Effects of Atriplex Halimus on Resistant Bacterial Strain of Different Origins

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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-lactamase (ESBL) extended-spectrum. The implementation of an alternative therapy represents the only solution to combat this health risk. The goal is interested in the mechanisms of action of the active principles of Atriplex halimus. The three extracts have a low yield linked to the characteristics of the plant itself. Antioxidant activity occurred in the three extracts which the methanolic extract has a stronger rate (76%). Initial results show that the three extracts of Atriplx halimus showed an inhibitory effect on the 8 strains tested pathogens and producing Beta-lactamase with zones of inhibition which vary from one strain to another (Staphylococcus aureus (hospital), Clostridium.sp (hospital), Salmonella.sp (hospital), E. coli (hospital), S. aureus (IMM), S. aureus (IVM), Shegilla.sp (IMM), E. coli (raw milk)). The essential oil owned activity against strains tested from a concentration of 200µl/ml while methanolic and aqueous extract showed an inhibitory effect with a concentration varied between 200 or 300 mg/ml according to the tested strain. A synergy between Atriplex halimus extracts and antibiotic test shows a good relationship between the two officers and the majority of the strains tested.

***Keywords*---**Atriplex halimus, bêta-lactamase, antioxidant effect, antibacterial activity.

I. INTRODUCTION

SINCE antibiotics are fundamental tools of modern medicine, the phenomenon of bacterial antibiotic resistance been a strong interest in the scientific community.

The discovery of antibiotics was a medical breakthrough. Already after the clinical introduction of these substances, certain disillusionment settled. Indeed, it has been found that the bacteria can become resistant to antibiotics. In 1944, the year of first use of penicillin, almost all strains of Staphylococcus aureus was sensitive to this new drug. In 1950 against only 30% of clinical isolates of this infectious germ responded to penicillin, this value currently being close to 15% Over time, as the bacteria have developed multiple resistors. Thus, there is known for example to resistant Gram negative 15 active substances. Fortunately, these multiple resistances are limited to a small number of strains, usually local to a single species of bacteria.

Antibiotic resistance of pathogenic bacteria to humans is a risk to public health because it reduces the effectiveness of antibiotics used as first-line and complicates the management of the patient. For subjects receiving antibiotic treatment, it leads to an excess risk of foodborne infections by resistant strains. The development of resistance in bacteria animals can lead to foodborne infections (Salmonella, Campylobacter) or opportunists (E. coli, Enterococcus sp., Staphylococcus aureus) is monitored in the context of an approach global public health.

Atriplex spicies (saltbushes) are dominant in many arid and semi-aride regions of the world, particularly in habitats that combine relatively high soil salinity with aridity (OSMOND and al 1980; Mc ARTHUR and SANDERSON, 1984)

Atriplex Halimus (Mediterranean saltbush) is a perennial shrub of the Mediterranean basin with an excellent tolerance to drought and salinity.

In northern Africa mainly in Algeria's population use it to curing certain diseases and infections.

II. MATERIALS AND METHODS:

A. Plant material

Atriplex halimus grows throughout the Mediterranean region, the

Middle East, North (very common in northern Africa and the Sahara mountains of the central Sahara), and in southern Europe, it is particularly common in areas where the soil is saline (HCDS, 1996).

Atriplex halimus is a perennial wild plant can grow close to the ground or take a living mainly in arid and semi-arid climate shrub (OZENDA., 1983).

This plant is characterized by a high degree of polymorphism. This polymorphism manifest at the morphology of plant structures at the level of structures reproductive. The shape of Atriplex halimus leaves may correspond to other species of the same genus (DUTUIT., 1999).

Fresh Atriplex halimus was collected from the region of Mascara in west Algerian

Extraction of the essential (EO)

The dried plant material 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

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essential oils (LUCCHESI, 2005).

The extraction is a process that takes three hours and produced as a result:

20 grams of cost sent a sufficient amount of distilled water, dried leaves were introduced into a flask (1000 ml 500) placed on a hot plate.

The ball is connected to a condenser at the end of what will be filed to recover an Erlenmeyer distillate; The entire plant, and water is heated to boiling for 2 hours and 30 minutes at 95 ° C.

The cooled distillate is emptied into a funnel with the addition of cyclohexane, and then dry with a spatula tip of MgSO4, cyclohexane was evaporated on a rotary evaporator. The recovered essential oil is transferred to a sterile tube with a Pasteur pipette.

