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In vitro evaluation of the antibacterial activity of *Atriplex halimus*

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Dedication

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Table of contents

Acknowledgement

Dedication

List of Tables

List of Figures

List of abbreviation

ملخص

Résumé

Abstract

Introduction

Part I: Literature Review

Chaptre 1*: *Presentation of the studied plant

1/ Presentation of the plant 5

2/ Taxonomy 6

3/ Nomenclature 7

4/ Geographical distribution of the species *Atriplex halimus* 7

In the world 7

In Algeria 7

5/ The chemical composition of *Atriplex halimus* 7

6/ Interest of the plant 8

Forage interest 8

Ecological and agronomic interest 8

Medical interest 9

7/ Traditional and medicine uses of the plant 9

Chapter 2: Secondary Metabolits

1/ Origin of secondary metabolites 12

2/ Classification, chemical and biological properties of secondary metabolites 12

a) Phenolic compounds 12

Part II: Experimental Part

Materials and Methods

1- Study Location 19

2- Type of Study. 19

3- Study Period 19

I- Materials 20
1/ Plant Material 20
2/ The bacterial strains tested 21
2.1. The culture media used 22
II- Methods 22
1/ Treatment of the Studied Plant 22
2/ *Atriplex halimus* plant hydroethanolic extraction 22
2.1. Hydroethanolic extract preparation 22
2.2. Maceration 23
2.3. Filtration 23
2.4. Evaporation 24
2.5. Determination of the Yield of the Hydroethanolic Extract 24
3/ Phytochemical Screening 25
3.1. Flavonoid Detection 25
3.2. Saponoside Detection 25
3.3. Tannin Detection 25
3.4. Quinone Detection 26
3.5. Alkaloid Detection 26
3.6. Terpene and Steroid Detection 26
4.Microbiological Revivification and Purity Control 27
4.1. Revivification and Subculturing of Tested Strains 27
4.2. Purity Control of Strains 27
5. Antibacterial Activity 28
5.1. Disc Diffusion Method 28
5.2. Preparation of the Stock Suspension 28
5.3. Preparation of the inoculum 28
5.4. Preparation of the discs 29
5.6. Inoculation 29
5.7. Preliminary Test 30
5.8. Quantitative study of the antibacterial activity of the hydroethanolic extract of *Atriplex halimus* 30
6/ Reading of results 31

7. Determination of the MIC of the hydroethanolic extract of Atriplex halimus using the microplate method 32

8. Determination of the Minimum Bactericidal Concentration (MBC) 32

Results and discussion

1/ Determination of the yield of the hydroethanolic extract of *Atriplex halimus* 34

2. Phytochemical Screening Results of *Atriplex halimus* 34

3.Strain purity control 37

4. Evaluation of the antibacterial activity of the pure and diluted hydroethanolic extract of *Atriplex halimus* 38

Conclusion and perspectives

References 45

Annexes 54

List of Tables

Table No.	Title	Page
01	Characteristics of the plant *Atriplex halimus*	7
02	Chemical compounds of the plant *Atriplex halimus*	9
03	Geographical coordinates of the plant collection area	21
04	Different bacterial strains tested	22
05	Culture media used	23
06	Diameter and Degree of Bacterial Sensitivity	32
07	Yield values of the hydroethanolic extraction of *Atriplex halimus*	35
08	Results of phytochemical screening of *Atriplex halimus*	36
09	Purity control of the tested bacterial strains	38
10	Evaluation of the antibacterial activity of pure and diluted hydroethanolic extract	39
11	The MBC/MIC ratio of the three tested strains	42
12	Laboratory Equipment, Materials, and Reagents Used	57
13	Composition of Reagents Used for Phytochemical Screening	58
14	Steps of Gram Staining	60
15	Preparation of Culture Media and Physiological Water	61

List of Figures

Figure No.	Title	Page
01	*Atriplex halimus*	06
02	Leaves of *Atriplex halimus*	06
03	*Atriplex halimus*	21
04	Location of the collection site of the plant *Atriplex halimus*	22
05	Maceration of the hydroethanolic extract of *Atriplex halimus*	24
06	Filtration of the macerate of *Atriplex halimus*	24
07	Evaporation of the hydroethanolic extract of *Atriplex halimus*	25
08	Revivification and subculturing of tested strains	28
09	Preparation of the mother suspension	29
10	Inoculation of bacterial strains in MH medium	30
11	Deposition and impregnation of discs	31
12	Measuring the diameters of the inhibition halos using a caliper	32
13	Experimental distribution of the microplate for MIC determination	41
14	The Rotavapor	59
15	Results of the Antibacterial Activity of the Hydroethanolic Extract	62

List of abbreviation

A. halimus: *Atriplex halimus*

S. saprophyticus: *Staphylococcus saprophyticus*

S. pneumoniae: *Streptococcus pneumoniae*

K. pneumoniae: *Klebsiella pneumoniae*

C. freundii: *Citrobacter freundii*

MIC: Minimum Inhibitory Concentration

MBC: Minimum Bacterial Concentration

ATCC: American Type Culture Collection

NB: Nutrient Broth

NA: Nutrient Agar

MHB: Mueller Hinton Broth

MH: Mueller Hinton (Agar)

DMSO: Dimethyl Sulfoxide

CFU: Colony Forming Unit

UV: Ultraviolet (light)

HCl: Hydrochloric Acid

NaOH: Sodium Hydroxide

$FeCl_3$: Ferric Chloride

H_2SO_4: Sulfuric Acid

Y%: Yield Percentage

Me: Mass of the dry extract

Ms: Mass of the sample

v/v: Volume per volume

G+: *Gram Positive*

G−: *Gram Negative*

ID: *Inhibition Diameter*

PE: *Petroleum Ether*

mg: milligram

ml : milliliter

mm : millimter

µL : microliter

min : minute

sec : second

ملخص

Atriplex halimus (الأسماء الشائعة: القطاف، الرغل، السُوَيْق البحري) هو شجيرة من فصيلة القطيفية، موطنها الأصلي شمال إفريقيا (المغرب، الجزائر، تونس، ليبيا، مصر). وهو نبات كثيف ذو أوراق فضية، تم تأقلمه حول حوض البحر الأبيض المتوسط، ثم تم إدخاله بنجاح على طول الساحل الأطلسي. هذا النبات منتشر جداً في الجزائر ويتميز بقدرته اللافتة على تحمل الظروف البيئية القاسية، لا سيما الملوحة العالية للتربة والجفاف. يهدف هذا البحث إلى إجراء تحليل فيتوكيميائي للمستخلص الهيدروإيثانولي للجزء الهوائي من هذا النبات، وتقييم إمكاناته المضادة للبكتيريا في المختبر. وقد تم تقدير مردود المستخلص الهيدروإيثانولي بنسبة 28%. في الواقع، مكّنت اختبارات التوصيف القائمة على التفاعلات الفيزيائية والكيميائية من إجراء تحرٍ فيتوكيميائي، كشف عن وجود الفلافونويدات، السابونينات، التانينات الكاتشينية، التربينات والستيرويدات، وعدم وجود الكينونات، القلويدات، وتانينات الغاليك. أظهر نبات أتركبلكس هاليموس فعالية مضادة للبكتيريا ضد نمو بكتيريا *Staphylococcus saprophyticus*، *Streptococcus pneumoniae*، *Klebsiella pneumoniae* و *Citrobacter freundii*.وهكذا، تراوحت أقطار مناطق التثبيط، المقاسة بطريقة الانتشار في الوسط الصلب (الأروماتوغرام)، بين 10.46 ملم و12.95 ملم، وتراوحت تراكيز التثبيط الدنيا (MIC) بين 12.50 و50 ملغ/مل. علاوة على ذلك، أشار معامل MBC/MIC إلى تأثير قاتل للبكتيريا على السلالات البكتيرية المختبرة. تظهر نتائج هذا البحث أن المستخلص الهيدروإيثانولي ل *Atriplex halimus* يحتوي على مركبات كيميائية متنوعة ويظهر نشاطاً قوياً مضاداً للبكتيريا، خصوصاً ضد البكتيريا إيجابية الغرام.

الكلمات المفتاحية : *Atriplex halimus* ، المستخلص الهيدروإيثانولي، التحري الفيتوكيميائي، النشاط المضاد للبكتيريا، التركيز المثبط الأدنى(CMI) ، التركيز القاتل الأدنى(CMB).

