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Master's Degree

Field : *Biological Sciences*

Option : *Applied Microbiology*

THEME

Evaluation of the biological activity of endophytic actinomycete isolated from medicinal plants of Laghouat's region.

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MCB

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DEDICATION

I dedicate this modest work to the dearest people in my life :

My parents

In memory of My dearest father Brahim (Allah Yarehmou)

My dear mother Zoulikha , who worked for my success, with her love, her support, all the sacrifices made and her precious advice, for all her assistance and her presence in my life, which allowed me to continue my studies in the best conditions

To My brother Yacine

To "My friend Nana"

To my uncle Djamel , who helped me throughout this work

To all my family

To my friends whom I lived with them beautiful moments during my studies at the
University

To my partner Amine

To all those who have contributed from near or far in the realization of this memoir.

I say thank you

SOFIANE

DEDICATION

*Dedicate this research to my mom and dad , all my family members
and my close friends especially my partner Sofiane for being
comprehensive during this period, in memory of grandpa slimane
,grandpa Ahmed, uncle Rachid
and si Tahar Laamri may they all rest in peace*

Mohammed

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This research was developed by the grace of Allah who gave the researchers the knowledge and wit to finish and established this research

we would like to thank all the people who helped to make this study possible.

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Thank you all we appreciate it sincerely.

ملخص

يعتمد هذا البحث المنجز في ولاية الأغواط على عزل و تحديد لبعض البكتيريا الداخلية (الاكتينومييسات الداخلية) من ثلاث نباتات محلية ذات أهمية طبية و التي يستفاد من شهرتها العلاجية و هي :

Retama reatam و الرتم *Rosmarinus officinalis*, و إكليل الجبل *Peganum harmala* , نبات الحرمل و قد تم أخذ العينات من ثلاث مواقع بالمنطقة : من غابة بلدية البريدة , بمحاذاة ملعب 18 فبراير , و بالقرب من بلدية سيدي مخلوف تم عزل سلالات البكتيريا الداخلية (الاكتينومييسات الداخلية) و تنقيتها إنطلاقا من السلق و الجذور على أربعة أوساط زراعية كالتالي:

Humic acid vitamin agar (HV media)/ Strach Casein Agar (CSA media)/ GAUZE' media (GM)/
Tap Water -Yeast Extract(TWYE)

كما تم أيضا إختبار النشاطات البيولوجية المضادة للميكروبات للسلالات المعزولة ضد البكتيريا و الفطريات ذات الأهمية السريرية وهي:
Staphylococcus aureus (ATCC 25923) Bacillus cereus (ATCC 11778) Klebsiella pneumonia (ATCC 70603) Escherichia coli (ATCC 25522) Bacillus subtilis (ATCC 6633) Candida albicans (ATCC 10231)
(fungi) Pseudomonas aeruginosa (ATCC 27853)

وفي سياق الاختبارات تم تطبيق تقنية الأسطوانة آجار و تقنية التركيز المثبط الأدنى أو ما يعرف بتقنية 96Puits

الكلمات المفتاحية : النبات ، إكليل الجبل ، الحرمل ، الرتم ، الساق ، الجذر ، الاكتينومييسات ، نشاط مضاد للميكروبات

ABSTRACT

This work, carried out in the wilaya of Laghouat was based on the isolation and identification of endophytic actinomycetes from three local plants of medical interest which are Harmal (*Peganum harmala*), rosemary (*Rosmarinus officinalis*) and R'tem (*Retama reatam*). The samples were taken at three sites respectively : near the "18th February stadium" in the city of Laghouat, the forest of the district of Brida, and near the district of Sidi Makhlouf.

Endophytic actinobacteria strains were isolated and purified on four culture media: **Humic acid vitamin Agar (HV media)**, **Starch Casein Agar (SCA media)**, **GAUZE's medium (GM)**, **Tap water-yeast extract (TWYE)**. The samples explored are the stems and the roots.

The antimicrobial activity of the isolated strains was tested against bacteria and fungi of clinical importance which are: *Staphylococcus aureus* (ATCC 25923) *Bacillus cereus* (ATCC 11778) *Klebsiella pneumonia* (ATCC 70603) *Escherichia coli* (ATCC 25522) *Bacillus subtilis* (ATCC 6633) *Candida albicans* (ATCC 10231) (fungi) *Pseudomonas aeruginosa* (ATCC 27853).

For the tests we used the cylinder agar technique and the minimal inhibitory concentration (MIC) technique.

Key words: plants, Rosemary, Harmal, R'tem, stems, roots, actinomycetes, antimicrobial activity.

RESUME

Ce travail mené dans la wilaya de Laghouat est basé sur l'isolement et l'identification des actinomycètes endophytes à partir de trois plantes locales ayant un intérêt médicale qui sont le Harmal (*Peganum harmala*), le romarin (*Rosmarinus officinalis*) et R'tem (*Retama reatam*). Les échantillons ont été prélevé au niveau de trois sites de la région : prêt de "stade de 18 février" dans la ville de Laghouat , la foret de la commune de Brida, et prêt de la commune de Sidi Makhoulf .

