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Research Article

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Assessment of *Atriplex halimus* extracts activity against multidrug resistant bacteria isolated from different environments

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ABSTRACT

The emergence of multi-resistant bacteria (BMR) represents a major health issue in the world. Among these bacteria, the most frequent encountered are those expressing a beta-lactamases (ESBL) extended-spectrum. The implementation of an alternative therapy represents the only solution to combat this health risk. The aim of this study is to investigate the bacterial diversity, antimicrobial resistance, types of beta-lactamases and to evaluate the antibacterial effect of the extract of Atriplex halimus on pathogenic multidrug resistant bacteria isolated from clinical infection and food samples. 13 pathogenic strains were isolated from different samples (5 Staphylococcus aureus, 5 E.coli, 2 Salmonella sp and 1 Clostridium sp) respectively. All isolates strains are Beta-lactamase producing. The three extracts from Atriplex hamilus, essential oil, hydrometanolic and acetate have a low yield linked to the characteristics of the plant itself. The extracts showed a higher antibacterial activity. The essential oil exerted an antagonist effect on all pathogens strains with 200µl/ml while hydrometanolic and acetate extract showed an inhibitory effect with a concentration of 300 mg/ml according to the tested strain. There results suggested that the extracts from Atriplex hamilus could be a potential alternative treatment of multidrug resistant bacteria.

Keywords: *Atriplex halimus* extract, beta-lactamase, antibacterial activity.

INTRODUCTION

The phenomenon of bacterial antibiotic resistance has been a strong interest in the scientific community since it represents a risk to public health. It reduces the effectiveness of antibiotics used as first-line and complicates the management of the patient. The development of resistance in bacteria animals can lead to food borne infections (Salmonella, Campylobacter) or opportunists (*E. coli*, *Enterococcus sp.*, *Staphylococcus aureus*) is monitored in the context of an approach global public health. It is time to allocate efforts to find an alternative to the emergence of microbial resistance by new bioactive natural compounds from traditional plant medicine. *Atriplex hamilus* species are dominant in many arid and semi-arid regions of the world, particularly in habitats that combine relatively high soil salinity with aridity [1][2]. The plant is traditionally used in Algeria especially in folk medicine in the treatment of many diseases and infections.

The aim of this study is to describe the bacterial diversity from clinical infection and food samples, antimicrobial

resistance, types of beta-lactamases and to evaluate the effect of the extracts of Atriplex *halimus* on pathogenic multi drug resistant bacteria isolated from clinical infection and food samples.

EXPERIMENTAL SECTION

1. Plant materials

Fresh Atriplex *halimus* leaves were collected from the region of Mascara in west Algerian in April 2014. The plant material was identified according to African flowering plants database, and by local expert. A voucher specimen (#226) (Fig.1) was deposited at the herbarium center of the department of Biology, Mascara University (North West of Algeria) for future reference. The leaves were dried for 3 weeks in darkness at room temperature.

Fig. 1 : Fresh Atriplex *halimus* cultivated Mascara (North West of Algeria)

1.1. Preparation of extract

For extraction of the essential oils, the dried plant material (1kg) was subjected to hydro-distillation using a Clevenger-type extraction device. This technique is based on the power which has water vapor transport to the essential oils by stream distillation of water for 2h at 95°C [3]. The methanolic and acetate extracts were prepared according to the method described by Escarpa and Gonzalez [4]with little modification .The plant (50gr) were grinded and homogenized in a blinder and extracted with 250 ml of 80% methanol or 60% acetone containing 1% of 2,6-du-tert-butyl-4-methylphenol, using an ultrasonic bath. The extraction was repeated three times. The same extracts were pooled and filtered through Whatman No 1 filter paper and evaporated by using a rotary evaporator to give the crude dried extract. The yield of each extract was estimated according the equation: Y (%)= (m/mo)x100 [3]. Finally the extracts obtained were sterilized by filtration, kept in sterile tube and protected from light at +4°C.

2. Microbial samples collection

Clinical samples were isolated from patients (aged between55 and 65 with varied pathology) in surgical service (Table). Food samples were carried out under aseptic conditions from three different sources (Table 1). All samples were caught during a period between Mars and September 2013. They were collected aseptically and transported to the laboratory in a cooler at +4 ° C for Microbial analysis.