Résumé

Atriplex halimus (noms communs : Guettaf, orache, sea purslane) est un arbrisseau de la famille des Chénopodiacées, originaire d'Afrique du Nord (Maroc, Algérie, Tunisie, Libye, Égypte). Plante buissonnante à feuillage argenté, elle a été acclimaté autour du bassin méditerranéen, puis introduit avec succès sur le littoral atlantique. Cette plante est très répandue en Algérie et se distingue par sa capacité remarquable à tolérer des conditions environnementales extrêmes, notamment la salinité élevée des sols et la sécheresse. L'objectif de cette étude est d'effectuer une analyse phytochimique de l'extrait hydroéthanolique de la partie aérienne de cette plante, et d'évaluer in vitro son potentiel antibactérien. Le rendement de l'extrait hydroéthanolique a été estimé à 28 %. En effet, des tests de caractérisation basés sur des réactions physico-chimiques ont permis un criblage phytochimique, révélant la présence de flavonoïdes, saponosides, tanins catéchiques, terpènes et stéroïdes, et l'absence de quinones, alcaloïdes et tanins galliques. Atriplex halimus a montré un potentiel antibactérien contre la croissance de *Staphylococcus saprophyticus* et *Streptococcus pneumoniae,* et une moindre capacité d'inhibition sur *Citrobacter freundii* et *Klebsiella pneumoniae*. Ainsi, les diamètres des zones d'inhibition, mesurés par la méthode de diffusion en milieu solide (aromatogramme), variaient entre 10,46 mm et 12,95 mm et les concentrations minimales inhibitrices (CMI) étaient comprises entre 12,50 et 50 mg/ml. De plus, le rapport CMB/CMI indiquait un effet bactéricide sur les souches bactériennes testées. Les résultats de cette recherche montrent que l'extrait hydroéthanolique *d'Atriplex halimus* contient divers composés chimiques et présente une forte activité antibactérienne, en particulier contre les bactéries Gram positives.

Mots clés : *Atriplex halimus*, extrait hydroéthanolique, screening phytochimique, activité antibactérienne, CMI, CMB.

Abstract

Atriplex halimus (common names: Guettaf, orache, sea purslane) is a shrub from the Chenopodiaceae family, native to North Africa (Morocco, Algeria, Tunisia, Libya, Egypt). A bushy plant with silvery foliage, it has been acclimatized around the Mediterranean basin, then successfully introduced along the Atlantic coast. This plant is very widespread in Algeria and is distinguished by its remarkable ability to tolerate extreme environmental conditions, notably high soil salinity and drought. The objective of this study is to conduct a phytochemical analysis of the hydroethanolic extract of the aerial part of this plant, and to evaluate its antibacterial potential in vitro. The yield of the hydroethanolic extract was estimated at 28%. Indeed, characterization tests based on physicochemical reactions enabled a phytochemical screening, revealing the presence of flavonoids, saponosides, catechin tannins, terpenes and steroids, and the absence of quinones, alkaloids, and gallic tannins. *Atriplex halimus* showed antibacterial potential against the growth of *Staphylococcus saprophyticus* and *Streptococcus pneumoniae*, and a lower inhibitory capacity on *Citrobacter freundii* and *Klebsiella pneumoniae*. Thus, the diameters of the inhibition zones, measured by the solid medium diffusion method (aromatogram), varied between 10.46 mm and 12.95 mm, and the minimum inhibitory concentrations (MICs) ranged between 12.50 and 50 mg/ml. Moreover, the MBC/MIC ratio indicated a bactericidal effect on the tested bacterial strains. The results of this research show that the hydroethanolic extract of *Atriplex halimus* contains various chemical compounds and exhibits strong antibacterial activity, particularly against Gram-positive bacteria.

Keywords: *Atriplex halimus*, hydroethanolic extract, phytochemical screening, antibacterial activity, MIC, MBC.

Introduction

Introduction

At the end of the 19th and beginning of the 20th century, numerous scientific investigations began to highlight the therapeutic properties of plant extracts. Since then, the use of plants has developed to become, for more than twenty years, a serious alternative to antibiotic medicine in infectious diseases. Indeed, many bacteria have become resistant to broad-spectrum antibiotics in the pharmaceutical arsenal. It is therefore necessary to look for another opportunity that will take over in anti- infectious treatments **(Chebaibi *et al*., 2015).**

Algeria is known for its abundant plant biodiversity. In particular, halophytes are plants that have been naturally selected in saline environments. Among the halophytes extensively used in physiological and molecular biological investigations is *Atriplex halimus*, which is among the medicinal plants that play a decisive role in traditional medicine in Algeria **(Hassaine *et al*., 2014)**.

Atriplex halimus is a perennial, halophytic, and nitrophilous shrub. Its variety of common names reflects its wide geographical distribution. It grows under semi-arid and arid conditions throughout Eurasia: from the Atlantic coasts, through the Mediterranean basin countries, and into the Middle East. This species is notable for its remarkable ability to tolerate extreme environmental conditions, particularly high soil salinity and drought. It has been planted for physical stabilization of the soil in the Mediterranean basin and beyond. In addition, it has significant agronomic and fodder value, as it is used as a feed supplement for livestock, especially during periods of forage shortage. Rich in protein and resistant to water stress, it is also being studied for its potential medicinal and nutritional applications. **(Walker & Lutts, 2014)**.

Historically, it has been sought for its medicinal properties, addressing a range of health issues from inflammation and hormonal imbalances to cardiovascular ailments. Emerging studies endorse its potential, highlighting its antioxidant, anticancer, antibacterial, and antidiabetic activities **(Mohammed *et al*., 2023)**.

This plant is widely used to treat digestive disorders, burns, flu-like coughs, bronchitis, and tumors, among others. The chemical composition of this plant, in terms of mineral salts and nitrogenous compounds, has already been determined in previous studies **(Guettouche, 2021)**.

In this work, we investigated the effects of the hydroethanolic extract of the Algerian aromatic plant *Atriplex halimus*.

The first part of our dissertation includes a literature review divided into two chapters.
The first chapter discusses the general botanical description, classification, chemical composition, and therapeutic benefits of the plant studied.
The second chapter discusses secondary metabolites. The experimental part presents the material and methods used for hydroethanolic extraction, phytochemical screening, the antibacterial activity, and the results obtained, as well as their discussion and finally a conclusion and perspectives.

Part I:

Literature Review

Chaptre 1:

Presentation of the studied plant

«*Atriplex halimus*»

1/ Presentation of the plant

Atriplex halimus (common names: guettaf, orache, sea purslane) is a forage species widespread throughout the Mediterranean region.

It is a shrub 1 to 3 meters tall, very branched, the leaves are alternate, briefly but distinctly petiolate, forming clumps that can reach 1 to 3 meters in diameter. more or less fleshy, glossy, covered with whitish vesicular hairs (trichomes), oval, entirely or slightly sinuate, 0.5 to 1 cm wide and 2 to 4 cm long. The flowering and fruiting period takes place from May to December. The floral architecture can be categorized into two fundamental types: one consisting of The female flowers with a single carpel inserted between two opposite bracts and the other of pentamerous male flowers **(Ouldkadour, 2019)**.

The plants are monoecious and bear inflorescences in terminal racemes, with male flowers at the top and female flowers at the base. The species *A. halimus* has two subspecies *halimus* and *schweinfurthii* **(Nedjimi *et al*., 2013)**.



Figure 01: *Atriplex halimus*.



Figure 02: Leaves of *Atriplex halimus*.

(Korpelainen *et al*., 2011).

Table 01: Characteristics of the plant *Atriplex halimus* **(Fatarna, 2019).**

Characteristics	**Extreme environment**
Deep root system	Drought
Mutual shading of the leaves	High light intensity
C4 Photosynthesis	High temperature, drought
High efficiency in water use	Drought
Salt accumulation	Salinity, drought, cold
Osmotic adjustment	Salinity, cold, drought
Accumulation of organic osmolytes	Drought, cold, salinity
Excess ion deposition in vesicular hairs	Salinity, Trace elements
Salt released by vesicular hairs	High light intensity

2/ Taxonomy

The systematic position of *Atriplex halimus L*. in the plant kingdom according to The Angiosperm Phylogeny Group is classified as follows **(Kadereit *et al*.,)**:

Kingdom: Plant

Sub-kingdom: Tracheobionta

Division: Magnoliophyta

Class: Dicotyledons or Magnoliopsida

Subclass: Caryphyliadae

Order: Caryphyllales

Family: Chenpodiaceae (amaranthaceae)

Genre: *Atriplex*

Species: *Atriplex halimus* (*Atriplex halimus L*).

3/ Nomenclature

Atriplex halimus is a species with different vernacular names:

- **In Arabic:** Guetaf, El gtaf
- **In French** : Pourpier de mer, arroche marine, épinard de mer
- **In English**: Sea orach, Mediterranean saltbush

4/ Geographical distribution of the species *Atriplex halimus*

In the world

Atriplex halimus is widely present in the world, found in temperate, Mediterranean, and subtropical zones, between 20 and 50° North and South latitude. It grows naturally throughout the Mediterranean basin up to Western Asia: including southern Portugal, France, southern and eastern Spain (and the Canary Islands), Italy, Greece, Malta, Turkey, Cyprus, Palestine, Syria, Lebanon, Jordan, Tunisia, Morocco, Algeria, Libya, Egypt, and Saudi Arabia **(Walker *et al.*, 2014).**

In Algeria

Among the medicinal plants that make up the vegetation cover of Algeria is *Atriplex.* It is spontaneous in arid and semi-arid bioclimatic zones and has been recorded in different regions of Algeria, with the largest areas corresponding to steppe zones (Tébessa, Batna, Mssila, Boussaâda, Biskra, Djelfa, Tiaret, Saida), and can be found in the northern regions of the northern Sahara and the mountains of the central Sahara (**Hassaine *et al*., 2014).**

5/ The chemical composition of *Atriplex halimus*

Chemical composition depends on several factors or parameters, such as climate, the age of the plant, and the season. **(Abbade, 2004).** This plant is known for its biological activities due to its various bioactive constituents. This plant material is very rich in proteins, fibers, mineral salts, vitamins A, C, and D, and secondary metabolites such as saponins, alkaloids, and flavonoids, with a low content of crude fats **(Benhammou *et al*., 2009; Nedjimi *et al.,* 2013)**.

