الجمهورية الجزائرية الديمقراطية الشعبية
DEMOCRATIC AND POPULAR REPUBLIC OF ALGERIA
وزارة التعليم العالي والبحث العلمي
MINISTRY OF HIGHER EDUCATION AND SCIENTIFIC RESEARCH
جامعة مولاي الطاهر سعيدة
UNIVERSITY MOULAY TAHER SAIDA
Laboratory of Biotoxicology, Pharmacognosy and Biological Valorization of Plants

Faculty of sciences
Department of biology

SUBMITTED FOR THE DEGREE OF:
MASTER OF SCIENCES

In Biology
Option: Applied Microbology

THEME

Antibiotic resistance and Antibacterial activity of *Sausurea lappa*, *Ajuga iva* and *Rosmarinus officinalis* extracts. Plus an expanding on Bioinformatics Data Integration & Annotattion.

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Discussed on: 17th September 2020

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2019/ 2020

In the name of Allah, the Beneficent, the Merciful

" But you may hate a thing although it is good for you, and may love a thing although it is evil for you. Allah knows, and you do not. "

AL-BAQARA (216)

Acknowledgements

In the name of Allah the most Merciful and Beneficent

First and Foremost praise is to **ALLAH**, the Almighty, the greatest of all, onwhom ultimately we depend for sustenance and guidance. I would like to thank Almighty Allah for giving me opportunity, determination and strength to do myresearch. His continuous grace and mercy was with me throughout my life andever more during the tenure of my study .

Now, I would like to thank and express my deep and sincere gratitude to my supervisor **Pr. RACHEDI** in Structural Biology & Bioinformatics, saida university, for the proposed theme. for his continuous support, guidance and encouragement. Inaddition to being an excellent supervisor.

I would also like to express my gratitude to my co-supervisor **Dr. BENREGUIEG** ,Assistant professor, of microbiology, Moulay taher saida university, for his continuous support and for his valuable guidance and suggestions. Inaddition to being an excellent co-supervisor, he is a man of principles and I appreciateall his contributions of time, support and ideas. Indeed,his quick feedback and constructive comments were really inspiring and helpful.

Although the work is not completed due to the propblems and delays imposed by the COVID-19 pandemic, I would like to thank the **laboratory technician** of microbiology for guide me and help me in the laboratory work in that short period .

Finally, I also appreciate the support of all teachers of microbiology, University of saida during my study years .

Dedication

To my family especially my mother and my late father who supported me all the way since the beginning of my life. To my brothers sisters and friends who havebeen a great source of motivation and inspiration.

Abstract

The emergence and spread of antibiotic resistance, as well as the evolution of new strains of disease causing agents, are of great concern to the global health community. Effective treatment of a disease entails the development of new pharmaceuticals or some potential source of novel drugs. Commonly used medicinal plants of our community could be an excellent source of drugs to fight off this problem.

It should be noted that this work falls under a larger project, started last year, researching the problem of Antibiotics Resistance seen in a number of dangerous bacteria isolated from local environment. While the project investigates the Antibiotics Resistance of the studied bacteria against commonly prescribed antibiotics, the project also researches for Antibacterial compounds of plant origins that would represent potent alternative antibiotics against the currently hard curring diseases. This project has a bioinformatics side to it which would work on annotating and database storing the various types of used data and generated results and provide them to the scientific community worldwide.

Three medicinal plants were selected based on medicinal reports belonging to two families in the form of leaves of *rosmarinus officinalis* (Lamiaceae), roots of *saussurea lappa* (Asteracea) and the aerial part of *ajuga iva* (Lamiaceae). Preliminary phytochemical screening of the crude extracts revealed the presence of alkaloids, tannins, flavonoids and quinones and essential oils in saussurea lappa . Several polyphenols (tannins and anthocyanins) and flavonoids in the aerial part of ajuga iva have been detected. Rosmarinus officinalis, contains tannins, flavonoids, quinones and essential oils .From the results obtained, we were able to get a general idea of the chemical composition of the three plant studied. Indeed, we can conclude that Saussurea lappa is more rich in secondary metabolites, and we noted that Rosemary is more productive than other plants in all the extraction processes.

Unfortunately, the COVID-19 pandemic problems have had its toll on many facets of this project, however, the data and results of this project would be valorized and the useful parts of would be annotated into the online dabatase BARID (Bacterial Antibiotic Resistance Investigation Database) and be made available to query by the scietific community via the web-address: http://www.bioinformaticstools.org/prjs/barid/

Keywords: Antibiotic resistance, Medicinal plants, Phytochemicals, Antibacterial activities, Secondary metabolites, Antibiotics.

Résumé

L'émergence et la propagation de la résistance aux antibiotiques, ainsi que l'évolution de nouvelles souches d'agents pathogènes, sont une préoccupation majeure pour la communauté sanitaire mondiale. Le traitement efficace d'une maladie implique le développement de nouveaux produits pharmaceutiques ou d'une source potentielle de nouveaux médicaments. Les plantes médicinales couramment utilisées dans notre communauté pourraient être une excellente source de médicaments pour lutter contre ce problème.

Il convient de noter que ce travail s'inscrit dans le cadre d'un projet plus vaste, lancé l'année dernière, qui étudie le problème de la résistance aux antibiotiques observée dans un certain nombre de bactéries dangereuses isolées de l'environnement local. Alors que le projet étudie la résistance aux antibiotiques des bactéries étudiées contre les antibiotiques couramment prescrits, le projet recherche également des composés antibactériens d'origine végétale qui représenteraient de puissants antibiotiques alternatifs contre les maladies actuellement difficiles. Ce projet a un côté bioinformatique qui travaillerait sur l'annotation et la base de données stockant les différents types de données utilisées et les résultats générés et les fournirait à la communauté scientifique du monde entier.

Trois plantes médicinales ont été sélectionnées sur la base de rapports médicinaux appartenant à deux familles sous forme de feuilles de *rosmarinus officinalis* (Lamiaceae), de racines de *saussurea lappa* (Asteracea) et de la partie aérienne d'*ajuga iva* (Lamiaceae). Le criblage phytochimique préliminaire des extraits bruts a révélé la présence d'alcaloïdes, de tanins, de flavonoïdes et de quinones et d'huiles essentielles dans la saussurea lappa. Plusieurs polyphénols (tanins et anthocyanes) et flavonoïdes dans la partie aérienne d'ajuga iva ont été détectés. Rosmarinus officinalis, contient des tanins, des flavonoïdes, des quinones et des huiles essentielles. À partir des résultats obtenus, nous avons pu nous faire une idée générale de la composition chimique des trois plantes étudiées. En effet, nous pouvons conclure que Saussurea lappa est plus riche en métabolites secondaires, et nous avons constaté que le romarin est plus productif que les autres plantes dans tous les processus d'extraction.