The essential oil is finally sterilized by filtration is kept in a flask protected from light at 4 ° C.

B. Extraction of polyphenols

Methanol and acetone extract

The sample were prepared according to the method described by Escarpa and Gonzalez (1998) 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% 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 dried extracts were stored at -20C until used. The obtained essential oil and polyphenol were sterilized by filtration and stored at 4°C until antibacterial tested against a pathogens.

C. Microbiological strains

All microorganisms were identified by recommendation of bergy's manual methods by culture in specific Medea followed by Gram coloration and biochemical test using automate microbiological system identification (API system). Inoculums for essays were prepared by diluting scraped cell mass in 0.85% NaCl solution, adjusted to McFarland scale 0.5 and confirmed by spectrophotometric reading at 580 nm. Cell suspensions were finally diluted to 10 6 UFC/ml. the microbial isolates were maintained on agar slant at 4°C in the laboratory LRSBG (faculty of S.N.V, University of Mascara) where the antimicrobial tests were performed.

D. Search beta-lactamase

Only one or more resistant strains are selected for antibiotic mark of beta-lactamases.

Search beta-lactamases in Enterobacteriaceae

Phenotypic demonstration of the presence of a β-lactamase extended spectrum is to highlight an image of a disk synergy between third-generation cephalosporin and clavulanic acid.

Apply on Mueller Hinton agar previously seeded with the test strain, a disc of ceftazidime, aztreonam or cefotaxime and amoxicillin + clavulanic acid disk (AMC), a 1 ½ cm.

a) Search beta-lactamases in Staphylococcus

Iodometric test (Courvalin et al., 1985)

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 β-lactamase.

- Preparation of gel by heating to boiling, 0.3 g of starch and 0.8 g agarose is dissolved in 50 ml of 0.5 M phosphate buffer pH 7; The solution was cooled in a water bath at a temperature of 55 ° C is then added with 1.2 ml of iodine-iodide and 30 mg of penicillin G solution; all is mixed and poured into Petri dishes and allowed to solidify; wells 4 to 5 mm in diameter are formed and separated by about 3 cm. The preparation of bacterial suspension from a culture 18 hours a box of strain to be studied, 4-5 colonies in 0.5 ml of sterile saline and vortex.

Fill wells gel 20 .mu.l bacterial suspension prepared extemporaneously.

The enzyme activity results in a discolored area that appears around the wells.

Antimicrobial activity assay

b) Antimicrobial activity was determined by the agar disc diffusion assay (NCCLS 2005).

The extracts were dissolved in dimethyl sulfoxide (DMSO) or distilled water.

Petri plates were prepared with 20ml of sterile Muller Hinton agar (Sigma, Paris, France) surface inoculate by suspension of cell (200µl) adjusted by McFarland 0.5 method (10 6).

The test cultures were swabbed on the top of the solidified media and allowed to dry for 10 min. the tests were conducted at different concentrations of the plant extract in sterile filter paper discs were placed on the surface of the medium and left for 30min at room temperature for compound diffusion. The inhibition zones produced by the plant extract were compared with the inhibition zones produced by commercial standard antibiotics : Ampicilline, Gentamicine, Aztréonam Colistine, Tetracycline, Acide nalidixique, Chloramphenicol, Amoxicilline Erythromycine, Pénicilline G, Oxacilline , Spriramycine.the zone of inhibition (mm) was measured from the boundary of the disc after cultivation at 37° for 48 hs.

c) Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) by micro dilution

MIC test were carried out according to Ell of using Muller Hinton Broth on a tissue culture test plate (96-well).briefly, 100 ml of each concentration from the substance (compounds or essential oils) was added in the first well, and serial dilutions were performed (1/2) and at the last 100ml of cell suspension was added to all wells. Hydro methanol extract was dissolved in DMSO (2%) , it used as negative control. The plate were agitated and incubated at 37°C for 48h.

The lowest concentration showing no culture was considered as the MIC and its express as (µg/ml, or ml/ml). for the MBC determination, 100ml of liquid from each well

that showed no change culture was plated on GNA and incubated at 37° C for 24h .the lowest concentration that yielded no growth after this sub-culturing was taken as the MBC.