The essential oil of this plant contains chemical compounds that were identified by (**Zian *et al.*, 2020)** and these compounds are in table 02:

Table 02: Chemical compounds of the plant *Atriplex halimus* **(Laouedj, 2017)**.

Octane	Camphene	Pelargonaldehyde
3-Furancarboxaldehyde	B-Pinene	P-Menthan-3-one, cis-p-
2-vinyl-5-methylfuran	Myrcene	Thiophene,2-ethynyl Germacrene D
1,1,4Trimethylcycloclohexane	A-Terpinene	Myristicin 9.

6/ Interest of the plant

Forage interest

Atriplex is a valuable forage plant due to its high resistance to drought, salinity, and sunlight, making it suitable for use by sheep, goats, and camelids. Species such as *A. halimus, A. nummularia, and A. canescens* can produce significant amounts of protein-rich forage under conditions of low annual rainfall **(Bouzid & Benabdeli, 2011)**. However, the high sodium chloride content and oxalate levels in *Atriplex* forage can increase water consumption and reduce palatability, which may limit its use in areas where water is scarce **(Lutts, 2004)**.

Ecological and agronomic interest

Species of the genus *Atriplex* are valuable for ecological and agronomic purposes, particularly for the rehabilitation of challenging environments due to their tolerance to high levels of boron and other heavy metals. It has been observed that *Atriplex canescens* and *Atriplex polycarpa* accumulate significant amounts of boron, iron, manganese, and aluminum in mining soils, while some species also store molybdenum and selenium. *Atriplex halimus,* in particular, seems promising for soil stabilization and potential desalination in arid regions, demonstrating its ability to accumulate cadmium and zinc without adverse effects on growth **(Lutts *et al.,* 2004)**, although it is sensitive to copper.

Medical interest

Interest in medicine (**Dey *et al.,* 2002)** found that sand rats (Psammomys obesis) that were denied this plant developed type II diabetes. **(Belhadj, 2016)** also found that the animals were healthy in their desert habitat when fed a low-energy diet consisting of *A. halimus*, but that when fed a more energetic diet in the lab, they developed metabolic abnormalities, including insulin resistance.

The only available information on its use to type II diabetes in humans comes from unpublished studies showing that consuming 3g of *A. halimus* daily lowers blood glucose levels **(Chikhi *et al*., 2014)**.

7/ Traditional and medicine uses of the plant

Plants of *A.halimus* have been used as traditional cures for many conditions for thousands of years.

It is used for traditional treatments for thoracic disorders such as laxatives, the relief of muscle and stomach pain and the regulation of secretions of the gallbladder **(Chikhi *et al.,* 2014)**. It is also used also to treat hypertension, urinary infections, eczema, and insect bites **(Miara *et al*., 2019)**.

The ash from burnt plants is used as an alkali to make soap, while the shoots can be burnt to yield an antacid powder, and arabic indigenous herbal practitioners employ the leaves to treat heart diseases and diabetes (decoction) and rheumatism (an extract prepared with boiling water is added to bath water) **(Calvo *et al*., 2015)**.

The fibre content may be beneficial for the quality of goat-derived dairy products demonstrated that the quality of *A.halimus* forage was related to the proportion of leaves and twigs eaten voluntarily by the animals **(Otal *et al*., 2010)**.

The plant also serves as a purgative and antiparasitic in veterinary medicine **(Ksouri *et al*., 2012; Stevanovic *et al*., 2019)**.

In Algeria, in particular, a decoction of its seeds is traditionally used in the treatment of breast cancer **(Benarba, 2015).** It would also have antitumor properties **(Alves-Silva *et al*., 2017; Bounouar *et al*., 2022).**

Its seeds are sometimes used as an emetic. Moreover, it presents also a traditional interest in the treatment of anemia **(Mohammedi, 2016)**, of thyroid diseases and various cysts **(Khadidja *et al*., 2020)**. Finally, the infusion or the decoction of *Atriplex halimus* leaves is used as an anti-sterility treatment in women, anti-lithiasis, anti-inflammatory, and for skin regeneration in deep burns **(Bounouar *et al*., 2022)**.

Chapter 2:

Secondary Metabolits

Secondary metabolites of aromatic and medicinal plants

1/ Origin of secondary metabolites

Secondary metabolites are components found in some plant species but absent in others, and they are thought to be indicators of how plants have adapted to various ecological conditions. Plants that evolve in an equatorial environment are subject to the same selection pressure, and secondary metabolites play a significant role in the plants' adaptive responses to environmental factors such as temperature, hygrometry, exposure to UV and visible light, oxygen concentration, atmospheric pressure, etc.

Secondary metabolites are just as important as primary metabolites for the survival of the species. Plants and vertebrates use phytoestrogens, chemical phytoestrogens that reduce estrogens and interfere with reproduction **(Alexandra, 2014).**

2/ Classification, chemical and biological properties of secondary metabolites

Plant secondary metabolites are categorized into three types of molecules based on their chemical characteristics: alkaloids, terpenoids, and phenolic chemicals **(Alexandra, 2014).**

a) Phenolic compounds

Phenolic compounds are considered as auxiliary metabolites, Display in all parts of higher plants (roots, stems, blooms, dust, natural products, seeds, and wood) **(Mahmoudi, 2013)**.

Characterized by the nearness of a fragrant ring bearing free hydroxyl bunches or those reinforced with carbohydratesThe most classes of phenolic components are phenolic acids (caffeic corrosive, hydroxycinnamic corrosive, ferulic corrosive, chlorogenic corrosive...), flavonoids, tannins, and coumarins.

Phenolic compounds are organically dynamic atoms and they are broadly utilized remedially as vasoconstrictors, anti-inflammatories, chemical inhibitors, cancer prevention agents, free radical foragers, and antimicrobials and they are included in various physiological forms too such as seed germination, and natural product maturing, cell development, root arrangement **(Belhadj, 2018).**

1. Flavonoids

Flavonoids constitute one of the most bunches of polyphenols, with more than 9000 diverse compounds and for the most part disseminated in all vascular plants **(Hernández, 2009).** The best know role of flavonoids is the production of flower color and bright colors recognized by plant pollinators **(Harborne and Williams, 2000).**

These compounds have colored pigments, yellow to red, giving these organisms their wide range of colors **(Elise, 2019).** The color of the natural products, blossoms, and clears out may be a characteristic of flavonoids **(ElGharras, 2009).**

Their common chemical skeleton has 15 carbon molecules, comprising of two benzene rings A and B associated by a central pyranic cycle C.

They vary from each other by the position of substitutions on the A and B rings, and the nature of C. Flavonoids are divided into diverse categories, the foremost imperative of which are flavonols, flavones, flavanols, isoflavones, flavanones, and anthocyanins.

These particles are found both in free shape, but are exceptionally frequently bound to sugars, in which case they are alluded to as heterosides comprising of phenolic aglycon or genin portion related with sugar.

They are found in different organs: blooms, natural products, stems, and roots. Aglycones are the most parts shown as wax shape in clears out, bark, and buds **(Benzahi, 2017).**

2. Alkaloids

Alkaloids are a broad and structurally varied class of secondary metabolites that are present in bacteria, fungi, mammals, and 300 plant families **(Bruno *et al*., 2020).**

Alkaloids are essential nitrogenous compounds of characteristic root, in which the nitrogen iota is included in a heterocyclic framework and its pharmacological action is critical.

Alkaloids have the property of responding with overwhelming metal salts, which permits for their simple characterization (Mayer's reagent, Dragendorff's reagent, Wasicky's reagent, Bouchardat's reagent).

The transformation of alkaloids found in a selection of food. Others seem to come from the metabolism of the animal and excreted by the exocrine glands and also play a role in communication (pheromones) **(Zakkad, 2016).**

3. Saponins

Throughout the Plant Kingdom, saponins are a significant class of secondary metabolites found in plants. These secondary metabolites often occur in plants as complex mixtures, and saponin content and composition may vary markedly depending on the genetic background of the plant material, the tissue type, the age and physiological state of the plant and environmental factors. Chemically, saponins are a class of chemicals that include triterpenoids, steroid alkaloids, and glycosylated steroids. Steroid aglycones come in two primary varieties: derivatives of furostan and spirostan. (**Wina *et al*., 2005)**.

It appears that saponins play a part in protecting the plant against microbial pathogens. The intuitive included with layer sterols result in hemolytic properties and spermicide action of certain particles **(Sabrina, 2003**).

A few saponin drugs are utilized for their antitussive properties (licorice rhizome), but moreover for their anti-edematous properties (cotyledons of horse chestnut seeds) or indeed for their pain relieving properties (Platycodon grandiflorum) **(Cherifi *et al*., 2016)**.

4. Tannins

The most prevalent secondary metabolites produced by plants are tannins, which typically make up 5% to 10% of the dry weight of tree leaves.