Malheureusement, les problèmes de pandémie de COVID-19 ont eu des conséquences néfastes sur de nombreuses facettes de ce projet, cependant, les données et les résultats de ce projet seraient valorisés et les parties utiles de seraient annotées dans la base de données en ligne dabatase BARID (Bacterial Antibiotic Resistance Investigation Database) et être mis à disposition pour interrogation par la communauté scientifique via l'adresse Web: http://www.bioinformaticstools.org/prjs/barid/

Mots clés: Résistance aux antibiotiques, Plantes médicinales, Phytochimiques, Activités antibactériennes, Métabolites secondaires, Antibiotiques.

ملخص

إن ظهور مقاومة المضادات الحيوية وانتشارها ، فضلاً عن تطور سلالات جديدة من العوامل المسببة للأمراض ، هي مصدر قلق كبير لمجتمع الصحة العالمي. يستلزم العلاج الفعال للمرض تطوير أدوية جديدة أو مصدر محتمل لأدوية جديدة. يمكن أن تكون النباتات الطبية شائعة الاستخدام في مجتمعنا مصدرًا ممتازًا للأدوية لمحاربة هذه المشكلة. تعود الفوائد المضادة للبكتيريا للنباتات المهمة طبيًا بشكل أساسي إلى المواد الكيميائية النباتية النشطة بيولوجيًا المنتجة في المستقلبات الثانوية في أنسجة النبات.

تجدر الإشارة إلى أن هذا العمل يندرج ضمن مشروع أكبر بدأ العام الماضي للبحث في مشكلة مقاومة المضادات الحيوية التي شوهدت في عدد من البكتيريا الخطرة المعزولة من البيئة المحلية. بينما يبحث المشروع في مقاومة البكتيريا المدروسة للمضادات الحيوية ضد المضادات الحيوية الموصوفة بشكل شائع ، يبحث المشروع أيضًا عن مركبات مضادة للبكتيريا من أصول نباتية من شأنها أن تمثل مضادات حيوية بديلة فعالة ضد الأمراض الصعبة الحالية. يحتوي هذا المشروع على جانب المعلوماتية الحيوية فيه والذي سيعمل على وضع التعليقات التوضيحية وقاعدة البيانات وتخزين الأنواع المختلفة من البيانات المستخدمة والنتائج المتولدة وتقديمها إلى المجتمع العلمي في جميع أنحاء العالم.

تم اختيار ثلاثة نباتات طبية بناءً على تقارير طبية تنتمي الى عائلتين في شكل أوراق بالنسبة الى اكليل الجبل ، جذور القسط الهندي و الجزء العلوي لشندقورة . تم تجفيف النباتات في الظل ، و سحقها واستخلاصها باستخدام مذيب الميثانول. الفحص الكيميائي النباتي الأولي للمستخلصات الخام عن وجود قلويدات، عفص، كينونات، وفلافونيدات وزيوت عطرية في القسط الهندي . تم اكتشاف العديد من مادة البوليفينول (التانينات والأنثوسيانين) والفلافونويد في الجزء الجوي من شندقورة . كما يحتوي اكليل الجبل على التانينات والفلافونويد والكينون والزيوت الأساسية. من النتائج التي تم الحصول عليها ، تمكنا من الحصول على فكرة عامة عن التركيب الكيميائي للنباتات الثلاثة المدروسة. يمكن ان نستنتج أن الأكثر ثراء في المستقلبات الثانوية هو القسط الهندي . و لاحظنا ان اكليل الجبل هو الأكثر أنتاجية في جميع عمليات أستخلاص.

لسوء الحظ ، أثر وباء كوفيد- ١٩ وتداعياته سلبًا على العديد من جوانب هذا المشروع ، ومع ذلك ، فإن بيانات المشروع، المتعلقة بالبكتيريا المختارة للبحث والمضادات الحيوية المختبرة والعلاجات العشبية والمركبات النشطة المضادة للبكتيريا، سيتم تقييمها لاحقا ثم تخزين ذا العلاقة منها في قاعدة بيانات "باريد" (قاعدة بيانات التحقيق في مقاومة المضادات الحيوية البكتيرية) وإتاحتها للاستعلام والإستخدام الأكاديمي عنوان الويب التالي: http://www.bioinformaticstools.org/prjs/barid/

الكلمات المفتاحية : مقاومة المضادات الحيوية ، النباتات الطبية ، المواد الكيميائية النباتية ، الأنشطة المضادة للبكتيريا ، المستقلبات الثانوية ، المضادات الحيوية.

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LIST OF ABBREVIATION

ABR : Antibiotic resistance

AACs : Chloramphenicol acetyltransferases

AGE's : Aminoglycoside modifying enzymes

AMEs : Aminoglycoside-modifying enzymes

AQE : Aqueous extract (distilled water)

ANSI : American National Standard Institute

AS : Aggregation substance

CDC : Centers for Disease Control and Prevention

CDI : Clostridium difficile infection

CF : Cystic Fibrosis

CGI : Common Gateway Interface

CRE : Carbapenem-resistant Enterobactericeae

CRPA : Carbapenem Resistant P. aeruginosa

DNA : Deoxyribonucleic acid

DBMS : database management system

EPEC : Enteropathogenic Escherichia Coli

EPs : Efflux pumps

EPS : extracellular polymeric substance

ERM : Erythromycin ribosomal methylation

ESBL : Extended-spectrum β-lactamases

ESP : Enterococcal Surface Protein

HGT : Horizontal Gene Transfer

HME : Hydromethanolic extract (methanol - water)

HTML : hypertext mark-up language

KIA : kligler iron agar

KP : Klebsiella pneumoniae

LPS : Lipopolysaccharide

MDR : Multidrug resistance

ME : Methanolic extract – (methanol)

MRSA : Methicillin-resistant Stapholococcus aureus

MTB : Mycobacterium Tuberculosis

PBPs : Penicillin binding proteins

PHP : Hypertext Preprocessor

PDR : Pandrug resistant or pan-resistant

PPL : Priority Pathogen List

QS : Quorum-sensing

RNA : Ribonucleic acid

RDBMS : Relational Database Management System

SCC : Staphylococcal chromosomal cassette

SQL : Structured Query Language

XDR : Extensive drug resistant

WHO : World Health Organization

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INTRODUCTION

Antibiotics have saved the lives of millions of people and have contributed to the major gains in life expectancy over the last century. However, the clinical efficacy of many existing antibiotics is being threatened by the emergence of multi-drug resistant (MDR) pathogens **(Bandow et al., 2003).** the recent appearance of strains with reduced susceptibility as well as, undesirable side effects of certain antibiotics . Infectious diseases caused by resistant microorganisms are associated with prolonged hospitalizations, increased cost, and greater risk for morbidity and mortality.