Mechanisms of action of extracts Atripex halimus on pathogenic strains

Lyse strains after contact with extracts of Atriplex halimus

To assess the release of the cell contents at 260 nm, the washed bacterial suspension was treated with inhibitory concentration of extracts (essential oil, polyphenol compound).

Technique (K.S Rosenthal et al., 1976).

1 Wash the strains tested

The bacterial culture obtained after 24 hours of incubation was centrifuged at 400g for 25 minutes, the supernatant is removed and the bacterial pellet is resuspended in a volume of sterile PBS phosphate buffer (Appendix) equal to the initial volume of bacterial culture. The suspension thus obtained is called washed suspension.

2 Attaching the ET on the tested strains

To check the adsorption of essential oils and their constituents on the strains studied, the washed suspensions were treated for 20 minutes with a concentration of ET subinhibitrice Atriplex halimus, then centrifuged at 12,000 g for 2 minutes.

The absorbance of the supernatant at 280 nm was measured in comparison with suspensions of EO or polyphenolic extracts centrifuged in the same manner without preliminary contact with the bacteria.

Method of synergy between the antibiotic and Atriplex halimus extract (FIC method) (Remmal A. et al. 1993)

In order to seek a possible synergy (plant extract + ATB), combinations between the plant extract (HE, EM, EA) e antibiotics (amoxicillin, penicillin) were tested for calculating the FIC index (Fractional Inhibitory Concentration).

Treatment in solid medium (pre-test)

The Petri dish was previously seeded area with a fresh suspension of the test strain (24 hour culture) and then proceeds to the development of cavities in a sterile cookie cutter in the agar cast and solidified in box . These cavities with a volume of 100 .mu.l of polyphenol extracts or HE and ATB (inhibitory concentration), which will diffuse into the agar and incubation is carried out after the observation of inhibition diameters were filled.

b) Micro method

Preparing extracts of different concentrations (A) and the antibiotic (B); the first column and the last row of the plate are provided for MIC determination of each antibacterial agent alone;

- Distribute horizontally 50 ml of each concentration of A;
- Distribute vertically 50 ml of each concentration of B;
-Add 90 .mu.l nutrient broth.

Preparation of bacterial inoculum

-Prepare From a pure culture of bacteria in the exponential phase, a suspension of the strain to be studied in 5-10 ml of sterile saline (or BN), a density equivalent to 0.5 MF (108 CFU / ml);

- Diluer Suspension opacity 0.5 MF 10-1;
- Inoculate the wells of the microtiter plate with 10 .mu.l bacterial suspension to obtain a final concentration of 5.105 CFU / ml;
- Incubate at 37 ° C.

-After 18 h incubation in each row note the first tube, comprising A + B, no visible growth;

-Determine, Each well in the FIC fractional inhibitory concentration of A and B or A and FIC FIC B;

-The Value of the association is quantified by the FIC index;

FIC A: is the ratio of the MIC of A and B on the MIC of A alone;

FIC B is the ratio of the MIC of B with A on the MIC of B alone;

FIC = FIC A + FIC B = (MIC of A with B / MIC of A alone) + (MIC B with A / MIC B alone)

If the index is less than 0.75 FIC, the combination is synergistic,

It is additive if it is equivalent to 1,

Results

I. Extraction of the active ingredients of Atriplex halimus

I.1. Yield, organoleptic properties and pH extracts of Atriplex halimus

1 essential oil

The essential oil of Atriplex halimus obtained from the aerial part is yellow, liquid appearance with a strong odor resulting from the salinity of the plant. Atriplex halimus has a low yield of 0.06%, which requires a large amount of hydroditillat for a considerable volume. The low yield may be due to various factors such as the characteristics of the plant as it grows in saline soils (LE HOUEROU., 1992) which may affect the growth and yield of the plant (-Paul Jean Legros, 2007). 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 (Longstreth and Nobel, (1979)). Salinity also reduces the intercellular space in the sheets (Parida and Das, 2005). 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 (CHIKHI ILYAS, 2013). These differences may be due to factors related to the ecosystem (climate, soil type, rainfall, etc.), the time of harvest and drying time. The essential oil of A. halimus has a neutral pH of 7.10 see.

2 Methanol extract

The result of this experiment allowed the production of two different extracts its appearance and color. The methanol extract is dark green with a solid look after evaporation.

E.M a yield of 0.084%. A study was made by

BELYAGOUBI .N (2011) 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. The methanol extract has a pH of 6.63.