Through toxicity and/or deterrent, tannins can protect leaves from insect herbivores (**Barbehenn, 2011).**

Tannins are known as shapeless yellowish or light brown aggregates that resemble powder, flakes, or sponges. It's interesting to note that practically every plant in every climate on Earth has tannins **(Ashok and Upadhyaya, 2012).**

In 1962, tannins were characterized as water-soluble phenolic compounds with an atomic weight between 500 and 3000, having the property of accelerating gelatin and other proteins and recoloring with ferric salts.

Nowadays, we recognize between:

- **Hydrolyzable tannins:** Esters of a sugar, which is exceptionally for the most part glucose, and **gallic** acid or **ellagic** acid **(Sabrina, 2003)**.

They are known for their astringent taste and are often found in fruits, flowers and leaves. As their name suggests, and they are easily hydrolyzed by acids and enzymes (tannase) to pyrogallol **(Rima, 2019)**.

- **Condensed tannins or proanthocyanidins**: Non-hydrolyzable coming about from the polymerization of flavan-3-ol units.They shape pseudo-solutions within the vacuoles. they have a higher molecular weight than hydrolyzable tannins **(Grasel *et al*., 2016)**.

Tannins have been reported to inhibit bacterial proliferation of *Staphylococcus aureus*, *Streptococcus pneumonia*, *Bacillus anthracis*, *Shigella dysenteriae*, and *Salmonella senftenberg* **(Sieberi *et al*., 2020**).

5. Coumarins

Coumarins are secondary metabolites that are present in a wide range of higher plants and also in some animal species and microorganisms, present in different plant organs including leaves, fruits, flowers and roots, but also in the exudates of plants roots **(Esseh *et al*., 2016).**

They are natural aromatic substances whose structure includes the benzo-a pyrone (coumarin) nucleus resulting from the lactonization of ortho-hydroxy-cis cinnamic acid, with odors similar to vanillin, widely used in perfumery **(Mahdjoub, 2020).**

It was found that it has pharmacological activities as antimicrobial, antiviral (including anti- HIV), anti-inflammatory, molluscicidal, anticancer, antioxidant, anticoagulant, cardiovascular and antidepressant **(Ashok and Upadhyaya, 2012).**

6. The quinones

Quinones are a class of natural and synthetic compounds found in a wide variety of plant and also present in fungi and bacteria **(Najjar *et al*., 2011).**

Quinones result from the oxidation of fragrant subsidiaries characterized by a 1,4-diketocyclohexa-2,5-diene theme (para-quinones) or a 1,2-diketocyclohexa-3,5-diene theme (ortho-quinones) **(Sabrina, 2003**).

They have a number of advantageous properties. They are involved in photosynthesis as electron carriers. As vitamins that help prevent and treat conditions like cardiovascular disease and osteoporosis **(Najjar *et al*., 2011).**

7. Terpenoids and steroids

Terpenoids and steroids Inferred from the same forerunners and shaped from the get together of branched 5-carbon units, determined from 2-methylbutadiene (isoprene polymers), Terpenoids and steroids likely constitute the biggest auxiliary compounds. Like greasy corrosive subsidiaries, such as acylglycerides, Terpenoids have a biosynthetic beginning in acetyl CoA or malonyl CoA. All things considered, they are not particular to plants since squalene, cholesterol are found in creatures **(Sabrina, 2003).**

Most terpenoids are defense agents against herbivores **(Alexandra, 2022).** The number of isoprene units characterizes the diverse classes of terpenes:

Monoterpenes (C10), Sesquiterpenes (C15), Diterpenes (C20), Sesterterpenes (C25), Triterpenes (C30), and Tetraterpenes (C40).

Steroids are derived from terpenes, more precisely triterpenes (C30). The precursor to these molecules is the squalene skeleton, which undergoes cyclization. During this process, the steroid loses carbon units and ultimately has a tetracyclic skeleton and 27 carbons **(Sophie, 2021)**.

Part II:
Experimental Part

Materials and Methods

1- Study Location

The study was conducted in a controlled laboratory setting at the University of Larbi Tebessi. This site was chosen because it allowed for controlled experimental conditions.

2- Type of Study

This research was an experimental study involving the manipulation of *Atriplex halimus* to observe its effects on Gram-positive and Gram-negative bacteria.

3- Study Period

The research was conducted from February 3rd to April 28th, covering the full process of plant collection, drying, extraction, and evaluation of its antibacterial effect. The duration was selected to ensure sufficient time for each stage of the experiment, including proper sample preparation, extraction optimization, and accurate testing of antibacterial activity.

Work objective

In recent years, there has been a big interest in the antibacterial properties of the widespread medicinal plants in Algeria, particularly those used in traditional medicine. This study, was carried out between February and April, 2025, at the Microbiology Laboratory of the Department of Applied Biology at Larbi Tebessi University, Tebessa.
The aim was to evaluate in vitro the potential of the plant Atriplex halimus, as a natural antibacterial agent.

To achieve this, we followed a structured approach:

- **Extraction Process**: We obtained a hydroethanolic extract and determined its yield.
- **Phytochemical Screening**: We identified the plant's secondary metabolites, which play a key role in its biological activity.
- **Antibacterial Testing**: Using the aromatogram method, the extract was tested against four bacterial strains*: Streptococcus pneumoniae, Staphylococcus saprophyticus***,** *Klebsiella pneumoniae***,** *Citrobacter freundii.*
- **Concentration Analysis**: We determined the minimum inhibitory and bactericidal concentrations of the extract to assess its effectiveness.

By conducting this study, we aimed to deepen our understanding of the antibacterial potential of *Atriplex halimus* and explore its possible applications in natural medicine which could offer alternative treatments in the face of growing antibiotic resistance.

I- Materials

1/ Plant Material

Atriplex halimus was identified by a local expert and collected respectively in February 3rd of 2025 from Bekerria tebessa in the east region of Algeria.



Figure 03: *Atriplex halimus* **(Personal photo, 2025).**

Table 03: Geographical coordinates of the plant collection area.

Scientific name of the plant	Region	Geographical coordinates	Date of collection
Atriplex halimus	Bekerria tebessa	35.3721° 8.24215°	3rd February of 2025

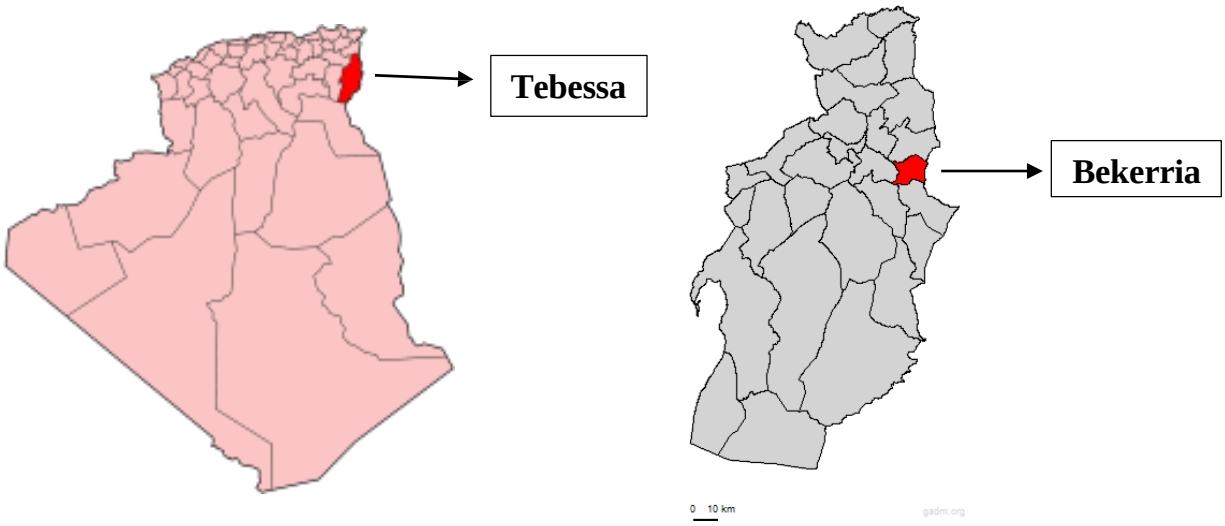


Figure 04: Location of the collection site of the plant *Atriplex halimus.*

2/ The bacterial strains tested

In our study, we used bacterial strains provided by the American Type Culture Collection (ATCC).

Table 04: Different bacterial strains tested**.**

Bacterial strain	Family	Shape	Gram	ATCC
Staphylococcus saprophyticus	*Staphylococcaceae*	Cocci (clusters)	Gram **positive**	15305
Streptococcus pneumoniae	*Streptococcaceae*	Cocci (chains/pairs)	Gram **positive**	49619
Klebsiella pneumoniae	*Enterobacteriaceae*	Rod-shaped	Gram **negative**	43816
Citrobacter freundii	*Enterobacteriaceae*	Rod-shaped	Gram **negative**	43864

2.1. The culture media used

Table 05: Culture media used.

Culture media	Use
Nutritious broth (NB)	• Revivification and enrichment of the tested bacterial strains • Replanting the colonies
Nutrient agar (NA)	
Muller Hinton Broth (MHB)	• Determine the minimum inhibitory concentrations (MIC) by the dilution method
Muller Hinton Agar (MH)	• Study of bacterial sensitivity to antimicrobial agents: Aromatogram

II- Methods

1/ Treatment of the Studied Plant

The plant material is first dried at room temperature, not exceeding 40°C, away from sunlight, in a dry and well-ventilated place for 15 days. Then, it is ground using an electric mill and sieved until a very fine green powder is obtained. Finally, it is stored in an airtight container.