Table 1: Origin of microbial strains

Site	Clinical infection	Food Samples
Sampling Nature	anal fissure	raw milk
	Urinary infection	poultry meat
		Water consumption
	Bone abscess	Fish intestine
		Bovine intestine

2.1. Identification of pathogenic strains

Samples were analyzed for aerobic bacterial content by cultures on a series of non-selective and selective media (Blood agar, Chapman medium, Hektoen medium, Nutritive agar medium, MRS medium), incubated at 37°C for 24 h, 48 h and up to72 h according to the investigated strains and with anaerobic condition in the atmosphere for *Clostridium.* Followed by Gram coloration and biochemical test using automate microbiological system identification (API system). The strains isolated from these standard culture procedures were identified with commercials kits (API Staph, API 20 E and others biochemical tests for *Clostridium sp* [5].

2.2. Inoculums' preparation

Nutriment broth [5] was used for growing strains and diluting suspensions. Bacterial strains were grown to exponential phase in nutriment broth at 37°c for 18 h and adjusted to a final density of 2x 10^8 CFU by diluting fresh cultures and comparison to Mac Farland standards (OD_{650}=0.7) [6]

2.3. Antibiotic susceptibility testing

Resistance towards antibiotics was assessed for each strain with the disc diffusion method [7] and bacterial growth on Muller Hilton Agar plates . The antibiotic tested for Staphylococaceae, were Oxacillin (10□g), Erythromycin (15µg), Spriramycin (10µg), Chloramphenicol (30µg), Tetracyclin (30 µg) , for Enterobacteriaceae were Ampicillin (10µg), Gentamicin (10µg), Aztreonam (30µg), Colistin (10µg), Tetracyclin (30µg), Chloramphenicol (30µg), for Clostridiaceae were Cefazolin(10µg) Nalidixic Acid (30µg), Amoxicillin. (10µg), Colistin (10µg).

2.4. Search of beta-lactamases

Only strain resistant to one or more than antibiotic was selected for beta-lactamases research. Phenotypic demonstration of the presence of a β-lactamase extended spectrum in Enterobacteriaceae is to highlight an image of a disk synergy between third-generation cephalosporin and clavulanic acid. Apply on Mueller Hinton agar [5]previously seeded with the test strain, a disc of ceftazidime, aztreonam or cefotaxime and amoxicillin + clavulanic acid disk (AMC), a 1 ½ cm.

Search beta-lactamases in *Staphylococcus sp* was conducted by iodometric test [8]. It consists of a complex of iodine discoloration-starch due to the reduction of iodine by penicilloic acid from the hydrolysis of penicillin G by beta -lactamase.

2.5. Antimicrobial activity assay byMinimum Inhibitory Concentration (MIC)

The minimal inhibition concentration (MIC) values were determined in all strains isolated from clinical infection and food using a microdilution method according to Howaida [9] using 96 well microtiter plates. The MIC was defined as the lowest concentration of the extracts inhibiting the growth of microbial strains. The growth media employed was nutrient broth. The highest concentration of extracts (essential oil = 500µl, methalolic and acetate=500mg). Nutrient broth (50µl) according to strain tested were distributed from the 2nd to the 12th well, a volume of sterile extracts of Atriplex hamilus was added into the 1st test well, and the 50µl of scalar dilution was transferred from 2nd to the 12th well. Finally, 50µl of calibrated bacterial suspension were added to each well. Erythromycin (10µg/ml) was used as positive control, methanol served as negative control. Growth was estimated by measuring well optical density at 620nm using a microplate Absorbance Reader Sunrise (Tecan Australia GmbH RC/TS/TS) comparatively to control wells (nutriment both + inoculum). The plate were agitated and incubated at 37°C for 48h. The lowest concentration showing no culture was considered as the MIC and it's express as (µl/ml, or mg/ml).

3. Statistical analysis

The tests were conducted twice for each sample and the mean of Colony Forming Unit (CFU) count was determined. The results are presented as the mean +- SD of three replicates. For antimicrobial activity, we consider Log CFU more than Log 1 as significant.

RESULTS AND DISCUSSION

1. Extraction of Essential oil from Atriplex *halimus*

The essential oil of Atriplex *halimus* obtained from the aerial part is yellow, liquid appearance with a strong odor resulting from the salinity. The plant has a low yield of 0.06% with neutral pH of 7.1. The low yield may be due to various factors such as the characteristics of the plant as it grows in saline soils [10] which may affect the growth and yield of the plant [11]. Furthermore, salinity causes an increase in the epidermal thickness, the thickness of the mesophyll, palisade cells of the length, the diameter of palisade cells of the leaves in Atriplex *halimus* [12]. Salinity also reduces the intercellular space in the sheets [13]. In addition, low levels of essential oil may be due to the method of extraction or the time and place of harvest. This species originated in Bechar provided with the same method of extracting essential oil content of 0.02% of the aerial part of the plant [14]. These differences may be due to factors related to the ecosystem (climate, soil type, rainfall, etc.), the time of harvest and drying time.