The promiscuous use of antibiotics accounts for a major part of the community burden of antibiotic use and contributes dramatically to the rising prevalence of resistance among major human pathogens. Vancomycin-resistant enterococci (VRE), methicillin-resistant Staphylococcus aureus (MRSA), MDR Mycobacterium tuberculosis and MDR Gram-negative bacteria are recognised as the most difficult healthcare-associated infections to control and treat. The development of extended-spectrum β-lactamases (ESBLs) and carbapenemases that target Gram-negative bacteria has resulted in infections that can be extremely difficult to treat leading to substantial increased illnesses and death rate. The effect is pronounced in third world as the costly replacement drugs for treating the highly resistant infectious diseases are unaffordable **(World Health Organization, 2008) .**

The resistance problem demands that a renewed effort be made to screen various medicinal plants for their potential antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds which are capable of producing definite physiological action on body. Another driving factor that encouraged scientists to search for new antimicrobial substances from various sources including medicinal plants has been the rapid rate of plant species extinction.

Despite abundant literature on the antimicrobial properties of plant extracts, none of the plant derived chemicals have successfully been exploited for clinical use as antibiotics. A significant part of the chemical diversity produced by plants is thought to protect plants against microbial pathogen. Hence, they have been proven to have antimicrobial importance both in vivo and in vitro **(Gibbons, 2004).**

This research was designed to study the antimicrobial potentiality of three medicinal plants Saussurea lappa (cust), Ajuga iva (Chendgoura) and Rosmarinus officinalis (rosemary). Phytochemical screening was carried out to identify major biologically active phytoconstituents. It is hoped that these active constituents will provide useful information for discovering new compounds with better activity against MDR bacteria than agents currently available .

The research has the following structure and specific focus:

- **Section I :** gives an overview of antibiotics work and how antibiotic resistance occurs in bacteria .
- **Section 2 :** summarizes the Resistance to antibacterial drugs in selected bacteria of international concern based on a systematic review of the scientific literature.
- **Section 3 :** Includes information briefly on medicinal plants used the roots of Saussurea lappa , the aerial part of Ajuga iva and leaves Rosmarinus officinalis
- **Section 4** : Bioinformatics part.
- **Section 5** : Includes the experimental part :
    1. The first part is related to the wet-laboratory research work which includes Bacteriology and Phytochemicals screening .
    2. This part was not completed due to the Covid 19 quarantine wich includes Anti-bacterial activity of the three plant extracts experiments.
- **Section 6 :** discusses the few results obtained in the experimental part.


Section
01

MULTIDRUG-RESISTANT

PSEUDOMONAS AERUGINOSA

Antimicrobial resistance (AMR): represents one of the most concerning threats to global health. AMR occurs when microorganisms are able to survive in the presence of drugs that would normally inhibit their growth.

I. ANTIBIOTICS:

1. A breif historical perspective :

Selman Waksman, a prominent researcher in the field of actinomycetes in the early part of the twentieth century, described the term antibiotic as a chemical compound generated from microorganisms that inhibits or destroys other microbes **(Hopwood, 2007; Davies and Davies, 2010).** Most antibiotics in use today originated from the phylum Actinobacteria with nearly 80% of actinobacterial-derived antibiotics produced by soildwelling bacteria of the genus Streptomyces **(Barka et al., 2016).** Before the discovery of natural antibiotics, synthetic compounds, including salvarsan, sulfa drugs and quinolones, were in use as chemotherapeutic agents (**Aminov, 2010).** Penicillin was the first natural antibiotic to be discovered accidentally by Alexander Fleming in 1928 when the Penicillium fungus contaminated a culture plate in his laboratory, however, penicillin was not developed for use until the late 1930s **(Hopwood, 2007).** Penicillin inhibits cell wall synthesis and was found to be very effective against Gram-positive but not against Gramnegative bacteria (due to the presence of the outer membrane) or the tubercle bacillus (because of the extra thick cell wall) **(Hopwood, 2007)**.

Following the discovery of penicillin by Fleming, other scientists, including Rene Dubos and Selman Waksman, started a deliberate search for antibacterial agents among soil microorganisms, including bacteria and fungi. It was soon realized that antibacterial activity was most often present in actinomycete cultures and less often in other bacteria or fungi. During this period, several antibiotics were discovered in the screens designed by these scientists but many of these were of little use in the clinic due to their toxicity in animals. The next biggest discovery came about in 1943, resulting in identification of streptomycin produced by Streptomyces griseus. Streptomycin inhibits protein synthesis by binding to the 30S subunit of the prokaryotic ribosome and was found to be effective not only against Gram-negative bacteria but also against the tubercle bacillus **(Hopwood, 2007).** With the discovery of streptomycin, the golden age of antibiotic discovery and development (1940–1990) ensued. This involved efforts of many academic institutions and major pharmaceutical companies in the United States and other countries. Currently, antibiotics affecting almost every process in the bacterial cell are known.

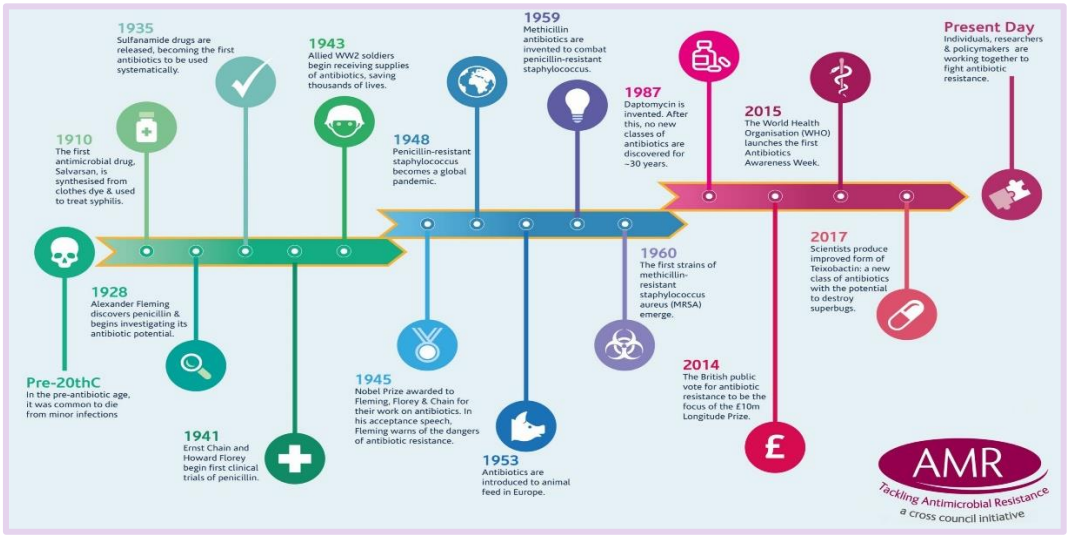


Figure 1: antimicrobial time line resistance.

2. Mechanisms of action :

Generally, antibiotics act by killing bacteria (bactericidal) or inhibiting their growth (bacteriostatic). Typical examples of bacteriostatic antibiotics are chloramphenicol, tetracyclines or macrolides, while ß-lactam antibiotics, fluoroquinolones or nitrofurantoin are bactericidal antibiotics. To enforce their inhibitory effects, antibiotics need to disturb central cellular processes, without exerting harmful effects to the patient. This can for example be achieved by inhibiting an enzyme or pathway that is essential in bacteria, but not in eukaryotic cells. Alternatively, antibiotics can inhibit targets that diverged from their homologues in eukaryotes to such an extent that a specific binding can be achieved to the bacterial variant alone. Figure 1 summarizes the most common cellular targets of antibiotics in bacteria.