Les souches d'actinobactéries endophytes ont été isolées et purifiées sur Quatre milieux de culture : Humic acid vitamin Agar (HV media), Starch Casein Agar(SCA media), GAUZE's medium (GM), Tap water-yeast extract (TWYE). Les échantillons explorés sont les tiges et les racines.

L'activité antimicrobienne des souches isolées a été tester contre des bactéries et champignons d'importance clinique qui sont : *Staphylococcus aureus* (ATCC 25923) *Bacillus cereus* (ATCC 11778) *Klebsiella pneumonia* (ATCC 70603) *Escherichia coli* (ATCC 25522) *Bacillus subtilis* (ATCC 6633) *Candida albicans* (ATCC 10231) (fungi) *Pseudomonas aeruginosa* (ATCC 27853)

Dans les tests On a appliqué la technique de cylindre d'agar et la technique de la concentration minimale inhibitrice appelé aussi la technique des 96 puits.

Mots clés : plantes, romarin, Harmal, R'tem, tiges, racines, actinomycètes, activité antimicrobienne

LIST OF ABBREVIATIONS

G+C	Guanine+ Cytosine
NO ₃	organic nitrogen(Nitrate)
NaHCO ₃	Sodium bicarbonate
HV	Humic acid-vitamin agar
SCA	Starch Casein Agar
GM	GAUZE's medium
TWYE	Tap water-yeast extract
Na ₂ HPO ₄	Sodium hydrogen phosphate
KCl	Potassium chloride
MgSO ₄ 7H ₂ O	Magnesium sulfate heptahydrate
FeSO ₄ 7H ₂ O	Ferrous sulfate heptahydrate
CaCO ₃	Calcium carbonate
KNO ₃	Potassium nitrate
K ₂ HPO ₄	Dipotassium phosphate
NaCl	Sodium chloride
FeSO ₄	Ferrous sulfate
NRRL	Northern Regional Research Laboratory (Agricultural Research Service Culture Collection)
CFU	Colony-Forming Unit
ATCC	American Type Culture Collection
GLM	Media glucose – yeast extract – malt extract
Rpm	Revolutions per minute
MIC	Minimal inhibitory concentration

List of Figures

List of Figures

Figures of chapter II

Figure II.1 : Shrub of <i>Peganum harmala</i>	8
Figure II.2 : Shrub of <i>Rosmarinus officinalis</i>	10
Figure II.3 : Shrub of <i>Retama raetam</i>	11

Figures of chapter III

Figure III.1 : Geographical distribution of the samples collection areas	14
Figure III.2 : Schematic of the Agar Cylinder Technique	17

List of Tables

List of Tables

Tables of chapter II

Table II.1 The classification of <i>Peganum harmala</i>	8
Table II.2 The classification of <i>Rosmarinus officinalis</i>	10
Table II.3 The classification of <i>Retama raetam</i>	12

Tables of chapter III

Table III.1 Places and areas of plant collection	14
Table III.2 Characteristics of the bacterial strains tests	16

Table of Contents

ملخص.....	I
Abstract	II
Résumé.....	III
List of Abbreviations	IV
List of Tables	V
List of Figures	V
Introduction.....	2
Chapter I. Endophytic Actinobacteria.....	5
I.1. Diversity of endophytic actinobacteria in medicinal plants	5
I.2. Endophytes as a source of secondary metabolites	5
Chapter II. Medicinal Plants.....	7
II.1. Generalities.....	7
II.2. Interest of medicinal plants	7
II.3. Botanical description of the species studied.....	7
II.3.1. <i>Peganum harmala</i>	7
II.3.2. <i>Rosmarinus officinalis</i>	9
II.3.3. <i>Retama raetam</i>	11
Chapter III. Material and Methods.....	14
III.1. Sample collection	14
III.2. Surface cleaning	15
III.3. cleaning efficiency	15
III.4. Isolation of Culturable Endophytic Actinobacteria	15
III.5. Evaluation of antimicrobial activity.....	16
III.6. Primary screening by cylinders agar method	17
III.7. secondary screening	18
Conclusion and Perspectives.....	19
Bibliography	20
Annex.....	

INTRODUCTION

INTRODUCTION

Introduction

Actinobacteria are Gram-positive bacteria with a high G+C content. They are an important component of microbial diversity, these microorganisms are mainly found in soil and isolated from diverse environments (*Akshatha J.V et al, 2016*). Recently, the discovery of new taxa of actinobacteria has decreased, while the re-isolation of known strains of actinomycetes has increased.

Therefore, one of the successful solution is the isolation of actinobacteria from untapped ecological niches, such as plant surfaces, roots and plant tissues (*Yuan et al., 2008*).

In this sense, endophytic microorganisms are the most potential source of new taxa of actinobacteria.

In fact, the term "endophytes" refers to microorganisms living in tissues of healthy host plants (*Akshatha J.V et al, 2016*). They are known for their beneficial effects on the host plant. Obligate endophytes are the most difficult to cultivate, because they require more specific conditions (*Eevers et al., 2015*).

Endophytic actinobacteria have been isolated from a wide variety of plants. The most frequently observed species belong to the genera *Microbispora*, *Nocardia*, *Micromonospora* and *Streptomyces*. They persist inside the plant host for long periods of time without causing observable disordered symptoms (*Ait Barka et al., 2016*). Endophytic actinobacteria possess the ability to inhibit or kill a wide variety of harmful pathogens, especially those from medicinal plants, (*Qin et al., 2011*).