2. Methanolic and Acetate extraction

The result of this experiment allowed the production of two different extracts its appearance and color with pH 6.63. The methanol extract is dark green with a solid look after evaporation, with a yield equal to 0.84%. A study was made by BELYAGOUBI [15] indicating that 24% yield of the same species and the same part used for extraction. This difference in the level of performance always goes to the origin and characteristics of the plant. While the acetate extract obtained is brown with a yield of 0.052% and a pH of 8.07. This results were similar than obtained by CHIKHI ILYAS,[14]

3. Bacterial Species distribution

13 Strains from clinical infection and Food samples were isolated and described in Table 2

Table 2: Pathogenic bacteria isolated from clinical infection and food

Site	samples	Pathogenic strain isolate	Denomination
Clinical infection	anal fissure	*Clostridium sp*	*Clostridium .sp*
	Urinary infection	*E.coli*	*E.coli .1*
	Bone abscess	*Staphylococcus aureus*	*S. aureus .1*
Food Samples	raw milk	*Staphylococcus aureus*	*S. aureus .2*
	poultry meat	*Staphylococcus aureus* *E.coli* *Salmonella sp*	*S. aureus.3* *E.coli. 2* *Salmonella sp .1*
	Water consumption	*Staphylococcus aureus* *E.coli*	*S.aureus.4* *E.coli. 3*
	Fish intestine	*Staphylococcus aureus* *E.coli*	*S. aureus.5* *E.coli.4*
	Bovine intestine	*Salmonella sp* *E.coli*	*Salmonella sp. 2* *E.coli. 5*

4. Antibiotic susceptibility test

According to the table 3, the bacterial strains isolates were multidrug-resistant to 100%. This level of resistance is a consequence of many factors, including misuse of antibiotics; increased severity of the status of hospitalized patients; lack of adherence; too short or sub-therapeutic dose; unconfirmed bacterial infection and improper use of antibiotics [16].

Table 3: Results of Antibiotic Susceptibility tests

bacteria	Antibiotics	Test sensibility
S. aureus 1, 2, 3,4,5	Ampicillin Oxacillin Erytromycin Gentamycin Spiramycin	R
E.coli 1,2,3,4,5	Gentamicin Colistin Amoxicillin	R
Salmonella sp 1,2	Gentamicin Colistin Amoxicillin	R
Clostridium.sp	Colistin Nalidixic Acid Cefazolin Amoxicillin	R

4. Determination of beta-lactamases

The search for beta-lactamase was performed only in *E. coli* and *Staphylococcus aureus* with resistance to

antibiotics are 100%. According to the results, all tested strains from *E.coli* of produce BLSEs. These enzymes are responsible for the multidrug resistance strains overlooked different antibiotics. This result shows the prevalence of the emergence of antibiotic-producing bacteria including BLSEs Enterobacteriaceae [17]. Producing strains BLSEs were often associated with nosocomial outbreaks associated with several risk factors such as ICU admission. The spread of plasmids (plasmid epidemics) and / or other mobile genetic elements is among the main causes for the emergence of BLSEs producing bacteria [18]. Because of the abundance and ubiquity of *E. coli*, several recent studies have mounted it is the species most affected by the emergence of new BLSEs [19]. While, in Staphylococcaceae, the presence of enzyme was achieved through the iodometric test which proved positive for strains with penicillinase phenotype. These enzymes may also acquire resistance acquired through mobile genetic elements (plasmids, transposons or integrons). Penicillin's production strains showed resistances to antibiotics belong to the family of amino pinicilines (AMX example) [20].

5. Antimicrobial activity of Atriplex hamilus extracts by micro dilution

According to the curves, the essential oil extracts from Atriplex *halimus* exhibited the strongest inhibitory effects on all pathogenic strains with MIC= 200 µl /ml (Fig 1a,b,c, d). Exceptionally, *E. coli* 1 isolated from clinical infection which noted a moderate activity with a MIC of 300 µl /ml (Fig 1e). The great resistance of this pathogen strain is due to its specificity and its character responsible for nosocomial infections [21].