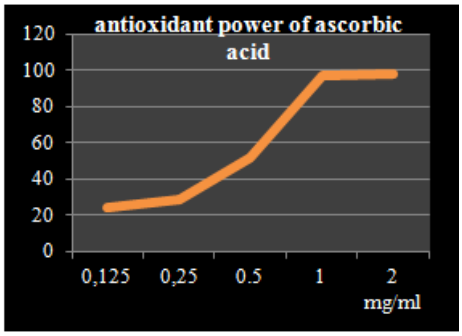
3 aqueous extract

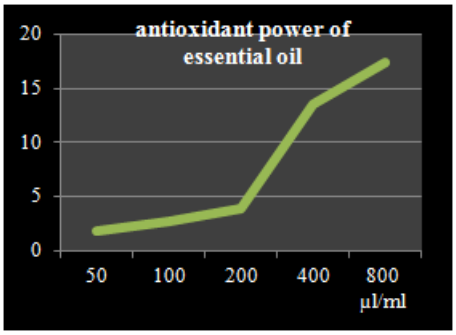
The aqueous extract obtained is brown with a powdery appearance after evaporating water (CHIKHI ILYAS, 2013). E.A a yield 0.052% and a pH of 8.07.

Calculation of IC 50

As based on the curves representing the percentage of antioxidant extracts power concentrations depending on the concentrations of 50% inhibition of DPPH as are subsequently

Extrait	E.A	E.M	H.E
IC50	5,8 mg/ml	1,25 mg/ml	2,3g/ml





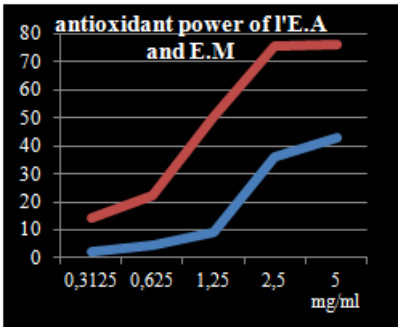


Fig.1 Representation of the antioxidant effect of different extracts of Atriplex halimus.

III. MICROBIOLOGICAL ANALYSIS

IV.1. Isolation

From the hospital

Analysis of original hospital levies can isolate 4 strains pathogenic (2 GRAM negative bacilli belonging to the family Enterobacteriaceae, 1 GRAM positive hull belongs to Staphylococaceae and another positive bacillus belongs to GRAM family Clostridiaceae).

From Animal origin

We selected four strains namely; Shégilla.sp two MRSA, E.coli .

IV.2. Microbiological and biochemical identification of isolates

Macroscopic observation of colonies on selective media and a microscopic observation which give the starting point for the identification was confirmed by a biochemical study.

IV.3. Susceptibility pattern of strains to antibiotics

All strains isolated and identified are resistant to multiple antibiotics tested (CASFM, 2012).

bacteria	Antibiotics	(mm)	R/I/S
Staphylococcus aureus	Pénicilline G	0	R
	Oxacilline	0	
	Gentamycine	0	
	Spiramycin	0	
E.coli	Gentamicine	0	R
	Colistine	9	
	Amoxicilline	0	
	Nalidixic Acid	0	
Salmonella sp	Gentamicine	10	R
	Colistine	09	
	Amoxicilline	0	
	Nalidixic Acid	0	
Clostridium.sp	Colistine	10	R
	Spiramycin	0	
	Céfazolin	0	
	Amoxicilline	0	

According to the table above, the four bacterial 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 (Rybak. MJ, 2004)

IV.4. Determination of beta-lactamases

The search for beta - lactamase was performed only for strains with resistance to antibiotics are 100%. In this test, it was verified for the production of ESBLs and penicillinases pathogenic strains in 8 (4 strains isolated and identified and 4 strains previously identified and sensitive to antibiotics).

1 Synthesis of beta-lactamases in Enterobacteriaceae:

According to the results, all tested strains of producing 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 (Philippon et al., 2006). 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 (Canton R et al., 2008 Pear IL et al., 2008).

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 (Rabaud C, 2010).