The search for new endophytes led to discovery of new bioactive secondary metabolites of various biological activities such as antimicrobial, antifungal, antitumoral, immunosuppressive and anti-inflammatory (bioactive molecules extracted from the *Streptomyces* NRRL 30562, *Streptomyces* NRRL 30566 , *Streptomyces* sp. TP-A0595, *Streptomyces laceyi* and *Streptomyces* sp. CS endophytic strains have shown a broad spectrum activity against many fungi, bacteria, tumors pathogenic to humans and plants) (*Strobel, 2003, Qin et al., 2011*).

In this study we aim to isolate endophytic actinobacteria from three medicinal plants, such as , harmal (*Peganum harmala*), Rosemary (*Rosmarinus officinalis*) and R'tem (*Retama raetam*), collected in the region of Laghouat. The purified strains will be characterized morphologically and then tested for their biological activity.

BIBLIOGRAPHIC REVIEW

CHAPTER I.
ENDOPHYTIC ACTINOBACTERIA

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I.1. Diversity of endophytic actinobacteria in medicinal plants

Since the discovery of the endophytic taxol-producing fungus, *Taxomyces andreanae*, yew (*Taxus brevifolia*) from Montana, USA, there has been a push to explore plant endophytic microbes with therapeutic potential and applications (*Strobel and Daisy., 2003; Akshatha J.V et al., 2016*).

Actinobacteria taxa are diverse, composed of streptomyces and nonstreptomyces. Stems, leaves and roots are the preferred parts for the isolation of actinobacteria.

I.2. Endophytes as a source of secondary metabolites

The rhizosphere is rich in resources, it is an extremely competitive environment. The bacteria must survive among competitors and predators, the microflora in the rhizosphere will likely produce a rich arsenal of antibiotics compounds and anti-nematodes. In the other hand, endophytic bacteria face much less competition, which can be observed in a arsenal less rich in metabolites, but they may produce other specific supporting metabolites or those necessary for interaction with the host. However, many endophytic bacteria are facultative colonizers, thus they have to compete with the rhizosphere before entering in the plant and could therefore be equipped with a rich arsenal of metabolites involved in defense as well as in interaction with the plant (*Brader et al., 2014*).

CHAPTER II.
MEDICINAL PLANTS

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II.1. Generalities

Most of the medicinal plants still used today were discovered as early as antiquity or the Middle Ages. Many of them are even known since prehistoric times. If a plant has medicinal properties, it is because it contains active ingredients to treat a specific condition. The doctors of antiquity or the Middle Ages did not have the means to prove the efficiency of a plant; they relied mainly on the appearance of a plant species given and on the experience passed down from their ancestors. In India for example or China, medicinal plants are widely used and studied they are even the subject of a regulated culture (*Janati-Idrissi, 2015*).

A medicinal plant is a plant that is cultivated or gathered in its natural environment for its medicinal properties. Researchers estimated that there are around 400,000 plant species in the world, of which about a quarter or a third have been used by companies for medicinal purposes (*Benayad, 2013*). Their action comes from their chemical compounds (primary or secondary metabolites) or the synergy between the different present compounds (*Sanago, 2006*).

II.2. Interest of medicinal plants

The interest that aromatic and medicinal plants still have is that they are applied in industrial, medical, pharmaceutical and agricultural fields. Their ability to synthesize extraordinarily diverse secondary metabolites allows them to defend themselves by various physical and chemical means.

Many molecules, which have a defensive action of the plant against pests, have been identified. Thus, more than 2000 plant species endowed with insecticides properties have been listed (*Benayad, 2013*).

II.3. Botanical description of the species studied

II.3.1. *Peganum harmala*

II.3.1.1. Description of the species *Peganum harmala*

Peganum harmala is a plant of the Zygophyllaceae family, called in Arabic (الحرمل) Harmal is a perennial, glabrous, bushy plant 30 to 90 cm high with a thick rhizome, it has a strong, unpleasant odor reminiscent of the street. Stems erect, very branchy disappear in winter; they bear alternate leaves, cut into narrow strips. The solitary, fairly large (25-30 mm), yellowish-white, green-veined flowers are formed of: five green, linear, persistent sepals which exceed the corolla, five petals elliptical, ten to fifteen stamens with a very widened thread in their lower part, the ovary, globular, rests on a fleshy disc

CHAPTER II. MEDICINAL PLANTS

and results in a fruit which is a spherical capsule, three-celled, 6 to 8 mm depressed at the top, surrounded by persistent and opening sepals by 3 or 4 valves to release the seeds. Seeds: numerous, small, angular, subtriangular, dark brown in color, including the outer seed coat is reticulate, has a bitter flavor; they are harvested in summer (*Chopra et al., 1960; Quezel & Santa, 1963; Ozenda, 1977*).



Figure II.1 : Shrub of *Peganum harmala* (*Rezzagui, 2012*).