2/ *Atriplex halimus* plant hydroethanolic extraction

2.1. Hydroethanolic extract preparation

One method that is frequently used to extract bioactive substances from medicinal plants is ethanolic extraction. It entails solubilizing and extracting the plant's active components using ethanol as a solvent. According to studies, ethanol extraction produces extracts that are rich in phenolic chemicals and have intriguing biological activity, most notably antibacterial and antioxidant qualities. This extraction technique was used in accordance with the guidelines provided by **(Hadjadj, 2017).**

2.2. Maceration

Macerate 70g of the powdered *Atriplex halimus* plant with 500 mL of a distilled water/ethanol mixture (80/20 v/v) in a beaker wrapped in aluminum foil, then let it stir using a magnetic stirrer for 40 minutes.





Figure 05: Maceration of the hydroethanolic extract of *Atriplex halimus* **(Personal photos, 2025).**

2.3. Filtration

The process of filtering using a Wattman No. 1 paper to filter the resulting macerate. Using 500 milliliters of the ethanol/distilled water solution, take the residue from the previous maceration and stir it for a full day. Using the second maceration, repeat the first filtration process.



Figure 06: Filtration of the macerate of *Atriplex halimus*

(Personal photo, 2025).

2.4. Evaporation

Transfer the two macerates of the *Atriplex halimus* into a round-bottom flask to evaporate them to dryness under reduced pressure, using a Rotavapor at a temperature of 44°C. Place the obtained hydroethanolic extract in an oven at 40°C for 48 hours to dry it completely. Weigh and store the extract in a refrigerator at a temperature of 4°C.



Figure 07: Evaporation of the hydroethanolic extract of *Atriplex halimus* **(Personal photos, 2025).**

2.5. Determination of the Yield of the Hydroethanolic Extract

The yield refers to the mass of the extract obtained after solvent evaporation. It is expressed as a percentage relative to the initial mass of the plant material subjected to extraction.
To calculate the yield, it is necessary to know the initial dry plant material mass and the final crude extract mass, then determine the percentage ratio. The hydroethanolic extraction yield is calculated using the formula below **(Boucherit, 2014)**:

$$Y\% = (Me / Ms) \times 100$$

- **Y%** : Yield percentage
- **Me**: Mass of the dry extract in grams
- **Ms**: Mass of the sample in grams

3/ Phytochemical Screening

3.1. Flavonoid Detection

- Pour 50 mL of boiling distilled water over 5 g of the previously prepared powder and let it infuse for 30 minutes
- After filtration, pour 6 mL of the infusion into 3 test tubes, distributing 2 mL per tube. Add 1 mL of NaOH, 1 mL of HCl, 1 mL of distilled water, and a few magnesium shavings to each tube.
- The appearance of an orange-pink color indicates the presence of flavonoids **(EL-Haoud *et al*., 2018).**

3.2. Saponoside Detection

- Since they are water-soluble, saponosides can be extracted with water.
- To prepare a 2% decoction, mix 5 g of the plant material powder with 50 mL of distilled water in an Erlenmeyer flask and maintain boiling for 30 minutes.
- After cooling and filtration, pour 5 mL of the filtrate into a test tube and shake it along the length of the tube for 15 seconds at a rate of 2 shakes per second.
- The appearance of a 1 cm high foam, persistent after 15 minutes, indicates the presence of saponosides **(Mecheri *et al*., 2023; EL-Haoud *et al*., 2018).**

3.3. Tannin Detection

Pour 50 mL of boiling distilled water over 5 g of the plant material powder and let it infuse for 30 minutes, then filter using filter paper.

Gallotannins: In a test tube saturated with sodium acetate, add 2 mL of filtrate and two drops of 1% Ferric Chloride ($FeCl_3$).
The appearance of a blue-black precipitate indicates the presence of gallotannins **(Mecheri *et al*., 2023).**

Catechic tannins: Evaporate 5 mL of the filtrate to dryness, then add 15 mL of $FeCl_3$ and maintain in a water bath at 80°C for 30 minutes.
The observation of a greenish-blue color indicates the presence of catechic tannins.

3.4. Quinone Detection

- Moisten 5 g of powdered plant material with a few drops of HCl, then add 10 mL of petroleum ether.
- Let it sit for 2 hours, then filter.
- In a test tube, add 2 mL of the filtrate and 2 mL of 10% NaOH.
- The presence of quinones is indicated when the aqueous phase turns red or violet **(EL-Haoud *et al*., 2018).**

3.5. Alkaloid Detection

- Alkaloids are nitrogenous substances acting as bases, giving precipitation reactions: in an acidic solution (pH 1 and 2), the interaction of alkaloid salts with iodized heavy metal compounds produces characteristic colored precipitates **(Mecheri *et al*., 2023)**.
- Mix 2g of *Atriplex halimus* powder with 10 mL of 10% sulfuric acid. Shake for 2 minutes, then filter.
- Place 2 mL of filtrate into 3 test tubes.
- Add 2 mL of Mayer's reagent to the first tube, 0.5 ml of Wagner's reagent to the second, and keep the third as a control.
- The appearance of a white or brown precipitate reveals the presence of alkaloids.

3.6. Terpene and Steroid Detection

- Macerate 5 g of *Atriplex halimus* powder in 100 mL of petroleum ether for 24 hours, then filter.
- Place the filtrate in a test tube and add 0.5 mL of concentrated sulfuric acid (H_2SO_4) to the triturate.
- The appearance of a purple or violet color, turning blue and then green, indicates a positive reaction **(EL-Haoud *et al*., 2018).**

4.Microbiological Revivification and Purity Control

4.1. Revivification and Subculturing of Tested Strains

The revivification process in microbiology involves rejuvenating microbial strains to obtain a young and pure culture. To use the strains:

- Aseptically take a sample of the preserved strains using a sterile platinum loop.
- Inoculate them into test tubes containing Nutrient Broth (BN) and incubate at 37°C for 24 hours.
- Subculture the strains from the nutrient broth cultures onto GN medium and incubate at 37°C for another 24 hours.



Figure 08: Revivification and subculturing of tested strains **(Personal photos, 2025).**

4.2. Purity Control of Strains

After revivification and subculturing a Gram staining was performed on each strain to confirm their purity.

- Spread a drop of physiological water and a bacterial colony onto a glass slide.
- Air-dry, then heat-fix the slide over a Bunsen burner flame.
- Apply Crystal Violet as the primary stain, leave for 60 seconds, then rinse with distilled water.
- Apply Lugol's iodine to fix the stain, leave for 60 seconds, then rinse.

- Add a few drops of alcohol to decolorize the bacteria, let it sit for 10 to 15 seconds, then rinse.
- For the final step, apply Fuchsin as the counterstain, leave for 60 seconds, then rinse.

5. Antibacterial Activity

5.1. Disc Diffusion Method

To determine the presence of antibacterial activities of the plant *Atriplex halimus* we used the disc diffusion method for antibacterial susceptibility testing that was carried out according to the standard method by **(Migheri *et al*., 2010).** A petri dish is inoculated with a bacterial suspension, and Whatman No. 1 paper discs are placed on the surface and impregnated with the hydroethanolic extract to be tested. After incubation, a zone of bacterial growth inhibition appears, known as the inhibition halo, forms around the discs. The effectiveness of the antibacterial activity of the hydroethanolic extract corresponds to the diameter of the halo.

5.2. Preparation of the Stock Suspension

Mix 1g of the crude hydroethanolic extract with 1mL of Dimethyl Sulfoxide (DMSO). Transfer the mixture into a sterile test tube and ensure proper homogenization using a vortex mixer.



Figure 09: Preparation of the mother suspension **(Personal photo, 2025).**

5.3. Preparation of the inoculum

- It is necessary to carry out antibacterial activity on young bacterial strains in the full growth phase.
- The strains are reactivated using the streaking technique on nutrient agar pre-poured into Petri dishes, then incubated at a temperature of 37°C for 18 to 24 hours.
- Collect a few isolated bacterial colonies using a platinum loop or a Pasteur pipette and transfer them into a tube containing approximately 10mL of sterile physiological saline.

- Homogenize the bacterial suspension by gently shaking the tube, adjusting the turbidity of the suspension to the desired concentration, generally 0.5 on the McFarland scale (Appendix), which corresponds to an optical density of 0.08 to 0.10 at a wavelength of 625 nm, equivalent to 10^8 CFU/mL **(Fenghour *et al.*, 2021).**

5.4. Preparation of the discs

- Cut Wattman No. 1 paper or blotting paper into disks with a diameter of 6mm.
- Place them in an airtight container and autoclave for 15 to 20 minutes at a temperature of 121°C to avoid any risk of contamination by exogenous germs.

5.6. Inoculation

- Aseptically pour the Mueller Hinton (MH) agar culture medium into Petri dishes at a rate of 20 mL per dish.
- Let it cool and solidify on the bench.
- Dip a sterile swab in the bacterial suspension of the tested strains.
- Squeeze it against the walls of the tube, then streak the agar surface in tight parallel lines while turning the plate gradually to cross the streaks, not forgetting to rotate the swab itself to cover the entire surface.
- Let it dry for about 15 minutes.