TABLE V
DIAMETER OF INHIBITION OF EACH EXTRACT STEM TESTED AREAS

H.E (µl/ml)	Diameters of the inhibition zones (mm)																							
	SA.IMM			SA.IVM			S.A			E.C			E.C (lait)			SALM			CLOST			SH		
	600	550	500	600	550	500	600	550	500	600	550	500	600	550	500	600	550	500	600	550	500	600	550	500
	10	6	-	**10**	-	-	**10**	6	-	**10**	8	7	**10**	6	-	**9**	-	-	**11**	7	-	**10**	6	5
E.M (mg/ml)	**9**	6	-	**10**	5	-	**9**	6	-	6	-	-	**8**	6	-	**9**	6	-	7	6	-	**9**	6	-
E.A(mg/ml)	6	-	-	6	-	-	6	5	-	6	-	-	**8**	-	-	7	6	5	7	6	-	7	6	-

2 Production pinicillinases in Staphylococcaceae:

The detection of 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) (W Sougakoff, Trystram D, 2003).

IV. STUDY OF THE ANTIMICROBIAL ACTIVITY OF ATRIPLEX HALIMUS

Dealing with bacterial antibiotic resistance, we focused our work towards an alternative evaluation of bacteriostatic and bactericidal different extracts of Atriplex halimus activity on pathogenic and producing bacteria BLSEs.

V.1. Diffusion method

This study has led us to detect the antibacterial activity of different extract it Atriplex halimus on pathogenic bacteria, producing multiple resistance to antibiotics and the BLSEs.

According to our results, the essential oil has antibacterial activity vis-à-vis the strains tested. It is noted that a concentration of 600 .mu.l / ml induced an effect on Clostridiums with a diameter of 11 mm and SA.IMM, SA.IVM, SA, E.coli, E.coli and dairy sources Shegilla .sp with an inhibition of 10 mm area. A concentration of 550 .mu.l / ml inhibited the growth of E. coli with a diameter of 8 mm.

This result is confirmed by CHIKHI ILYAS (2013) it was found that the essential oil Atriplex halimus has an effect on some bacteria including E. coli. It should be noted that the

antimicrobial activity of an essential oil is due mainly to its chemical composition, and in particular, the nature of its major constituents (Lawrence, 1993; Dorman and Deans, 2000). It can be attributed also to one or more molecules present (s) in low (s) share (s) in essential oils (Belaiche et al., 1995; Tzakou et al, 2007).

For the methanol extract, a well estimated effect appeared on various pathogenic strains. A diameter of 10 mm is measured using the effect of a concentration of 600 mg / ml while on the same SA.IVM concentration induced affect SA.IMM SA, Salmonella.sp, with Shegilla.sp inhibition zone reached 9 mm. An inhibitory effect on E. coli is applied to milk origin with a diameter of 8 mm.

A study was conducted by Tarek A Elbashiti and these

collaborators (2011) which shows that the extract of A. halimus has no effect on E. coli and S. aureus.

For the aqueous extract, it has felt a little effect since the activity is induced only on E. coli of dairy origin with a diameter of 8 mm, the concentration reaches 600 mg / ml. This effect may be due to the presence of terpenoids, saponins, alkaloids, flavonoids and tannins that have a wide range of biological activity, and are probably the source of the medicinal virtues of A. halimus.

According to Tarek A Elbashiti et al. (2011), the aqueous extract of Atriplex halimus of Palestinian origin (Gaza Strip Champs Alzawaida) has auqun effect on E. coli and S. aureus. This result shows the effectiveness of our plant because it has additive properties that have this beneficial effect on pathogenic bacteria.

The high salinity can also be in a second biological factor involved in the inhibition of the strains tested with equal percentage of Na + to 12%.

V.2. The method of micro dilution

Achieving micro dilution method gives us information about the inhibitory effect of extracts of Atriplex halimus compared to the diffusion method. According to our results, the bacterial growth is reduced as a function of time in presence of different extracts (essential oil, and the aqueous methanol extract) compared to the control which proves their inhibitory effect vis-à-vis the strains tested.

According to the curves, wholes extracts (HE, EM, EA) Atriplex halimus showed inhibitory activity on all pathogenic strains with an MIC of 200 .mu.l / ml for the essential oil and an MIC of 200 mg / ml on the aqueous extract and methanol. Exceptionally, only the E. coli strain hospital-was inhibited by an MIC of 300 mg / ml of the aqueous extract due to the great resistance of this strain pathogen responsible for nosocomial infections.