II.3.1.2. Classification in botanical systematics

According to (*Moghadam et al., 2010*), the classification of this species is as follows:

Table II.1 The classification of *Peganum harmala*

Kingdom	Plants
Phylum	Spermaphytes
Sub-phylum	Angiosperms
Class	Eudicots
Subclass	Malvides
Order	Sapindales
Family	zygophyllaceae
Genus	Peganum
Species	Peganum harmala

II.3.1.3. Origin and habitat

Cosmopolitan species very common on sandy soils and a little nitrated, it grows in Southern and Austro-Eastern Europe, Minor Asia, Tibet, Iran, Turkestan, Syria, Egypt and North Africa. (*Yousefi et al., 2009*).

CHAPTER II. MEDICINAL PLANTS

In Algeria, *P. harmala* is common in the high plateaus, in the northern, southern and the mountains of the central Sahara. It is renowned for sandy soils, in the wadi beds and inside towns (*Maire, 1933; Chopra et al. 1960 and Ozenda, 1991*).

II.3.1.4. Properties and uses

The plant has a medicinal interests where the seeds have long been used: they are narcotics, anthelmintics, antispasmodics and used in some cases against rheumatism and asthma (*Siddiqui et al., 1988; Bellakhdar, 1997*). It is known in traditional medicine as a remedy for a range of complaints (*Shi et al., 2000*). A red dye obtained from its seeds is widely used in Turkey and Iran for coloring carpets (*Baytop, 1999*) it has also been used traditionally to treat diabetes (*Bnouham et al., 2002*). *Peganum harmala* has been used by certain populations to treat certain system disorders such as Parkinson's disease (*Leporatti, 2009*), in psychiatric conditions like nervousness (*González et al., 2010*).

In traditional medicine from North Africa, harmel is a renowned panacea treated most disorders. Some "recipes" collected in the Maghreb to illustrate this diversity of jobs, are reported (*Hammiche et al., 2013*).

- Fresh plant juice: in liniment based on sheep fat, against articular pain.
- Poultices of chopped leaves, applied in situ and held in place by a scarf, against rheumatism, headache, fever, cough and lung disease, bites of snakes; applied to the soles of the feet as an emmenagogue and abortifacient.
- Fumigations based on dry plants or seeds for neonatal tetanus, rheumatism, female genital disorders, mental and nervous illnesses, adults and children insomnia (*Hammiche et al., 2013*).

II.3.2. *Rosmarinus officinalis*

II.3.2.1. Description of the species *Rosmarinus officinalis*

Rosmarinus officinalis grows spontaneously at the seaside. It is a shrub of the Lamiaceae family, it measures 50 cm to 1 meter and more, always green, very aromatic, very branchy, very leafy (leaves in needle-like whitish and fluffy from below). The flowers are pale blue or whitish. Its bark peels off older branches and its odor is extremely odorous and tenacious (*Makhloufi, 2009*) Flowering begins in February and continues until April-May. Like most Lamiaceae, the fruit is a tetramer (brown in color), ovoid, is surrounded by a persistent and dry calyx (*Belkhodja, 2016*).

CHAPTER II. MEDICINAL PLANTS



Figure II.2 : Shrub of *Rosmarinus officinalis* (Makhloufi, 2009)

II.3.2.2. Classification in botanical systematics

Table II.2 The classification of *Rosmarinus officinalis* (Touafek, 2010)

Kingdom	Plants
Phylum	Phanerogams
Class	Dicotyledons
Order	Lamiales
Family	Lamiaceae (Labies)
Subfamily	Nepetoideae
Tribe	Mentheae
Genus	Rosmarinus
Species	Officinalis

II.3.2.3. Origin and habitat

Rosemary (*Rosmarinus officinalis*), French name: Le romarin, Arabic name: الإكليل, also called the incense tree. It lives in the scrubland on limestone soil where it forms large clumps. It is also cultivated as a condiment herb, honey plant, phytotherapeutic plant as well as for perfumery. Its name means sea dew in Latin. The producing countries of this category of plant are: Spain, France, Algeria, Tunisia, Morocco, North America, Portugal, Yugoslavia and Egypt. This plant can withstand low temperatures when it snows and lasts for a long time, it is resistant to high degrees of salinity especially when it grows near coastal regions (Touafek, 2010).

CHAPTER II. MEDICINAL PLANTS

II.3.2.4. Properties and uses

In traditional medicine, rosemary helps digestion, treats headaches and migraines, bleaching, colic, improves liver and biliary functions and digestive disorders. It is used externally to treat rheumatism and circulatory disorders. It is hypoglycemic, it treats eye conditions and is used as an antiseptic, cholagogue, antispasmodic, vulnerary and diuretic (*Makhloufi, 2009*).

Rosemary is often cultivated for its aromatic oil and considered useful in controlling soil erosion. In traditional medicine, its aerial parts are used to relieve renal colic, dysmenorrhea (*Belkhodja, 2016*).

II.3.3. *Retama raetam*

II.3.3.1. Description of the species *Retama raetam*

Retames are shrub legumes, occupying arid, semi-arid and coastal, called dune fixing plants, their name derives from the biblical name (ROTEM) which was changed by the Arabs to (R'tem) or (retam) (*ZOHARY, 1962 ; SHALLABY et al, 1972*).