Figure 10: Inoculation of bacterial strains in MH medium **(Personal photos, 2025).**

5.7. Preliminary Test

Using sterile forceps**,** gently place three sterile discs onto the previously inoculated Petri dishes containing the tested bacterial strains.

- Soak the first disc with 10µl of the stock solution of the prepared hydroethanolic extract.
- Soak the second disc with 10µl of DMSO**.**
- Leave the third disc empty as a control**.**

Incubate the plates at 37°C for 24 hours**.**

5.8. Quantitative study of the antibacterial activity of the hydroethanolic extract of *Atriplex halimus*

Place four Whatman paper discs onto the inoculated petri dishes containing the tested bacterial strains:

- first disc: leave dry as control
- second disc: soak with 10µL of DMSO (negative control)
- third disc: soak with 10µL of the crude hydroethanolic extract
- fourth disc: soak with 10µL of a diluted extract solution
- Incubate the plates in an incubator at 37°C for 24 hours
  (Yapo *et al.*, 2020).



Figure 11: Deposition and impregnation of discs **(Personal photos, 2025).**

6/ Reading of results

After incubation of the plates, use a caliper to measure the diameters of the inhibition halos that have formed around the discs where bacterial growth is prevented. these diameters, in millimeters, are proportional to the sensitivity of the studied bacteria.

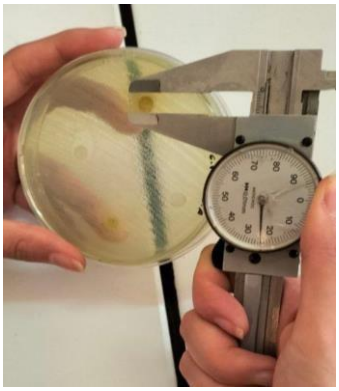


Figure12: Measuring the diameters of the inhibition halos using a caliper **(Personal photo, 2025).**

The bacterial sensitivity to the different compounds is classified based on the inhibition halo diameters mentioned in the table:

Table 06: Diameter and Degree of Bacterial Sensitivity **(Mouas *et al.*, 2017).**

Diameter	**Sensitivity Level**	**Symbol**
X< 8 mm	Not sensitive / Resistant	-
8 mm <X< 14 mm	Sensitive	+
14 mm<X< 20 mm	Very sensitive	++
X>20 mm	Highly sensitive	+++

7. Determination of the MIC of the hydroethanolic extract of Atriplex halimus using the microplate method

We opted for the protocol by **(Fenghour *et al*., 2024**) with some modifications.

The minimum inhibitory concentration (MIC) is defined as the lowest concentration (expressed in micrograms/mL) that prevents visible growth of the tested strains **(Hacini *et al.*, 2017).**

- The determination of MIC against bacterial strains is carried out using the liquid microdilution technique in a 96-well microplate.
- Prepare the stock solution by mixing 1 g of the crude hydroethanolic extract with 10 mL of DMSO. Dispense 100 µL of Mueller-Hinton broth (MHB) into all wells of the microplate.
- Add 100 µL of the previously prepared stock solution into the first well of the first four rows. Take 100 µL from the first well and dilute it into the next well, and so on, to obtain a 1/2 dilution series.
- Add 10 µL of the bacterial suspension, and each row is reserved for a single bacterial strain. The 5th row is reserved for the positive control containing MHB and the inoculum, while the 6th row is for the negative control (extract and MHB).
- Incubate the microplate for 24 hours at 37°C.

8. Determination of the Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) is the lowest concentration of a substance that leaves at most 0.01% of surviving germs.

- Take the contents of the wells that show no visible turbidity and streak them onto MH agar.
- Incubate for 24 hours at 37°C **(Fenghour *et al.*, 2024).**

Streaks with no microbial growth represent the MBC. Based on the MBC/MIC ratio, the antimicrobial effect can be assessed as follows:

- **MBC/MIC ratio < 4**: bactericidal effect.
- **MBC/MIC ratio > 4**: bacteriostatic effect **(Yapo *et al*., 2020).**

Results and discussion

1/ Determination of the yield of the hydroethanolic extract of *Atriplex halimus*

The obtained yield values are presented in Table 07.

Table 07: Yield values of the hydroethanolic extraction of *Atriplex halimus*.

Powder weight (g)	Dry hydroethanolic extract weight (g)	Extraction yield (%)	Extract color
70	**19.6**	**28**	**Dark green**

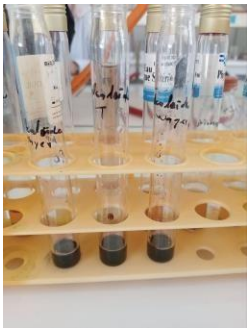
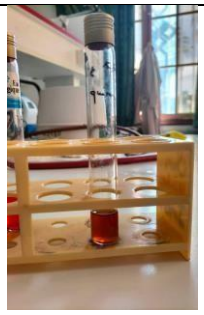
The hydroethanolic extract obtained from the leaves and flowering tops of *Atriplex halimus* yielded is 28%. This result is higher than that reported by **(Nabila *et al*., 2009)**, who obtained a yield of 24%, and significantly higher than the yield reported in the Bir Hani region of Boussaâda by **(Merikhifa *et al*., 2022),** which was 18.425%. It is also higher than the 19.6% yield reported by **(Moha, 2021)** from the Guelmim region in southern Morocco and **(Gharbi & Slamani, 2017)** that achieved a hydroethanolic extract yield of 32.54%.

The yield variability of the extract of vegetal material is generally influenced by a number of factors, including geographic location, harvest season and stage, harvesting conditions, and extraction technique **(BOUZIANE, 2015).**

2. Phytochemical Screening Results of *Atriplex halimus*

The phytochemical screening results of dry powdered leaves extracts of *A.halimus* are presented in Table 08. The used symbols (+/-) are presented to provide a presence or absence of the phytochemical according to the tested experiment.

Table 08: Results of phytochemical screening of *Atriplex halimus.*

Secondary Metabolites	Presence (+) / Absence (–)	Indicators (Color / Observation)	Results
Flavonoids	**+**	**Yellow to reddish color**	
Saponins	**+**	**Persistent foam**	
Alkaloids	**–**	**No white or brown precipitate**	
Quinones	**–**	**No violet color**	

Terpenes and Steroids	**+**	**Purple color turning blue**	
Gallic Tannins	**–**	**No blue-black precipitate**	
Catechin Tannins	**+**	**Bluish-green color**	

The results obtained reveal that the plant contain tannins Catechin, saponins, flavonoids, and terpenes and Steroids. Moreover, quinones, Gallic Tannins and Alkaloids were found to be absent in the extracts of *A.halimus.*

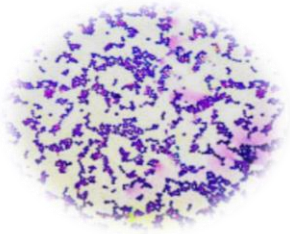
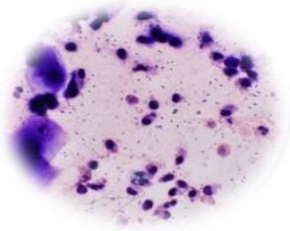
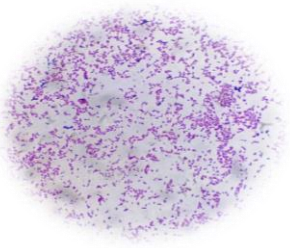
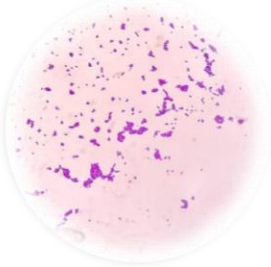
Previous studies on the phytochemical analysis of *Atriplex halimus* **(Mkaddem *et al*., 2023)** have also reported the presence of flavonoids, saponins, tannins and terpenes and steroids, while it has been noted a total absence of quinones and alkaloids in the same plant species. These findings support and validate our own results.

In the other hand **(Chaouche *et al*., 2121)** marked a presence of alkaloids, tannins and flavonoids and absence of coumarins, terpenoids and saponins.

The difference in chemical composition between the same plants being studied, as well as those in another region, can be attributed to various factors that affect the presence, absence and distribution of active ingredients. These factors include climate, soil type, water, altitude, extraction solvent etc **(Nurcholis *et al*., 2022).**

3.Strain purity control

Table 09: Purity control of the tested bacterial strains.

Tested strain	**Morphology / Microscopic appearance after Gram staining**	
Staphylococcus saprophyticus ***ATCC: 15305***	**Cocci (clusters)**	
Streptococcus pneumoniae ***ATCC: 49619***	**Cocci (chains/pairs)**	
Klebsiella pneumoniae ***ATCC: 43819***	**Rod-shaped**	
Citrobacter freundii ***ATCC: 43864***	**Rod-shaped**	

4. Evaluation of the antibacterial activity of the pure and diluted hydroethanolic extract of *Atriplex halimus*

Two Gram-positive strains and two Gram-negative strains were used to study the antibacterial potential of the hydroethanolic extract of *Atriplex halimus*.