V.3. Lyse strains after contact with extracts of Atriplex halimus

In order to test the adsorption of essential oils and their constituents on bacterial isolates, the test for which fastening principle measuring the absorbance of the extracts or components thereof in a concentration subinhibitris before and after brief contact with the bacteria was achieved.

Dissemination method

In order to improve the efficiency and the antibacterial effect of antibiotics on pathogenic and producing bacteria BLSEs, we conducted a test of combination of extracts of Atriplex halimus and antibiotics tested in the antibiogram. According to primary results shows improvement in the inhibition zones, one can deduce that there is a synergy between extracts and antibiotics.

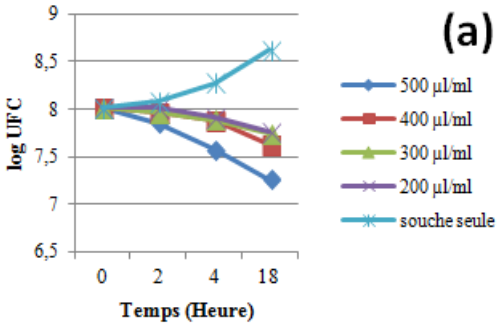


Fig. 3 Growth curves of S.aureus with essential oil

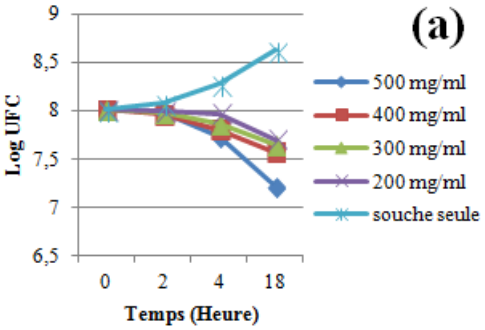


Fig. 3 Growth curves of S.aureus with methanol extract

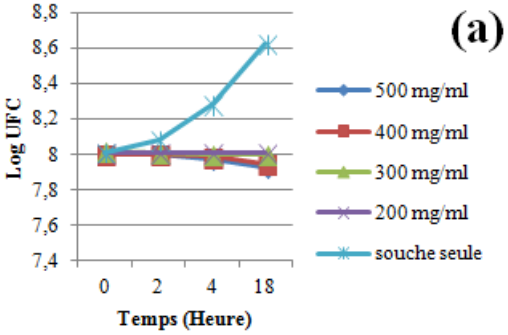


Fig. 3 Growth curves of S.aureus with aqueous extract

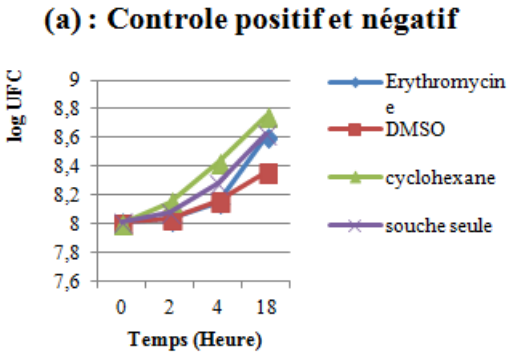


Fig. 3 Positive and negative control microdilution.