Retames are perennial plants, they are monoecious shrubs, which can reach up to 3 meters long, characterized by a stocky and short trunk, bearing many branches dense, arched, flexible and drooping, strongly furrowed and little leafy, the young shrubs are silky green to silvery gray (*BENISTON, 1985 et OZENDA, 1958*)

The root system is a swivel type that can reach several meters deep (*STOCKER, 1974*).

Adventitious roots are also present on the twigs and colonize the dune surface

The Retames have a great symbiotic capacity, being part of the fabaceae family, their roots end in small swellings called nodules, which are home to a very diverse microbial fauna. This symbiotic association allows them to fix atmospheric nitrogen and convert it to assimilable organic nitrogen (NO₃).



Figure II.3 : Shrub of *Retama raetam* (*Mekki, 2016*)

CHAPTER II. MEDICINAL PLANTS

II.3.3.2. Classification in botanical systematics

Table II.3 The classification of *Retama raetam* (Ozenda, 1991)

Kingdom	Plants
Phylum	Phanerogams
Sub-phylum	Angiosperms
Class	Eudicots
Subclass	Malvides
Order	Fabales
Family:	Fabacees
Genus:	Retama
Species:	<i>Retama raetam</i>

II.3.3.3. Origin and habitat

The retames are characterized by a wide geographical distribution, originating in North-West Africa (ZOHARY, 1959).

In Algeria the retames occupy a considerable area from North to South (THOMAS, 1968) et (STOCKER, 1974).

Retama raetam is located in the south of Oran, Bechar, south of Djelfa, Ain Safra, Touggourt, central Kabylia, east of Biskra (IGHIL, 1962) . also in Ouargla (ALLAL BENFEKIH, 2006).

It is a common plant in the arid ecosystems that surround the sea Mediterranean. This plant uses partial dormancy as a strategy for withstand long periods of drought (MITTLER., 2002).

II.3.3.4. Properties and uses

The retames play a very important role in maintaining balance natural environments and ecosystems, recognized as plants of arid and semi-arid areas.

Retames are dune fixing species, thanks to their system highly developed root. The roots of Rétama raetam penetrate up to 20 m deep in the ground (ZOHARY , 1962)

retames contribute to the bio-fertilization of saline and poor soils, and play an important role in the nitrogen cycle (FARCHICHI. A, 1996).

Retama has been listed as a medicinal plant from the regions arid

In traditional medicine, Retama raetam is used in the treatment of several diseases such as eczema, it is used in the south in the care of snakebites (El HAMROUNI A, 2001)

In addition *Retama reatam* has antioxidant, antimicrobial and cytotoxic activity (MAGHRANI.M et al., 2005).

CHAPTER III.
MATERIAL AND METHODS

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III.1. Sample collection

In order to isolate endophytic actinobacteria, we selected three plants, harmful (*Peganum harmala*), Rosemary (*Rosmarinus officinalis*) and R'tem (*Retama raetam*) using the help of an agronomic engineer **Mr Soukhal Djamel** in the sampling and identification.

The samples were collected from three different sites located in the wilaya of Laghouat (**Figure III.1**), **Table III. 1** shown the exact places of plants collections.

Healthy samples of roots and stems of each plant were collected and placed in sterile plastic bags, and brought immediately to the laboratory for further analysis.

Table III.1 Places and areas of plant collection

Plant	Family	Plant part	Collection location
<i>Peganum harmala</i>	<i>Zygophyllaceae</i>	Stem and Roots	Near Laghouat Stadium (33°47'10"N 2°51'24"E)
<i>Rosmarinus officinalis</i>	<i>Lamiaceae</i>	Stem and Roots	Brida forest (33°56'56"N 1°44'99"E)
<i>Retama raetam</i>	<i>Fabaceae</i>	Stem and Roots	Near Sidi Makhoulouf (34°01'33"N 2°41'59"E)