The bacterial cell wall is a major target for many antibiotics, including all ßlactams. It is essential to bacteria but not present in eukaryotic cells which reduces the risk of strong cytotoxic effects. The bacterial cell wall consists of an inner membrane, a periplasmic space and a thick peptidoglycan layer (Gram-positives) or a thin peptidoglycan layer followed by an outer membrane (Gram-negatives). The peptidoglycan layer itself is composed of Nacetylglucosamine and N-acetylmuramic acid disaccharides which are crosslinked via pentapeptides **(Vollmer and Bertsche, 2008)**. The enzymes involved in peptidoglycan biosynthesis are inhibited by ß-lactam antibiotics causing a loss of structural integrity of the bacterial cell and eventually cell lysis. Besides inhibition of enzymatic catalysis, some antibiotics can cause cell death by disruption of membrane integrity **(Sato and Feix, 2006).**

This is a common mechanism for antimicrobial peptides, including polymyxins. These molecules can directly interfere with the physical properties of the membrane and cause bacterial lysis **(Epand et al., 2016) .**

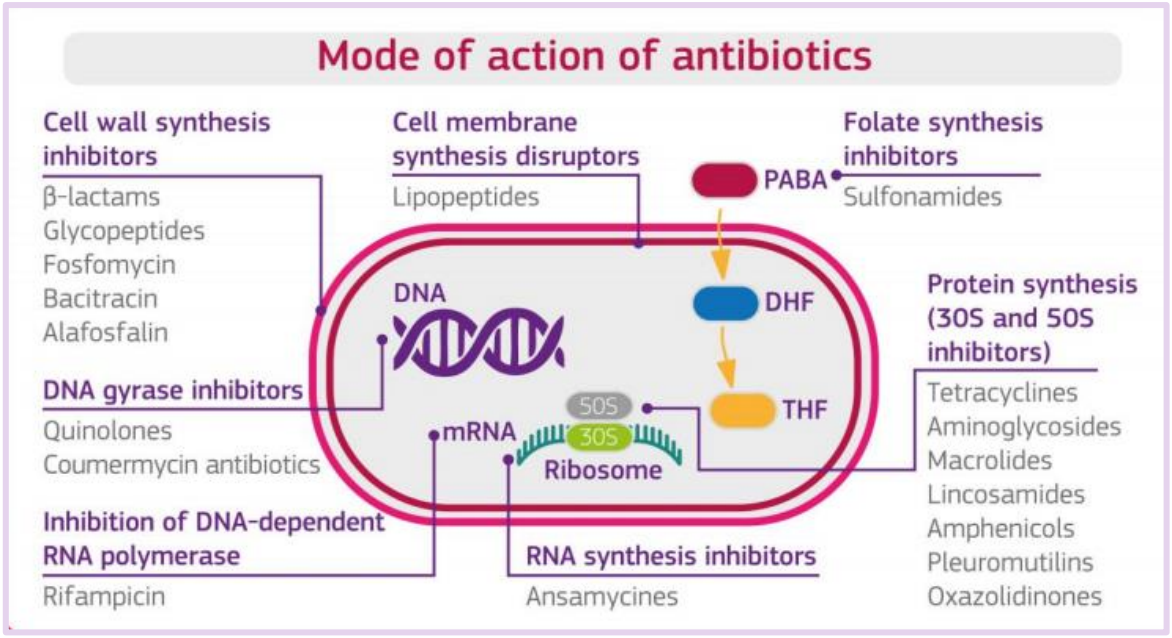


Figure 2 : Common antibiotic targets in the bacterial cell.

Proteins involved in transcription and translation are also targeted by antibiotics. Rifampicin, for example, binds to the bacterial RNA polymerase and inhibits elongation of the newly synthesized transcripts **(Campbell et al., 2001)** . Aminoglycoside antibiotics bind the small ribosomal subunit and disturb the elongation of newly synthesized amino acid chains resulting in accumulation of mis-translated proteins and eventually cell death **(Mingeot et al., 1999).** Another group of antibiotics that target the ribosome are the macrolides. Unlike aminoglycosides, these molecules bind to the large subunit and block the exit tunnel. This binding also disrupts protein synthesis and inhibits growth **(Menninger 1995)**. Fusidic acid prevents the translocation of elongation factor G, which is an essential step during protein synthesis. Quinolone antibiotics directly target DNA replication by inhibiting DNA gyrases and topoisomerase IV enzymes. This results in erroneous unwinding of DNA, introduction of double strand breaks and cell death **(Andriole 2000).** Trimethoprim and sulfonamides interfere with the synthesis of tetrahydrofolic acid, an essential precursor for thymidine synthesis, by inhibition of dihydrofolate reductase or dihydropteroate synthetase, respectively **(Brogden et al., 1982).**

II. ANTIBIOTICS RESISTANCE :

The origin of genes for antibiotic resistance is due to a natural process . The source could be genes encoding resistance in the antibiotic producing bacteria themselves as a mechanism for their own protection or generally due to spontaneous mutations in the bacterial chromosome. The spontaneous mutation frequency for antibiotic resistance is on the order of about 10^8 -10^9 . Whilst mutation is a rare event, it does not take long for resistance to develop in a bacterial population owing to the fast growth rate of bacteria and the absolute number of cells attained **(Tiwari and Tiwari 2011).**

Once the development of resistance has occurred, the mutated gene is directly transferred to the bacteria's progeny during replication. In the selective environment of the antibiotic, the wild type are killed and the resistant mutant allowed to flourish, influenced by the rate and pattern of antibiotic use (selective pressure) and influence of the particular resistance on bacterial fitness . Resistance to penicillin in S. aureus was observed as early as 1942 after penicillin came into use **(Davies and Davies 2010).**

As the next generations of antibiotics were developed to overcome the problems of resistance against the available ones, bacteria developed resistance mechanisms to the newer antimicrobial agents **(Behera, 2010).** For example, the production of an enzyme penicillinase by S. aureus led to penicillin resistance initially. To resist penicillinase, cloxacillin was developed. To contest this antibiotic, the bacteria altered the target site for binding of b-lactam antibiotics i.e. the penicillin binding proteins (PBPs) and this led to the development of MRSA. Presently the bacteria have been reported to be resistant to not only methicillin but also chloramphenicol, macrolides, aminoglycosides, tetracycline and lincosamides **(Nikaido, 2009).**

A recent database lists more than 20,000 potential resistance genes of nearly 400 different types, predicted mainly from available bacterial genome sequences **(Liu and Pop, 2009).** Fortunately, the number existing as functional resistance determinants in pathogens is much smaller **(Davies and Davies, 2010).**

1. Type of antibiotic resisteance :

Resistance is the ability of a bacteria against the antogonizing effect of an antibacterial agent upon reproduction prevention or bactericidal. The development of resistance to antibiotics in bacteria often develop as a result of unnecessary and inappropriate use of antibiotics.