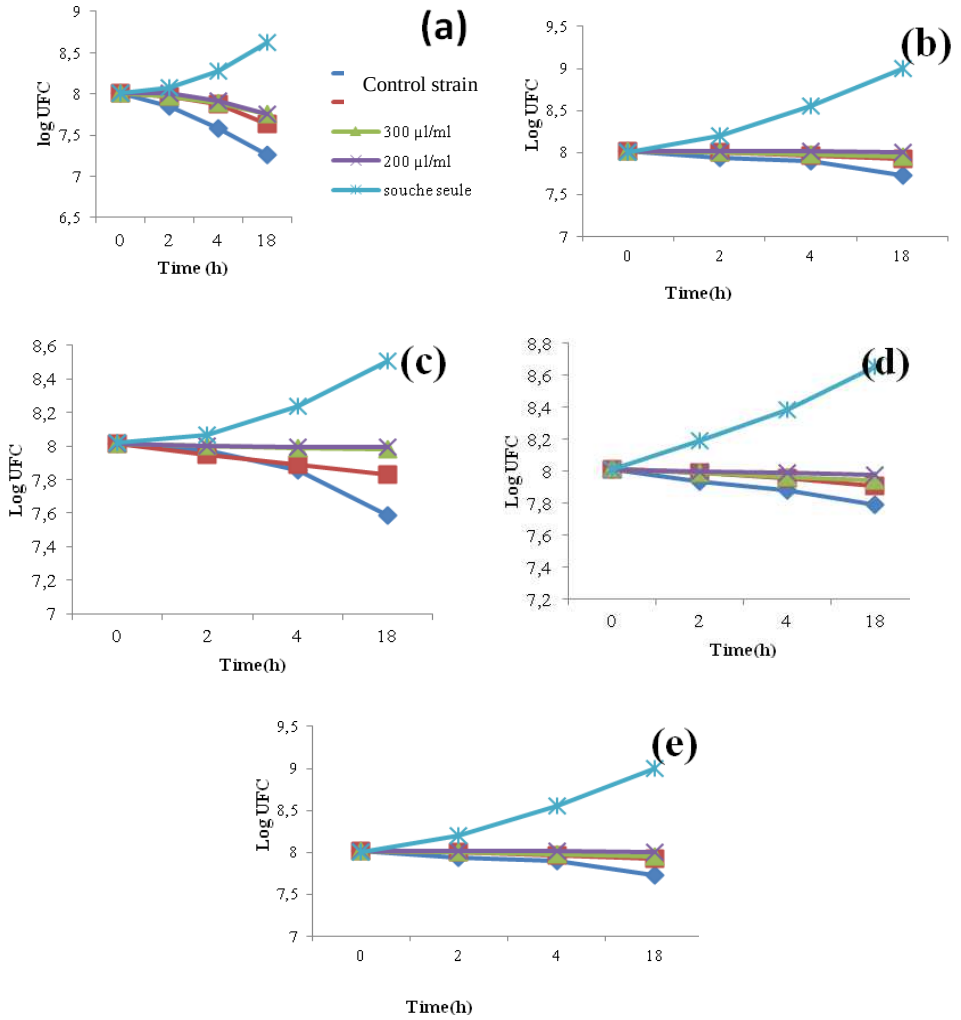

Fig.1. Antibacterial effect of essential oil of Atriplex Hamilus on some strains isolated from clinical infection and food samples. (a) ***Staphylococcus aureus*** **1, (b)** ***Staphylococcus aureus 2*****, (c)** ***Clostridium sp*****, (d)** ***Salmonella sp*** **1, (e)** ***E.coli*** **1**

From this result, it appeared also, all 13 Gram positive and Gram negative pathogens species were sensitive to methanolic and acetate extracts at 300 mg / ml, indicating the very interesting antibacterial potential of this compounds (Fig 2 a,b,c,d,e,f). The strongest inhibitory effects can be due to various chemical substances contained in the three extracts such as several phenolic and non phenolic alcohols, tannins, aldehydes, ketones, alkaloids saponins and terpenoids [**22]**. Further purification and characterization of the actives compounds from the essential oil, methanolic and acetate extracts will provide a good understanding of the antibacterial activity.

