubes.

Cubes of 10 g fresh cheese were put in sterile boxes. The content of each box was covered with 13 ml of the ASP that showed the best activity (previously sterilized by filtration as indicated above). The cheese samples were thus kept soaked overnight at 4°C before removing excess of the extract. Fresh cheese samples not treated with the extract (ASP) were also prepared to serve as a negative control. The cheese samples were kept at 4°C for 15 days.

Microbiological analyses

The microbial counts were carried out at days 0, 5, 10, and 15 of storage at 4°C. Ten grams of cheese were dissolved in 90 mL of sodium

citrate buffer previously heated to 45°C. After homogenization, resulting solution represents dilution 10^{-1} form which aliquots were prepared at decimal dilutions (10^{-2}, 10^{-5}) physiological water. 1 mL of the appropriate dilution was spread on different selective media. Plate count agar (PCA) for total mesophilic aerobic count, oxytétracycline agar (OGA) for yeasts and molds counts, Man, Rogosa and Sharpe (MRS) agar for Lactobacillus and violet red bile lactose (VRBL) agar for coliform counts were used. All agar plates were incubated at 37°C for 24 h and the results of microbial counts were expressed as the log10 of colony forming units per gram of cheese (log10 CFU/g) [28].

Statistical analysis

Mean values of various parameters were calculated and compared by analysis of variance by STATGRAPHICS (2009) program. The significant difference was evaluated at p-value ≤ 0.05.

Results and Discussion

Antibacterial activity of ammonium sulfate precipitates

A crude extract was prepared by soaking of the dry leaves of A. herba-alba in phosphate buffer and in order to obtain concentrated fraction in active molecules, a series of ammonium sulfate precipitation was carried out. The precipitates obtained were dissolved in phosphate buffer and their antibacterial activity was examined by demonstrating the zones of inhibition on agar medium inoculated with a target strains. Seven strains were tested: *B. subtilis*; *L. innocua*; *L. monocytogenes*; *E. feacalis*; *S. aureus*, *M. leteus* and *E. coli*.

Results of peptide extracts antibacterial activity evaluation obtained at different levels of ammonium sulfate (ASP 20, ASP 40, ASP 60 and ASP 80) are presented in Table 1. The inhibition zones (mm) values are given as comparison with those of the antimicrobial agent taken as a positive control (Nisin).

The extracts showed antibacterial activity against *E. feacalis*, *M. leteus* and *L. monocytogenes* strains with clear zones of growth inhibition ranging from 10 to 23 mm. However, the best activity is noted for ASP 60 with inhibition zones of 23.67 ± 0.44 mm for *E. faecalis*, *M. leteus* and 18.00 ± 0.67 mm for *L. monocytogenes* (Figure 1).

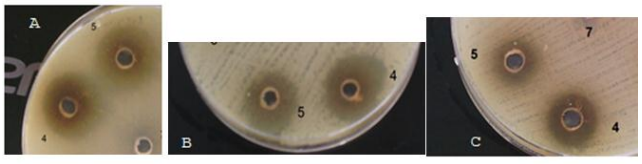

Figure 1: Antibacterial activity of the *A. herba alba* ammonium sulfate precipitates (ASP), against *Micrococcus leteus* (a), *E. feacalis* (b) and *L. monocytogenes* (c) determined by agar-well diffusion test. (4: PSP 40, 5: ASP 60).

MIC was determined ASP 60 and for only two strains; *E. feacalis* and *L. monocytogenes*. The lowest MIC value was noted for *E. feacalis* with 0.23 mg/ml against 0.9 mg/ml for *L. monocytogenes*. These results are correlated with the agar-well diffusion.

However, no activity was obtained against Gram (-) tested strains. These results are consistent with those obtained by Fedhila et al. [26] who reported antibacterial activities of *A. herba-alba* aqueous extracts of towards Gram (+) strains which are: *L. monocytogenes*, *S. aureus, B. cereus*, and *Bacillus cytotoxicus*. And without showing any activity towards Gram (-) strain tested such as *S. arizona*, *E. coli* and *P. aeruginosa.*

Several extracts and essential oils of *A. herba-alba* have shown biological activities, such as anti-malarial, anti-viral, anti-tumor, anti-hemorrhagic, anti-coagulant, anti-oxidant, antidiabetic activities and strong antibacterial activities against several human pathogens [29-31]. *A. herba-alba* essential oil contains mainly aromatic substances such as terpenoid, flavonoid, coumarin, acetylenes, caffeoylquinic acids and sterols [32]. Furthermore, it contains antimicrobial peptides [22].

Extracts antibacterial activity obtained by soaking dry leaves of *A. herba-alba* in phosphate buffer indicates hydrophilic and polar properties of the active molecules. In addition, the proteolytic treatment of these active extracts with proteases (Proteinase K and Trypsin) results in a loss of the antimicrobial activity of 40 to 60% [22]. Thus, these authors, suggest that antibacterial activity are due to plant agents of proteinaceous nature. Similar studies on 'Oudneya africana'; a spontaneous plant from arid regions of Tunisia, shows that the treatment of active extracts obtained from this plant by chymotrypsin and proteinase K induces a loss of about 73% and 71% of the antimicrobial activity [26]. These results show the presence of proteinaceous molecules with an antimicrobial potential in these plants.