Through the intense use of antibiotics, resistant microorganisms have emerged over the years, and problems were started to be experienced for the treatrment of these infections emerged with these resistant microorganisms. Today, on the one hand trying to develop new drugs, on the other hand, there are difficulties in treatment as a result of development of resistance to these drugs rapidly. The development of resistance to antibiotics is a major public health problem in all over the world **(Yüce, 2001; Gold, 1996).** The main four types of resistance to antibiotics develops **:**

a. Natural resistance:

This kind of resistance is caused by the structural characteristics of bacteria and it is not associated with the use of antibiotics It has no hereditary property. It develops as result of the natural resistance, or the microorganisms not including the structure of the target antibiotic, or antibiotics not reaching to its target due to its characteristics. For example, Gramnegative bacteria vancomycin does not pass in the outer membrane so Gram-negative bacteria is naturally resistant to vancomycin. Similarly, L-form shape of bacteria which are wall-less forms of the bacteria, and the bacteria such as cell wall-less cell Mycoplasma and Ureaplasma are naturally resistant to beta-lactam antibiotics that inhibit the cell wall synthesis **(Yüce, 2001; Jawetz et al., 1995; Nikaido, 2009).**

b. Acquired resistance:

As result of the changes in the genetic characteristics of bacteria, an acquired resistance occurs due to its not being affected from the antibiotics it has been responsive before. This kind of resistance occurs due to mainly structures of chromosome or extrachromosomal (plasmid, transposon, etc.).

c. Cross resistance:

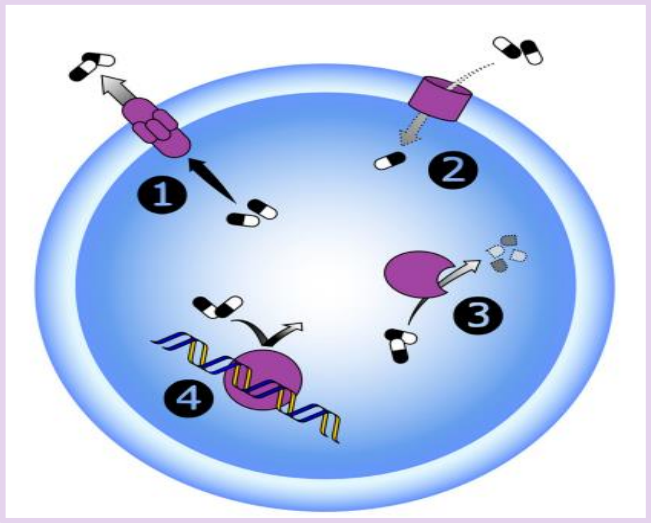
Some microorganisms which are resistant to a certain drug, that acts with the same or similar mechanism and also resistant to other drugs. This condition is usually observed in antibiotics whose structures are similar: such as resistance between erythromycin, neomycin-kanamycin or resistance between cephalosporins and penicillins. However, sometimes it can also be seen in a completely unrelated drug groups. There is an example of cross-resistance between erythromycin-lincomycin. This may be chromosomal or extrachromosomal origin **(Jawetz et al., 1995 ; Mayer et al., 1995).**

d. Multi-drug resistance and pan-resistance:

Multidrug-resistant organisms are usually bacteria that have become resistant to the antibiotics used to treat them. This means that a particular drug is no longer able to kill or control the bacteria. Inapropriate use of antibiotics for therapy resulted in the selection of pathogenic bacteria resistant to multiple drugs(resistant to at least three classes of antibiotics). Multidrug resistance in bacteria can be occured by one of two mechanisms. First, these bacteria may accumulate multiple genes, each coding for resistance to a single drug. This type of resistance occurs typically on resistance (R) plasmids. Second type of resistance, namely multidrug resistance may also occur by the increased expression of genes that code for multidrug efflux pumps, enzymatic inactivation, changes in the structure of the target etc. If the bacterial strains resistant to three or more classes of antimicrobials, it is considered as multi-drug resistant. If the strains, resistant to all but one or two antibiotic gruops, they are considered as extensively-drug-resistant, if the strains resistant to all available antibiotic, they are classified as pan-drug-resistant. For example, multidrug resistance (MDR) *Acinetobacter* species (spp.) can be defined as the isolate resistant to at least three classes of antimicrobial agents (namely, all penicillins and cephalosporins (including inhibitor combinations), fluroquinolones, and aminoglycosides). Extensive drug resistant (XDR) *Acinetobacter* spp.' shall be the Acinetobacter spp. isolate that is resistant to the three classes of antimicrobials described above (MDR) and shall also be resistant to carbapenems. Pandrug resistant or pan-resistant (PDR) Acinetobacter spp. shall be the XDR *Acinetobacter* spp. that is resistant to polymyxins (colistin) and tigecycline **(Nikaido, 2009; Eliopoulos et al., 2008).**

2. Mechanismes of antibiotic resistance :

There are four main molecular mechanisms by which bacteria may resist the effects of antibiotics; modification of the target site, modification or destruction of the antibiotic, antibiotic efflux via efflux transporters and reduced antibiotic influx through decreased membrane permeability (Figure 3) **(Munita and Arias, 2016).** These resistance mechanisms can be present together in different combinations in one bacterial cell, potentially allowing high level resistance to multiple antibiotic compounds simultaneously **(Nikaido, 2009).**



Bacterial resistance mechanisms to antibiotics.

- Increased drug efflux;
- decreased drug uptake;
- drug modification/destruction and
- target modification.

Figure 3 : Bacterial resistance mechanisms to antibiotics.

2.1. Inactivation of antibiotics :

One of the main mechanisms of antibiotic resistance is the bacterial ability to produce enzymes capable to inactivate the drug by hydrolysis or chemical modifications (Figure 3). The biochemical reactions catalysed by the enzymes include: acetylation, phosphorylation and adenylation**.**

There are three main enzymes that inactivate antibiotics such as β-lactamases, aminoglycoside-modifying enzymes, and chloramphenicol acetyltransferases (AACs) **(Dockrell et al., 2004).**

Beta-lactamases :

β-lactamases hydrolyze nearly all β-lactams that have ester and amide bond, e.g., penicillins, cephalosporins, monobactams, and carbapenems. About 300 β-lactamases are known till date. β-lactamases are broadly prevalent enzymes that are classified using two main classification systems: Ambler(structural, table 1) and Bush–Jacoby–Medeiros (functional) **(Alekshun and Levy, 2007).** Ambler classification system is described below:

- **Class A β-lactamases:** Also referred as penicillinase; these are clavulanic acid susceptible. Two commonly encountered ClassA β-lactamases found in members of Enterobacteriaceae are designated as TEM-1, SHV-1. These are penicillinase with little or no activity against cephalosporin **(Rice et al., 2003).** These are progenitors of extended-spectrum β-lactamases (ESBL). ESBL are enzymes that have changed substrate profile because of amino-acid substitution allowing hydrolysis of most

cephalosporins. ESBL are resistant to penicillins, third-generation cephalosporins (e.g.,ceftazidime, cefotaxime, ceftriaxone), aztreonam, cefamandole, cefoperazone, but are sensitive to methoxy-cephalosporins, e.g., cephamycins and carbapenems and are inhibited by inhibitors of β-lactamases, e.g., clavulanic acid, sulbactam, or tazobactam **(Jacoby and Munoz-Price, 2005; Bonnet, 2004) .**

- **Class B β-lactamases:** These are metallo-β-lactamases. These require enzymes such as zinc or heavy metals for catalysis and their activity is inhibited by chelating agents. These classes of enzymes are resistant to inactivation by clavulanate, sulbactam, aztreonam, and carbapenems. E.g., New Delhi metallo-β-lactamase **(Rasmussen and Bush, 1997).**
- **Class C β-lactamases:** These are also called cephalosporinases. These are produced by all Gram-negative bacteria with exception of Salmonella and Klebsiella. Class C hydrolyzes cephalosporins including extended spectrum cephalosporins, in comparison to class A β-lactamases, these have large cavities, and as a result, they are able to bind the bulky extended spectrum penicillin. An example of this type is Amp C β-lactamases. This class of enzymes is resistant to all β-lactams except carbapenems. They are not inhibited by clavulanate **(Crichlow et al., 1999; Lobkovsky et al., 1994).**
- **Class D β-lactamases:** These are oxacillin hydrolyzing enzymes – found most commonly in Enterobacteriaceae and in P. aeruginosa. Oxacillin-hydrolyzing enzymes confer resistance to penicillin, cloxacillin, oxacillin, and methicillin. They are weakly inhibited by clavulanic acid but are inhibited by sodium chloride **(Naas and Nordmann, 1999).**

Table 1 : Ambler classification of β-lactamases (**Sanseverino et al., 2018) :**

Ambler class	Representative enwye type	Exemples of enwym
Class A	penicillinases**,** carbapenemases**,** cephalosporinases**,** extended spectrum**,** β-lactamases**,** broad spectrum β-lactamases	cefotaximase-M **,** sulfhydryl variable enzymes **,** Klebsiella pneumoniae**,** carbapenemases

Class B	metallo-beta-lactamase	imipenemase metallo-β-lactamases**,** Verona integron encoded metallo-β-lactamases **,** New Delhi metallo-β-lactamase-1
Class C	penicillinases**,** cephalosporinases	AMPC
Class D	oxacillin hydrolysing enzymes	oxacillin hydrolyzing enzymes.

Aminoglycoside modifying enzymes (AGE's) :

AG are neutralized by specific enzymes: Phosphoryl-transferases, nucleotidyl-transferases or adenylyl-transferases, and AACs. These aminoglycoside-modifying enzymes (AMEs) reduce affinity of a modified molecule, impede binding to the 30S ribosomal subunit **(Strateva and Yordanov, 2009)** ;and provide extended spectrum resistance to AG's and FQ **(Maurice et al., 2008).** AMEs are identified in S. aureus, E. faecalis, and S. pneumoniae strains.

Chloramphenicol-acetyl-transferases :

Few Gram-positive and Gram-negative bacteria and some of Haemophilus influenzae strains are resistant to chloramphenicol, and they have an enzyme chloramphenicol transacetylase that acetylates hydroxyl groups of chloramphenicol. Modified chloramphenicol is unableto bind to a ribosomal 50S subunit properly **(Tolmasky, 2000).**

2.2. Decrease of antibiotic penetration :

Many of the antibiotics used in clinical practice have intracellular bacterial targets or, in case of gram-negative bacteria, located in the cytoplasmic membrane (the inner membrane). Therefore, the compound must penetrate the outer and/or cytoplasmic membrane in order to exert its antimicrobial effect. Bacteria have developed mechanisms to prevent the antibiotic from reaching its intracellular or periplasmic target by decreasing the uptake of the antimicrobial molecule. This mechanism is particularly important in gram-negative bacteria (for the reason specified above), limiting the influx of substances from the external milieu. In fact, the outer membrane acts as the first-line of defense against the penetration of multiple toxic compounds, including several antimicrobial agents. Hydrophilic molecules such as β-lactams, tetracyclines and some fluoroquinolones are particularly affected by changes in

permeability of the outer membrane since they often use water-filled diffusion channels known as porins to cross this barrier **(Pagès et al., 2008).** The prime example of the efficiency of this natural barrier is the fact that vancomycin, a glycopeptide antibiotic, is not active against gram-negative organisms due to the lack of penetration through the outer membrane. Likewise, the innate low susceptibility of Pseudomonas and Acinetobacter baumanii to β-lactams (compared to Enterobacteriaceae) can be explained, at least in part, to a reduced number and/or differential expression of porins **(Hancock and Brinkman, 2002).**

Several types of porins have been described, and they can be classified according to their structure (trimeric vs. monomeric), their selectivity and the regulation of their expression. Among the best-characterized porins, the three major proteins produced by E. coli (known as OmpF, OmpC and PhoE) and the P. aeruginosa OprD (also known as protein D2) are classical examples of porin-mediated antibiotic resistance. Alterations of porins could be achieved by 3 general processes, **i)** a shift in the type of porins expressed, **ii)** a change in the level of porin expression, and **iii)** impairment of the porin function. Importantly, changes in permeability through any of these mechanisms frequently result in low-level resistance and are often associated with other mechanisms of resistance, such as increased expression of efflux pumps (see below) **(Nikaido, 2003).**

2.3. Efflux pump :

An antibacterial agent can be effective upon reaching the specific site of action and accumulate at specific concentrations. Efflux pumps (EPs) act as an export or efflux system that can cause resistance to the wide ranges of antibacterial agents. Throughout this mechanism, the antibacterial agent is pumped out faster than the time it requires to be diffused in bacterial cell and consequently, the intrabacterial concentration becomes much less than the effective value. For example, the protein-synthesis systems such as ribosome are located in the cytoplasm. So that, inhibitors of protein synthesis are forced to pass through the cell membranes and then accumulate up to a sufficient concentration to induce the blockade of protein synthesis. By reducing the intrabacterial concentration of inhibitors, which are mediated by EPs, the procedures of bacterial protein synthesis can be performed without any interruptions **(Levy, 1992; Paulsen et al., 1996).**