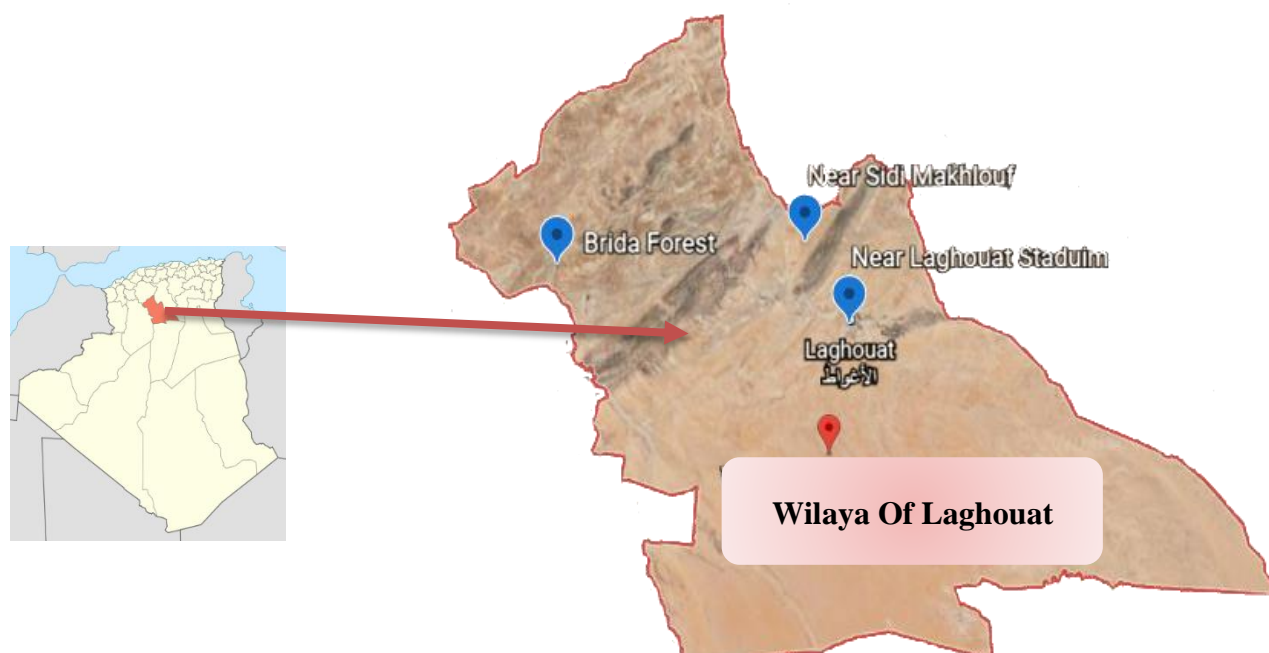


Figure III.1 : Geographical distribution of the samples collection areas

CHAPTER III. MATERIAL AND METHODS

III.2. Surface cleaning

According to *Hallmann et al. 2006* and *Coombs and Franco 2003*, the samples were air dried for 48 h at room temperature. Then washed with running tap water to completely remove the soil debris from the surface and adherent epiphytes. After drying, the samples were subjected to a surface sterilization procedure in five steps:

1. The samples were immersed in 70% ethanol for 5 minutes,
2. then soaked in (3% to 10%) of sodium hypochlorite for 2 minutes,
3. after that , they were immersed back in hydrogen peroxide for 1 minutes.
4. The fragments are then rinsed with distilled water three times.
5. Lastly, the plants tissues were soaked in 10% NaHCO₃ in order to inhibit fungal growth (*Nimnoi et al., 2010*)

III.3. cleaning efficiency

To check the efficiency of the surface cleaning, the plant tissue samples were cut, aseptically, with sterile scissors into small fragments, which are directly placed on the surface of the Tryptica soja agar medium : 17.0 g of Pancreatic digest of casein, 3.0 g of Papaic digest of soyabean meal, 5.0 g of Sodium chloride, 2.5 g of Dextrose (Glucose), 2.5 g of Dipotassium hydrogen phosphate, and 15.0 g of Agar , PH 7.4). The plates were then incubated at 28 ° C for 2 to 4 weeks (*Yuan et al., 2008*).

If no microbial growth has occurred on the surface of the media, the sterilization is considered as successful (*Schulz et al., 1993; Qin et al., 2009*)

III.4. Isolation of Culturable Endophytic Actinobacteria

In order the isolate endophytic actinobacteria, 10 g of the clean sample were crushed using mortar, to which, 90 ml of sterile physiological water were gradually added and well mixed.

The suspension was transferred into a screw-cap test tube (16.5×105 mm) and centrifuged at room temperature for 20 min at 1,500×g to eliminate plant debris.

Aliquots of 0.1 mL of supernatant phase were spread on duplicate onto agar plates of four different mediums:

Humic acid-vitamin agar (HV media) : 1.0 g of Humic acid, 0.5 g of Na₂HPO₄, 1.71 g of KCl, 0.05 g of MgSO₄.7H₂O, 0.01 g of FeSO₄.7H₂O, 0.02 g of CaCO₃, minor B-vitamins, 50 ng of Cycloheximide, 18 g of Agar per liter of distilled water pH= 7.2.

CHAPTER III. MATERIAL AND METHODS

Starch Casein Agar(SCA media): 1.0 g of Casein powder, 10.0 g of Starch, 37.0 g of Sea water, 15.0 g of Agar , pH=7.3.

GAUZE's medium (GM) : (20.0 g of soluble starch, 1.0 g of KNO₃, 0.5 g of K₂HPO₄, 0.5 g of MgSO₄ 7H₂O, 0.5 g of NaCl, 0.01 g of FeSO₄, 10.0 g of Agar and 1000 mL of distilled water ,pH=7.4.

Tap water-yeast extract (TWYE): 5.0 g of yeast extract, 2 g of K₂HPO₄, and 18 g of Agar per liter of tap water. All media were supplemented with 50 mg/l of cycloheximide to inhibit fungal growth.

The plates were incubated at 30°C for 4 weeks. The actinomycetes were selected, based on their morphological characteristics; the powdery and filamentous colonies suspected of being actinobacteria were spotted, and then transferred to a new culture medium to obtain a pure culture (*Yuan et al., 2008*).

Unfortunately, our research project was stopped at this stage due to the covid-19 pandemic. The following titles are the tests that we were planning to do achieve.