Microbiological growths on cheese during the storage

To test the effect of ASP 60 addition on the fresh cheese "Takammérite", several counts have been carried out during the storage at 4°C and the results are shown in the Figure 2.

For untreated cheese, the growth kinetics of total mesophilic aerobic population showed a gradual increase during the 15 days of storage (from 4.19 ± 0.06 to 4.44 ± 0.05 log10 CFU/g). However, their level was significantly lower for treated cheese (4.28 ± 0.04 log10 CFU/g, p=0.0072) (Figure 2a).

A significant difference is also noted for the coliform bacteria level between the two cheeses (4.02 ± 0.11 log10 CFU/g for untreated cheese *vs.* 3.68 ± 0.13 log10 CFU/g for treated cheese) after 15 days of storage (p=0.0496) (Figure 2b).

The growth kinetics of Lactobacilli shows an increase over the 15 days in untreated cheese (3.2 ± 0.22 to 3.79 ± 0.08 log10 CFU/g). On the other hand, the growth rate is significantly lower in the case of treated cheese at the end of the experiment (3.39 ± 0.32 log10 CFU/g) (p=0.0040).

The growth of yeasts and molds can lead to organoleptic deterioration of the fresh cheese due to their high lipolytic and proteolytic activity. The increase in their growth is favored by the pH decrease of the medium.

Yeast and mold are observed from the first day with a count of 3.74 ± 0.09 log10 CFU/g. After 15 days of storage, the rate reaches 4.40 ± 0.15 log10 CFU/g in cheese without extract. However, the treated cheese, showed significant slowest increase in the number of yeasts and molds (4.06 ± 0.13 log10 CFU/g) (p=0.0150) (Figure 2c). It is necessary to note that the processed fresh cheese is prepared by raw milk that has not undergone any heat treatment to reduce its initial microbial load.

We remind that, addition of conventional food preservatives is usually combined with heat treatment or other treatments ensuring their microbiological stability during storage.

Studies showing, *in vitro*, the antimicrobial activity of peptides are uncountable. However, there is no much works treating activity of antibacterial peptides in the food medium whose complex composition may affect the efficiency of these peptides. Added to skimmed milk and to the carrot juice, the peptides resulting from αs2-casein hydrolysis have showed substantial loss of activity. The latter seems to be influenced by the presence of metals cations [33]. So, studies on the factors influencing the activity of these peptides and appropriate methods for efficient applications are necessary.

In this study we applied the *A. herba-alba* extract on the surface of the cheese. The use of this extract in the mass of cheese could provide better protection of its microbiological quality and deserves to be tested. Besides, the study of the effect of this addition on the sensory quality of cheese is also necessary. (Figure 2d).

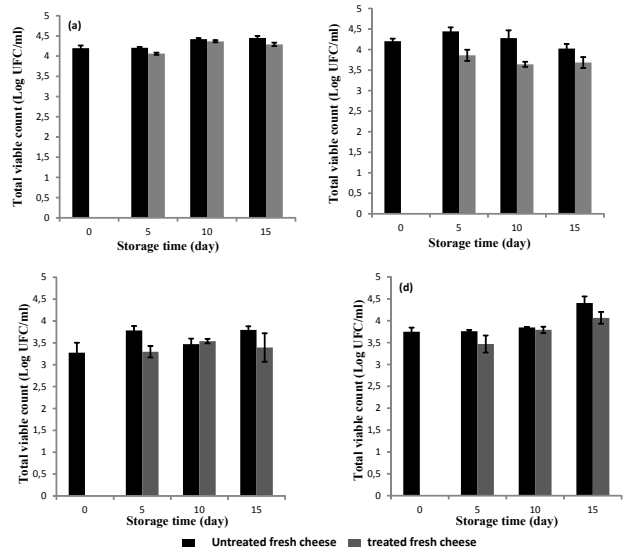

Figure 2: Microbial changes in fresh cheese "Takammrite" untreated and treated by *A. herba-alba* ASP60, during storage under refrigeration. (a) total mesophilic aerobic population, (b) coliform counts, (c) *Lactobacilli* counts, (d) yeasts and molds counts.

Conclusion

Fractional precipitation by ammonium sulfate made it possible to carry out a first purification step of the active molecules resulted at 60% salt saturation of maximum antibacterial activity.

In the current context of food safety and protection using natural molecules, the application of ASP60 on traditional fresh cheese "Takammérite", as preservative, causes a significant slowdown in microbial growth during storage by refrigeration. Nevertheless, it is necessary to specify that the tested extract underwent only a first purification step by ammonium sulfate precipitation where the extract still contains impurities and compounds without any antimicrobial activity that might affect the action of active peptides. Indeed, the purity of these molecules is necessary for their activity.

Following these results, and in the context of the study of the potential of plants as a new source of natural preservatives for the bio preservation of food, it appears that the active molecules present in the leaves of *A. herba-alba* are promising natural additives as synthetic preservative substitutes currently used.

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