The results of the evaluation of the antibacterial activity of the pure and diluted hydroethanolic extract of *Atriplex halimus* are presented in the table below.

Table 10: Evaluation of the antibacterial activity of pure and diluted hydroethanolic extract of *Atriplex Halimus*.

	Bacterial Strains	**Pure Hydroethanolic Extract (10 µl) mL**		**Diluted Hydroethanolic Extract 10−1/mL**		**Negative Control (DMSO)**	**Results pictures**
G+	***Streptococcus pneumoniae ATCC: 49619***	**11.87 ± 0.19**	**+**	**10.46 ± 0.23**	**+**	**0**	
	Staphylococcus saprophyticus ATCC: 15305	**12.95 ± 0.21**	**+**	**11.84 ± 0.17**	**+**	**0**	

G-	***Klebsiella pneumoniae ATCC: 43819***	**0**	**-**	**0**	**-**	**0**	
	Citrobacter freundii ATCC: 43864	**6.73 ± 0.16**	**-**	**5.33 ± 0.18**	**-**	**0**	

(–): Resistant. (+): Sensitive.

According to the results obtained in the table, the variability of the inhibition zone diameters expressed by the hydroethanolic extract of *Atriplex halimus* against the tested strains indicates that they react differently to the tested extract.

Our preliminary test results showed that the hydroethanolic extract of *Atriplex halimus* was effective in inhibiting the growth of *Staphylococcus saprophyticus*, *Streptococcus pneumoniae*, in the other hand *Citrobacter freundii*, and *Klebsiella pneumoniae were resistant*.

The largest inhibition zone was that of *Staphylococcus aureus* with a diameter of 12.95± 0.21 mm, followed by *Streptococcus saprophyticus* with a diameter of 11.87 ± 0.19mm, then *Citrobacter freundii* with a small zone with a diameter of 6.37 mm± 0.16.

The results of the study on the antibacterial potential of the extract we obtained showed that the hydroethanolic extract of *Atriplex halimus* has a more or less significant effect on all strains. The highest zones are those of the strains *S. saprophyticus* and *Streptococcus pneumoniae*, thus our extract of *Atriplex halimus* is more effective on Gram (+) than on Gram (-).

In the study by **(Chaouche *et al*., 2021)** the antibacterial activity of *Atriplex halimus* extracts from two Algerian regions—El Oued and Tlemcen demonstrated notable antibacterial effects, with inhibition zones of 15 mm against *Staphylococcus aureus* and 12 mm against *Listeria monocytogenes*. In contrast, the Tlemcen extract did not exhibit significant antibacterial activity against the tested strains.

The geographical origin of the plant material affects its antibacterial activity. Environmental factors such as soil composition, climate, and altitude can lead to variations in the concentration of active constituents **(Tagnaout *et al*., 2016).**

5/ Determination of the MIC and MBC of the hydroethanolic extract of *Atriplex halimus*

After 24 hours of microplate incubation, we observed a clear and transparent appearance in some wells, and turbidity indicating bacterial growth in other wells.

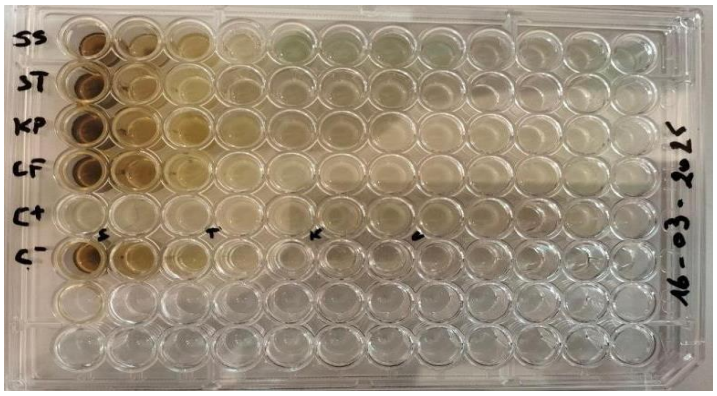


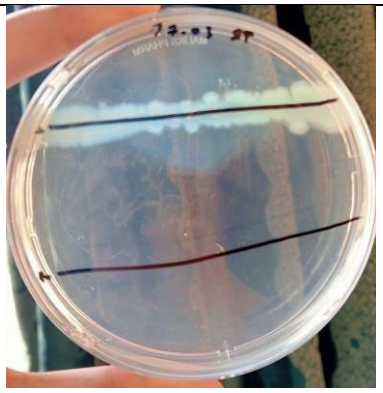
Figure 13: Experimental distribution of the microplate for the four tested strains for MIC determination **(Personal photo, 2025).**

Ss: *Streptococcus pneumoniae* / **St**: *Staphylococcus saprophyticus* / **Kp**: *Klebsiella pneumonia*
CF: *Citrobacter freundii*

C (-): Control negative / **C (+)**: Control positive

The MIC and MBC results of the five bacterial strains are presented in the table 11**.**

Table 11: The MBC/MIC ratio of the three tested strains.

Bacterial strains	**Hydroethanolic extract of *Atriplex halimus* (mg/ml)**		**MBC/MIC ratio**	**Antibacterial activity**	**BMC**
	MIC	**MBC**			
Streptococcus pneumoniae ATCC: 49619	***12.5***	***25***	***2***	**Bactericidal**	
Staphylococcus saprophyticus ATCC: 15305	***12.5***	***25***	***2***	**Bactericidal**	
Citrobacter freundii ATCC: 43864	***50***	***50***	***1***	**Bactericidal**	

The MIC of *Staphylococcus saprophyticus* and *Streptococcus pneumoniae* is equal to 12.5 mg/ml, our results are higher than those who reported MIC values of 2.5 mg/mL (**Chaouche *et al*., 2021)**, while that of *Klebsiella pneumoniae* and *Citrobacter freundii* is equal to 50 mg/mol. The difference in results could be attributed to the type of extract, (**Chaouche *et al*., 2021)** used a hydromethanolic extract.

There are several factors influencing the determination of antibacterial activity as the extraction methods, the type of the tested strains, the type and concentration of the extract, the method of evaluation of the antibacterial activity the nature and chemical structure of bioactive molecules present in the extract **(Tagnaout *et al*., 2016)**. It is crucial to emphasize that when the minimum inhibitory concentration is low, the extract's antimicrobial action is higher **(Famuyide *et al*., 2019)**. The impact is regarded as bacteriostatic if the MBC/MIC ratio is larger than 4, and bactericidal if it is less than 4 **(Yapo *et al*., 2020).**

Our results agree with those of **(Karima *et al*., 2020)** who showed a high efficacy of the hydroethanolic extract of the same plant on gram + strains and a lack of antibacterial activity against gram negative, which confirms our results.
Indeed, the outer membrane of Gram-negative bacteria is specifically composed of phospholipids and lipopolysaccharides (LPS) for this reason it's more resistant.
The importance of this structure lies in its ability to protect bacteria against antibacterial agents **(Colucci and Cimaglia, 2020)**.

Conclusion and perspectives

Algeria, due to its exceptional geographical location, has a significant wealth of medicinal plants, many of which are used in traditional treatments. Some medicinal plants possess highly important biological properties that find numerous applications in various fields, namely in medicine, pharmacy, cosmetics, and agriculture. This study focuses on the promising potential of *Atriplex halimus* extracts, the yield of the extracts was within an acceptable range 28 %, suggesting efficient extraction, especially when ethanol was used. Phytochemical screening revealed the presence of several bioactive compounds such as flavonoids, saponins, tannins. These secondary metabolites are likely responsible for the observed antibacterial activity.

In vitro results showed that the *Atriplex halimus* extract possesses a significant broad-spectrum antibacterial potential against both Gram-positive, including *Staphylococcus saprophyticus*, *Streptococcus pneumoniae*, and Gram-negative bacteria *Citrobacter freundii*, and *Klebsiella pneumoniae* were mostly resistant, with the degree of inhibition varying based on the bacterial strain and extract concentration. Meanwhile the MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) values varied across the different strains. Lower MIC and MBC values were recorded for *Staphylococcus saprophyticus* and *Streptococcus pneumoniae*, indicating a higher sensitivity to the extract, while *Citrobacter* and *Klebsiella* showed relatively higher values, reflecting moderate to low susceptibility.

Finally, *Atriplex halimus* demonstrated significant antibacterial activity in vitro, making it a promising candidate for further investigation as a natural antimicrobial agent. Its efficacy, especially against resistant strains, along with its phytochemical richness, supports its potential use in the development of alternative or complementary therapeutic strategies.