III.5. Evaluation of antimicrobial activity

III.5.1. Preparation of test bacteria suspension

To screening of antimicrobial activity, seven bacteria, such as: *Staphylococcus aureus* (ATCC 25923) *Bacillus cereus* (ATCC 11778) *Klebsiella pneumonia* (ATCC 70603) *Escherichia coli* (ATCC 25522) *Bacillus subtilis* (ATCC 6633) *Candida albicans* (ATCC 10231) (fungi) *Pseudomonas aeruginosa* (ATCC 27853), were used as test microorganisms.

Table III.2 Characteristics of the bacterial strains tests

Bacterial strains	Gram
<i>Staphylococcus aureus</i> (ATCC 25923)	+
<i>Bacillus cereus</i> (ATCC 11778)	+
<i>Klebsiella pneumonia</i> (ATCC 70603)	-
<i>Escherichia coli</i> (ATCC 25522)	-
<i>Bacillus subtilis</i> (ATCC 6633)	+
<i>Pseudomonas aeruginosa</i> (ATCC 27853)	-
<i>Candida albicans</i> (ATCC 10231) (fungi)	

The preparation of the suspension for each strain was undertaken, after cultivation of bacteria and fungi in nutrient agar and Sabouraud Dextrose Agar respectively, for 24 hour. After 24 h incubation at 37 °C for bacteria, and 48 h incubation at 30 °C for fungi, the optical density were adjusted between

CHAPTER III. MATERIAL AND METHODS

0.08 to 0.1 (measured at 600 nm), which corresponding to the microbial charge rang between 10^6 CFU/ml to 10^8 CFU/ml.

III.6. Primary screening by cylinders agar method

The actinomycetes strains were inoculated on **5254** agar plates medium: Glucose : 15,0 g/l ; Soymeal 15,0 g/l ; Corn steep 5,0 g/l ; CaCO_3 2,0 g/l ; NaCl 5,0 g /l ; Distilled water: 1000 ml. pH = 7,2). Actinomycetes strains are seeded in tight streaks on the surface of 15 ml of **5254 medium**. After 7 days incubation at 28 ° C, 6 mm in diameter agar cylinders were taken and deposited on the surface of the Muller-Hinton medium previously seeded with bacteria tests. The plates were then placed at 4°C for two to four hours to allow diffusion of the active substances, then they are incubated at the temperature of 37°C for 24 hours.

After incubation, the presence of an inhibition zone is observed around the discs of actinomycetes, which indicate the production of active secondary metabolites against the test strain. The diameter of inhibition is measured in millimeters, using a graduated ruler. The absence of inhibition zones around the agar discs, indicates a negative result which shows that bacteria are resistant to substances produced by actinomycetes. More the inhibition zone is big, the greater the antibacterial activity as well. (*Petrosyan et al., 2003*).

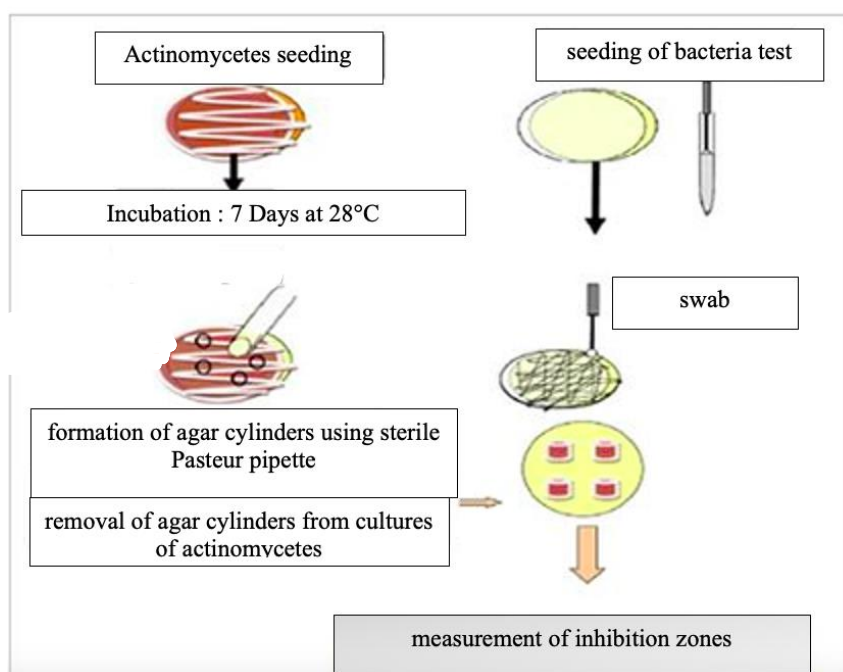


Figure III.2 : Schematic of the Agar Cylinder Technique

CHAPTER III. MATERIAL AND METHODS

III.7. secondary screening

III.7.1. Extraction of secondary metabolite from actinomycetes culture

The strains which showed good antibacterial activity were transferred into **5254 liquid medium** using a sterile inoculating loop. The cultures were incubated at 30°C for 5-10 days at 160-180 rpm on the shaker. After the incubation period, the sterility is tested via light microscopy. In the case of a sterile culture, the following procedures is used for extract:

1. 20ml of a 5 day old culture is mixed with 20ml of ethyl acetate in a 50ml Falcon tube
2. After a 30 min shaking step, the tubes are once again mixed for 10 min in a rotary shaker
3. Afterwards the samples are centrifuged at 9000rpm for 10 min and the upper phase is transferred into a 50ml evaporation flask
4. At about 40°C the ethyl acetate is evaporated in a rotary evaporator
5. Finally the extract is solved in 1 ml of ethyl acetate: acetone: methanol (1:1:1) and centrifuged at 14000 rpm for 10 min.