EPs are capable of conveying both lipophilic or amphipathic molecules out of the bacteria. In another aspect, they have been also able to transport one type of substrate and/ or the range of structurally dissimilar antibacterial agents , which had been detected and found in

multiple drug-resistant bacteria **(Webber and Piddock, 2003).** Five major families of EPs have been recognized in bacteria, which includes major facilitator superfamily (MFS), multidrug and toxic efflux (MATE), resistance-nodulationdivision (RND), small multidrug resistance (SMR), and ATP binding cassette (ABC) **(Lomovskaya et al., 2001).** The MFS, ABC, SMR, and MATE families are mainly found in both Grampositive and -negative bacteria, while the RND superfamily is specifically found in Gram-negative bacteria **(Mahamoud et al., 2007).** The group of RND families always consists of a tripartite complex that spans across both membranes of Gram-negative bacteria. In regards to Gram-positive bacteria, the MFS family has been reported as the most abundant EPs while their well-known members are known to be NorA from Staphylococcus aureus and PmrA from Streptococcus pneumoniae. Antibiotic resistance via this mechanism can be observed in a wide range of pathogenic Gram-positive and -negative bacteria and fungi such as S. aureus, Pseudomonas aeruginosa, Acinetobacter baumannii, and Candida albicans **(Tegos et al., 2011).**

2.4. Change in target site

A large number of target replacement and protection mechanisms are also found in clinical isolates. The classical example of target modification is seen in MRSA strains where resistance to β-lactams is conferred by an exogenous PBP, known as PBP2a, whose transpeptidase domain is insensitive to the action of several different β-lactams. Acquisition of PBP2a facilitates bypass of the original sensitive target, however, since it does not contain the transglycosylase activity it functions together with the transglycosylase domain of the native PBP2 to perform cross-linking reaction in the presence of β-lactams. PBP2a is coded by the mecA gene, which is located on a large MGE called SCCmec (Staphylococcal chromosomal cassette) in S. aureus. Many different types of SCCmec cassettes have been described, which contain varying numbers of accompanying resistance elements **(Fishovitz et al., 2014; Liu et al., 2016).**

Other target modification examples in clinical strains include point mutations or enzymatic alteration of the target **(Munita and Arias, 2016).** For examples of point mutations in the target, see **(Hooper, 2002; Floss and Yu, 2005).** Enzymatic alteration of the target is best understood in the case of macrolide resistance conferred by a large group of erythromycin ribosomal methylation (erm) genes. These enzymes methylate a specific adenine in the 23S rRNA **(Weisblum, 1995).** The erm genes in clinical strains are present on mobile genetic elements and are widespread among both Gram-positive and Gram-negative bacteria **(Roberts, 2008).**

Significant similarities between the methylation enzymes found in the clinical isolates and the producers have been observed, suggesting a common ancestral origin **(Uchiyama and Weisblum, 1985; Doi et al., 2016).** Finally, known examples of target protection in clinical strains include the Tet(M) and Tet(O) proteins commonly encoded by genes located on MGEs in S. aureus. Interestingly, these proteins are homologous to the elongation factors EF-G and EF-Tu, and their binding to the ribosome facilitates removal of tetracycline in a GTP-ase activity-dependent manner **(Burdett, 1996; Trieber et al., 1998).**

2.5.Biofilm formation :

Biofilm is formed by a complex aggregation of microbes, wherein the cells are embedded matrix of extracellular polymeric substance (EPS) (self-produced). Production of biofilms through adherence of bacteria to human tissues and medical devices is a major virulence factor associated with increased antibiotic resistance, reduced phagocytosis, and overall persistence of the microorganisms **(Hoiby et al., 2010).** Additionally, these biofilms being difficult to eradicate, are a source of many intractable infections. The inherent resistance of biofilms to the antibiotics can be attributed to failure of antibiotic to penetrate or slow growth rate of organisms owing to slower metabolism **(Kostakioti et al., 2013).**

3. Mechanisms of antibiotic resistance gene acquisition :

Bacteria are characterised by a genetic plasticity that allows them to adapt to different environmental threats including the presence of antibiotic molecules that may compromise their survival. Antibiotic resistance (ABR), developed as a strategy to respond to the antibiotic occurrence, can be genetically mediated through either the acquisition of resistance genes from other bacteria or through the occurrence of spontaneous resistance mechanisms which favour the survival of microorganisms **(Toma and Deyno, 2015).** While some bacterial strains display intrinsic resistance, a bacterial population can gain resistance to antibiotics by the recombination of foreign DNA into the chromosome or via the mutation in key genes during replication. This mutation can then be passed to the subsequent generations leading to a population of resistant bacteria (vertical transmission), as shown in (Figure 4A). More commonly, resistance genes can be acquired from other strains and species (horizontal transmission) through different mechanisms (Figure 4B): transformation (uptake of the free DNA from the environment), transduction (transfer of DNA from a virus to bacteria) and conjugation (transfer of DNA between bacteria by direct cell-to-cell contact) (Figure 4B) **(Munita and Arias, 2015).**

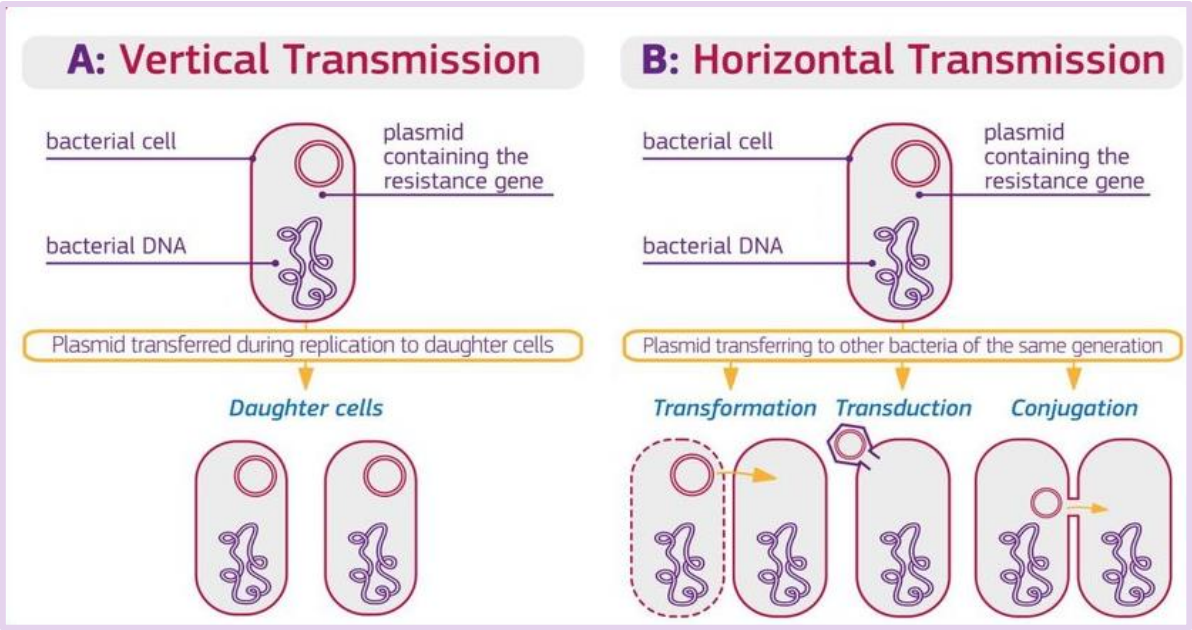


Figure 4 : Mechanism of vertical and horizontal transmission in bacteria.