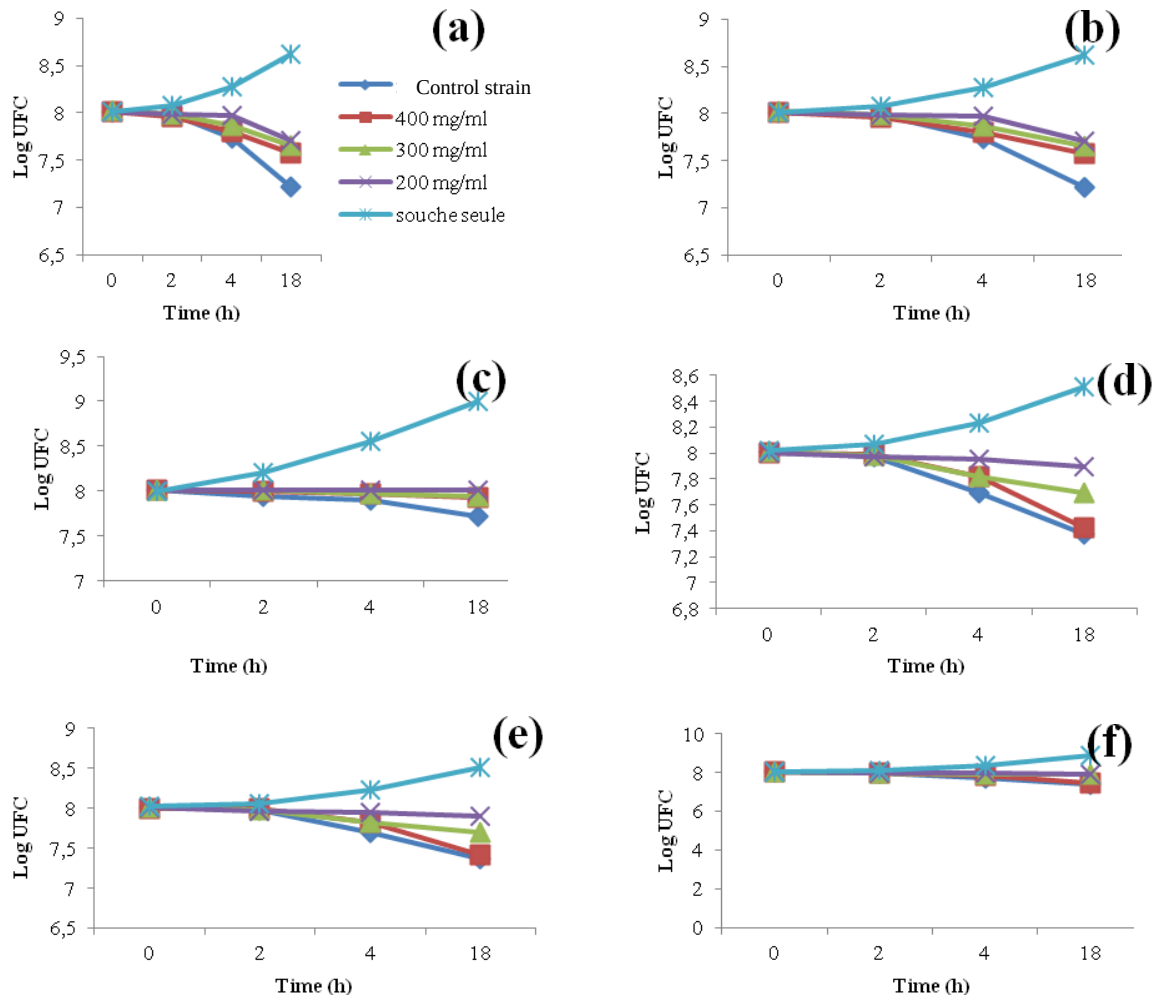

Fig. 2. Antimicrobial effect of methanolic and acetate extract of Atriplex Hamilus on some strains isolated from clinical infection and food samples

(a) ***Staphylococcus aureus*** **1, (b)** ***Staphylococcus aureus*** **3, (c)** ***Clostridium sp*****, (d)** ***E.coli 1*****, (e)** ***E.coli 3*****, (f)** ***Salmonella sp***

CONCLUSION

In conclusion, the emergence of multidrug-resistant bacteria infections is a serious threat to humans and environment worldwide. In this study, samples from Clinical infection and Foods allowed to isolate different strains Gram positive and Gram negative bacteria pathogenic, multidrug resistant and producing beta-lactamases.

The results of the antibacterial activity of different extracts of Atriplex *halimus* demonstrate strongest inhibitory effect against all pathogenic strains producing beta-lactamase. Further physiochemical researches are required to

identify the active molecules responsible for the inhibition effect, and to determine the mechanism of action of the plant extract on the pathogenic strains for further application as an alternative natural therapeutic antimicrobial agent .

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