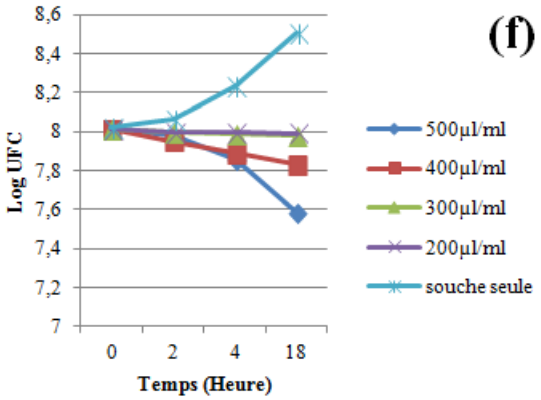


Fig. 3 Growth curves of E coli with essential oil

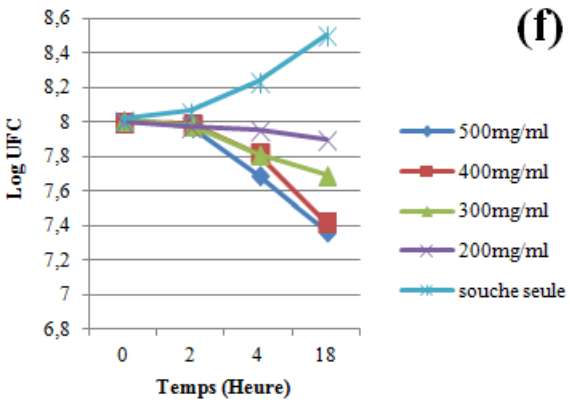


Fig. 3 Growth curves of E coli with methanol extract

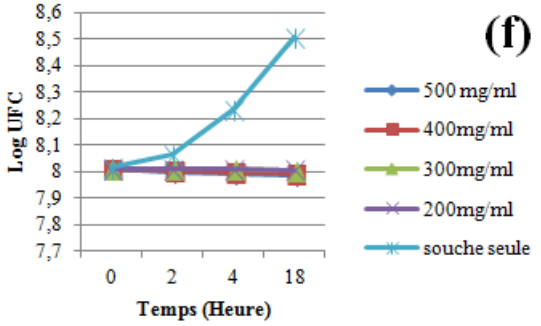


Fig. 3 Growth curves of E coli with aqueous extract

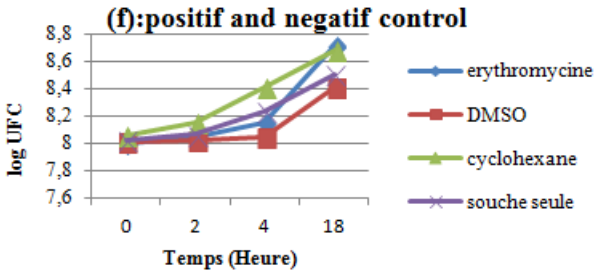


Fig. 3 Positive and negative control microdilution.

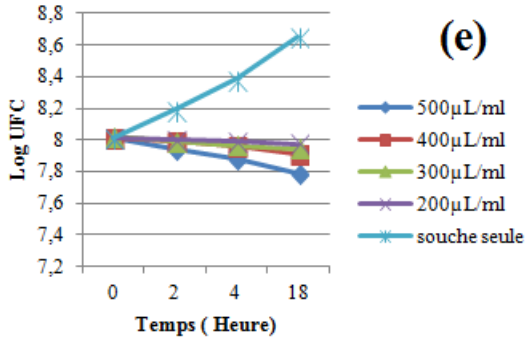


Fig. 3 Growth curves of Clostridium.sp with essential oil

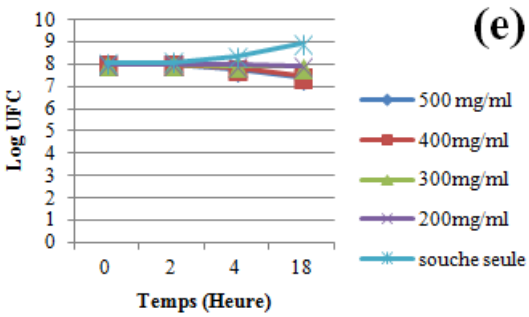


Fig. 3 Growth curves of Clostridium.sp with methanol extract

V. Conclusion

Today, the emergence of multidrug-resistant bacteria and producing beta-lactamases because a major health problem worldwide broadcasts.

These enzymes are the main cause in the majority of health disturbances from the ineffectiveness of antibiotics including beta-lactams.

Over the past decade, extensive research has been conducted on the antioxidant and antimicrobial effects of extracts of medicinal plants which are a potential source of biomolecules.

Algeria is its geographical location is home to a unique biodiversity occupied by a large wealth of Medicinal and Aromatic Plants "PAM". Many WFP are used in traditional medicines and not scientifically evaluated to date. From the perspective of valuation extracts A. halimus, we became interested in studying their antioxidant and antimicrobial effects. Isolation and identification from clinical specimens allowed us to obtain different strains producing beta-lactamases and penicillinases

The extracts of A. halimus have a very important antioxidant capacity compared to the reference (ascorbic acid) methanol extract of which has the highest percentage in this activity. This power is probably due to the high salinity that contains the plant.

The study of the antibacterial activity of different extracts of the plant allowed us to demonstrate the presence of extracts from this inhibitory effect against various plant pathogenic strains producing beta-lactamase positive and negative GRAM whose aqueous extract has a small effect compared .to the methanol extract and essential oil.

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