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Annexes

1/ Laboratory Equipment and Materials

Table 12: Laboratory Equipment, Materials, and Reagents Used

Equipment	Small Materials	Reagents & Chemicals	Glassware
• Electronic balance (Scout Pro 600g) • Rotary evaporator (Büchi-R210) • Refrigerator • Incubator (Memmert) • Spectrophotometer (UniLine 9300) • Magnetic stirrer (LabTa) • Water bath • Optical microscope (OPTIKA) • Vortex (VWR) Autoclave • Sand bath (WITEG CAT 7813 Staufen)	• Filter paper • Aluminum foil • Gauze band • Micropipette (5µl–50µl) • Inoculating loop Bunsen burner • Whatman discs (6 mm) • Forceps • Sterile swabs • Black background • Vernier caliper • Magnetic stir bars Spatula • Tube rack • Yellow tips Eppendorf tubes • Cuvette • Glass pipettes • Absorbent paper	• Gentian violet • Lugol's solution • Alcohol • Fuchsin • $BaCl_2$ • H_2SO_4 (Sulfuric acid) • DMSO • NaOH • HCl • Acetic acid • $FeCl_3$ • TCA – • Mayer's reagent • Magnesium shavings Acetic anhydride • Sodium acetate • Ethanol • Sterile distilled water • Petroleum ether • Chloroform	• Erlenmeyer flasks • 1000 ml graduated cylinder • Graduated pipettes (1 ml, 10 ml) • Pasteur pipettes • Glass flasks Funnels • Test tubes • Graduated beakers • Petri dishes • Airtight glass containers • Slides and cover slips

2.Composition of Reagents Used for the Phytochemical Screening of the Plant

Table 13: Composition of Reagents Used for Phytochemical Screening

Reagent	Composition
Sodium Hydroxide (1/10)	Dissolve 0.2g of NaOH in 50ml of distilled water
Mayer's Reagent	• 0.136g of mercury chloride • 0.5g of potassium iodide • Distilled water to make 10ml
Wagner's Reagent	• 0.2g of potassium iodide • 0.127g of iodine • Distilled water to make 10ml
Ferric Chloride (FeCl3 at 1%)	• 0.1g of ferric chloride in 10ml of distilled water
Trichloroacetic Acid (TCA)	• Dissolve 0.5g of trichloroacetic acid in 20ml of distilled water

Description and Principle of the Rotavapor

1.Principle

The principle of the Rotavapor, also known as the rotary evaporator, is based on:

- Reducing the pressure to lower the boiling point of the solvent,
- Rotating the sample to increase the effective surface area, and
- Heating the solution.

2.Description of the Rotavapor

The Rotavapor consists of the following components:

- A glass evaporation flask containing the sample to be processed
- A water or oil bath that evenly heats the evaporation flask
- A motor that rotates the flask at a constant speed
- A high-efficiency glass condenser cooled by water
- A receiving flask to collect the condensed distillate
- A pressure reduction system (such as a water jet pump or vacuum pump)



Figure 14: The Rotavapor **(personal photo).**

3. Operation

The main steps of its operation are:

- The sample is placed in a glass evaporation flask, which is evenly heated by a water bath.
- The flask rotates at a constant speed, creating a thin film of solution on the inner walls. This increases the surface area in contact with air.
- The pressure in the system is reduced using a water jet pump or vacuum pump, which lowers the boiling point of the solvent.
- The heat from the water bath causes the solvent to boil and evaporate. The vapor is then directed to the condenser.
- The condenser, made of two glass tubes with water circulating between them, cools the vapor, which then condenses into liquid form.
- The condensed liquid flows into a receiving flask.

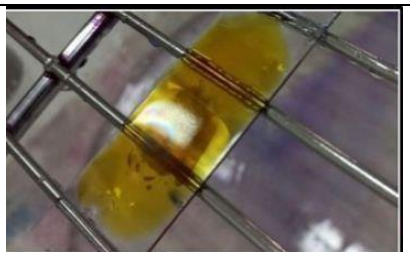
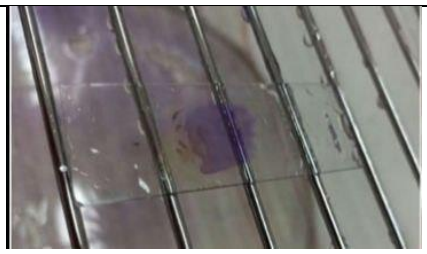
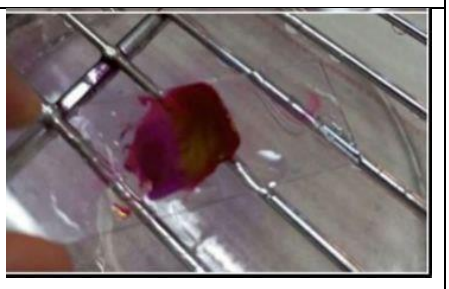
3.Steps of Gram Staining

1.Principle

The principle of Gram staining is based on a differential staining technique that allows visualization of bacterial morphology, arrangement, and classification as Gram-positive or Gram-negative based on their cell wall composition.

2.Staining Steps

Table 14: Steps of Gram Staining (Personal Photos, 2024).

Step	Preparation	Purpose	
01	Staining with gentian violet or crystal violet (30 sec to 1 min)	Stain the bacterial content purple	
02	Mordant with Lugol's solution (iodine-iodide) (30 sec to 1 min)	Fix the violet stain inside the bacteria	
03	Rapid decolorization with alcohol (+ acetone)	Remove the stain from thin-walled bacteria (Gram–), retain it in thick-walled bacteria (Gram+)	
04	Counterstaining with safranin or fuchsin (30 sec to 1 min)	Stain decolorized bacteria pink (Gram–) to differentiate them from purple ones (Gram+)	

4. Preparation of Culture Media and Physiological Water

Tableau 15 : Préparation des milieux solides et liquides

Solid Media		
Medium	**Composition**	**Preparation**
Nutrient Agar (GN)	- Meat extract: 1g/L - Yeast extract: 2.5g/L - Peptone: 5g/L - Sodium chloride: 5g/L - Agar: 15g - pH = 7	Dissolve 20g of dehydrated GN in 1L of distilled Autoclave at 121°C for 15–20 min
Muller Hinton Agar (MH)	- Acid hydrolysate of casein: 17.5g - Meat extract: 2g - Corn starch: 1.5g - Beef infusion: 300ml	Ready to use
Liquid Media		
Nutrient Broth (BN)	- Peptone: 10g - Dehydrated beef extract: 1g - Yeast extract: 2g - Sodium chloride: 5g - pH = 6.8 ± 0.2	Dissolve 13g of dehydrated BN in 1L of distilled water. Autoclave at 121°C 20 min
Muller Hinton Broth (BMH)	- Casein hydrolysate: 17.5g - Meat infusion: 2g - Starch: 1.5g	Dissolve 21g of dehydrated MH in 1L of distilled water. Autoclave at 121°C for 15–
Physiological Water (Saline Solution)		
Sodium chloride (NaCl)	9g	Preparation: Dissolve 9g of sodium chloride in 1000g of distilled water. Adjust the pH if needed. Sterilize in an autoclave at 121°C for 30 minutes.
Distilled water	1000g	

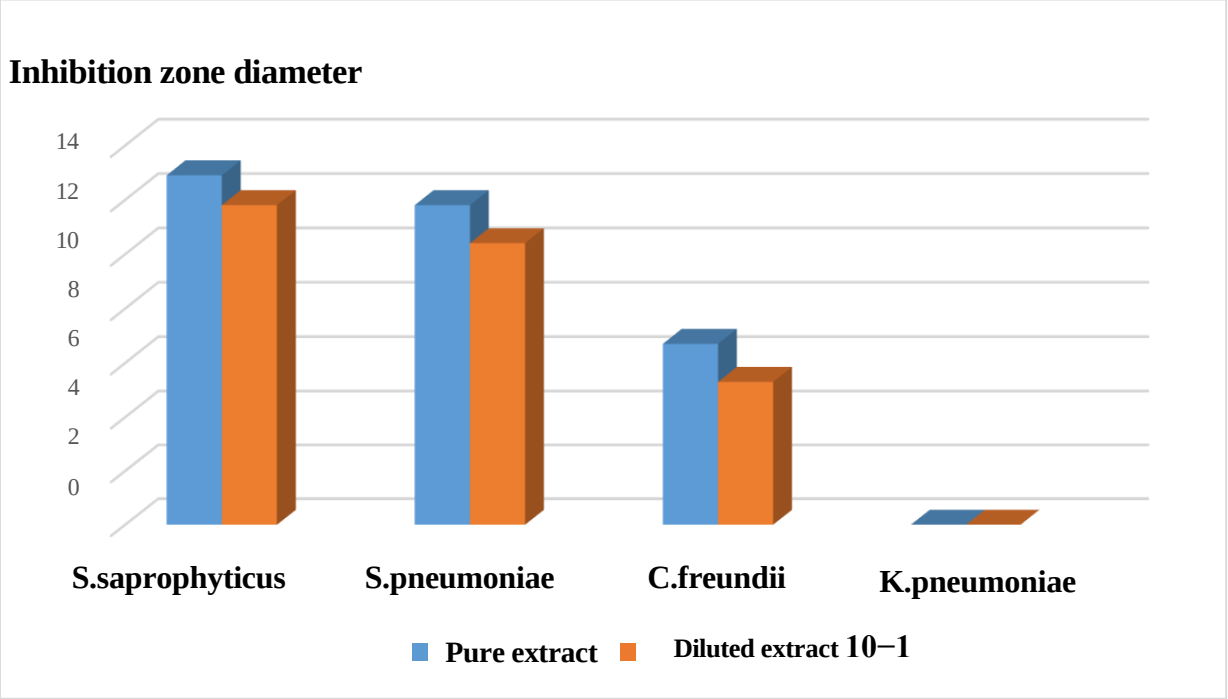


Figure15: Results of the Antibacterial Activity of the Hydroethanolic Extract.