Note: the evaporation flask is weighed before and after the evaporation in order to determine the extract concentration .

III.7.2. MIC by manual microdilution in liquid medium (96 well microplate technique)

This technique is based on the cultivation of a standardized bacterial inoculum in the presence of series of 1:2 dilutions of the extract. The 96 well microplate allow the testing of 8 increasing concentrations of different antibiotics. After 18 to 24 hours of incubation, the well were examined, with naked eyes, for visible bacterial growth This technique provides quantitative results. The MIC corresponds to minimal inhibitory concentration of crud extract, which inhibit visible growth of the microorganisms (*Jorgensen JH et al .,2009*)

CONCLUSION AND PERSPECTIVES

CONCLUSION AND PERSPECTIVES

This study consists on the isolation and purification of endophytic actinobacteria from three medicinal plants {Harmal (*Peganum harmala*), Rosemary (*Rosmarinus officinalis*) and R'tem (*Retama reatam*) }, collected from the wilaya of Laghouat.

Given the health situation of our country and that of the whole world because of the covid-19 pandemic our work has stopped at the purification phase , but the results of previous researches revealed that, endophytic actinobacteria has antibacterial power with varying inhibition degrees , on the selected test bacteria.

Given the immense biodiversity that our country has , where each plant is characterized by a fairly large reservoir of secondary metabolites with specific therapeutic and pharmacological characteristics that need to be exploited by researchers, and the great potential offered by endophytic actinobacteria, we propose as perspectives to:

- Finish the undone tests of anti tick and insecticidal activity in order to get proper results about the biological activity of endophytic bacteria isolated from the previously mentioned plants;
- Identify new natural bioactive substances that could respond to different health problems and also offers an alternative to synthetic drugs, which allows to avoid the ecological consequences that can be caused by the overexploitation of certain plant species;
- Orient scientific researches towards carrying out complementary studies of the antibacterial activity of isolated endophytic actinobacteria in order to develop drugs based on these plants;
- Explore new strategies and molecular techniques in order to isolate new strains or improve the production efficiency of their bioactive substances by acting directly on the strains or by optimizing the conditions of their culture.

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PBqqmoUjaKu7horRfhZ5MWsx-OFHMQYBTLUBHbcWDsboILUGg5Q#metadata_info_tab_contents

ANNEX

	Media Name	Media Composition	References
1	Humic acid vitamin Agar (HV media)	1.0 g of Humic acid, 0.5 g of Na ₂ HPO ₄ , 1.71 g of KCl, 0.05 g of MgSO ₄ .7H ₂ O, 0.01 g of FeSO ₄ 7H ₂ O, 0.02 g of CaCO ₃ , minor B-vitamins, 50 ng of Cycloheximide, 18 g of Agar per liter of distilled water, PH= 7.2	<i>Hayakawa (1990)</i>
2	Starch Casein Agar (SCA media)	1.0 g of Casein powder, 10.0 g of Starch, 37.0 g of Sea water,15.0 g of AgarPH=7.3	<i>Ku`ster and Williams (1964a,1964b)</i>
3	GAUZE's medium(GM)	20.0 g of soluble starch, 1.0 g of KNO ₃ , 0.5 g of K ₂ HPO ₄ , 0.5 g of MgSO ₄ 7H ₂ O, 0.5 g of NaCl,0.01g of FeSO ₄ , 10.0 g of Agar and 1000 mL of distilled water, pH=7.4	<i>Ivantiskaya et al. (1978)</i>
4	Tap water yeast extract (TWYE)	5.0 g of yeast extract, 2 g of K ₂ HPO ₄ , and 18 g of Agar per liter of tap water	<i>Crawford et al. (1993a,1993b); Qin et al.(2009a,2009b,2009c)</i>
5	Tryptic soya Agar (TSA)	17.0 g of Pancreatic digest of casein, 3.0 g of Papaic digest of soyabean meal, 5.0 g of Sodium chloride, 2.5 g of Dextrose (Glucose), 2.5 g of Dipotassium hydrogen phosphate and 15.0 g of Agar)	<i>Li et al. (2008a,2008b)</i>
6	Muller Hinton	3g of Meat extract, 17.5g of Acid hydrolysate of casein, 1.5g of Starch, 16g of Agar, 1000ml of Distilled water, pH = 7.3	<i>Mueller, J. H.; Hinton, J. (1 October 1941)</i>
7	Sabouraud broth	Peptones 10,0 g Glucose 40,0 g Agar 15,0 g, pH = 5,6	<i>LARONE D.H 1995</i>
8	5254 media	Glucose : 15,0 g/l ; Soymeal 15,0 g/l ; Corn steep 5,0 g/l ; CaCO ₃ 2,0 g/l ; NaCl 5,0 g /l ; Distilled water : 1000 ml, pH = 7,2	<i>Wink, J 2009</i>