Any antibiotic use can lead to antibiotic resistance. Antibiotics kill germs like bacteria and fungi, but the resistant survivors remain. Resistance traits can be inherited generation to generation. They can also pass directly from germ to germ by way of **mobile genetic elements**.


Section
02

3. Resistance to antibacterial drugs of international concern :

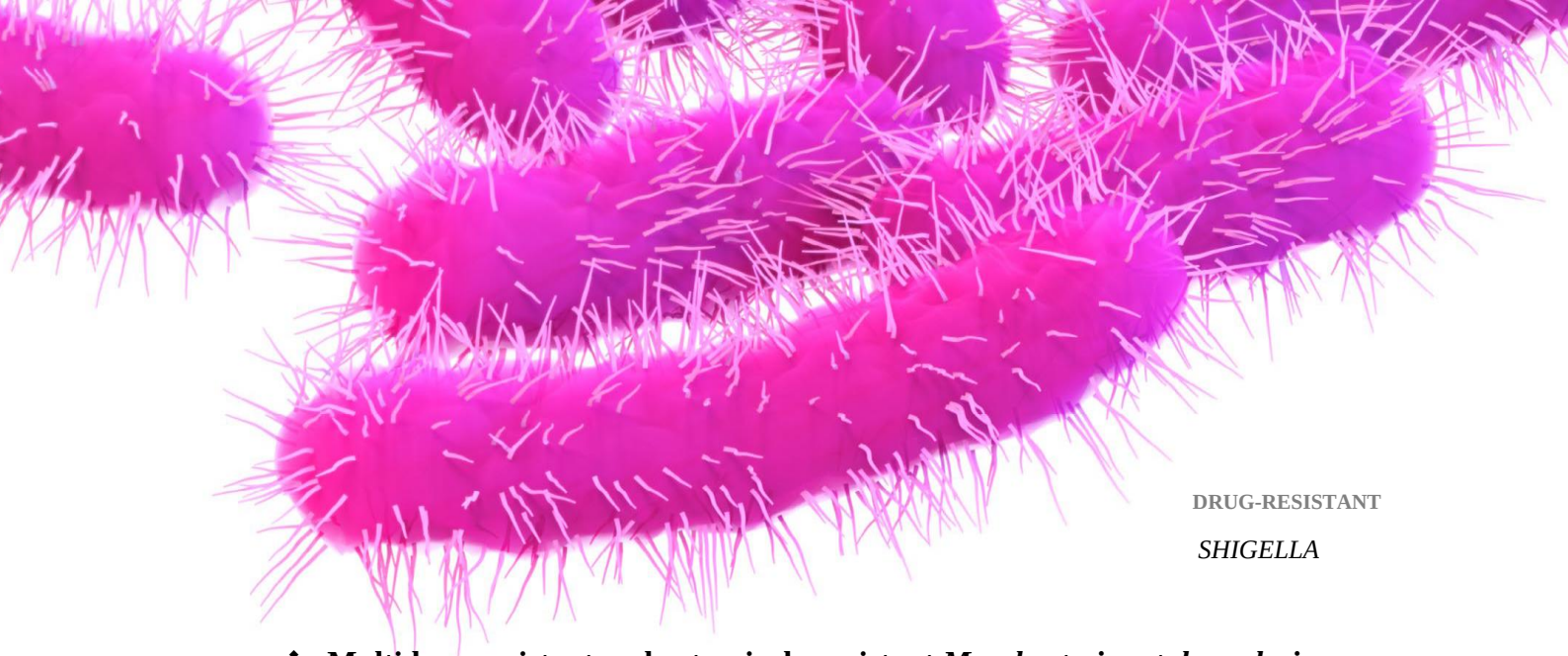
Numerous important organizations, like the Centers for Disease Control and Prevention (CDC), Infectious Diseases Society of America, World Economic Forum, and the World Health Organization (WHO) have declared antibiotic resistance to be a "global public health concern.

The CDC assessed antibiotic-resistant bacterial infections according to seven factors: clinical impact, economic impact, incidence, 10-year projection of incidence, transmissibility, availability of effective antibiotics, and barriers to prevention **(Centers for Disease Control and Prevention, Office of Infectious Disease. Antibiotic resistance threats in the United States, 2013).**The threat level of each bacteria was then classified as "urgent," "serious," or "concerning" **(Rossolini et al., 2014).** In general, threats that are urgent or serious require more monitoring and prevention activities, whereas those considered concerning require less. A summary of information regarding the resistant bacteria mentioned above follows. Information regarding other strains of resistant bacteria that have been identified as threats by the CDC can be found **(www.cdc.gov, January 28, 2015).**

In 2016, in the wake of the increasing global awareness of the need for new antibiotics, WHO's member states mandated that WHO create a priority pathogen list (PPL) of antibiotic resistant bacteria to direct research and development of new and effective drugs. Due to the high prevalence of multidrug resistance among ESKAPE bacteria, defined by the Infectious Diseases Society of America as *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp.*, these pathogens feature prominently in the global PPL of antibiotic-resistant bacteria.

The mandate also followed recommendations of the 2016 UN report of a high-level panel on the global response to health crises, which emphasised the threat posed to humanity from a number of under-researched antibiotic-resistant bacteria that urgently require enhanced and focused research and development investments .

The global PPL stratifies bacterial pathogens into three priority tiers: critical, high and medium.3 Carbapenem-resistant *A. baumannii*, *P. aeruginosa* and *Enterobacteriaceae* spp., which includes *K. pneumoniae*, feature in the critical priority tier. Methicillinresistant, vancomycin intermediate and resistant *S. aureus*, in addition to *E. faecium*, are featured in the high priority tier **(OPGA/WHO/FAO/OIE. At UN, global leaders commit to act on antimicrobial resistance. Collective effort to address a challenge to health, food security, and development. 2016).**



DRUG-RESISTANT

SHIGELLA

- **Multidrug-resistant and extensively-resistant *Mycobacterium tuberculosis***
- **Priority 1: critical**
  - *Acinetobacter baumannii*, carbapenem resistant
  - *Pseudomonas aeruginosa*, carbapenem resistant
  - Enterobacteriaceae, carbapenem resistant,
  - E, thirdgeneration cephalosporin resistant
- **Priority 2: high**
  - *Enterococcus faecium*, vancomycin resistant
  - *Staphylococcus aureus*, methicillin resistant, vancomycin resistant
  - *Helicobacter pylori*, clarithromycin resistant
  - *Campylobacter* spp, fluoroquinolone resistant
  - *Salmonella* spp fluoroquinolone resistant
  - *Neisseria gonorrhoeae*, third-generation cephalosporin
  - resistant, fluoroquinolone resistant
- **Priority 3: medium**
  - *Streptococcus pneumoniae*, penicillin non-susceptible
  - *Haemophilus influenzae*, ampicillin resistant
  - *Shigella* spp, fluoroquinolone resistant

WHO priority list for research and development of new antibiotics for antibiotic-resistant bacteria

1. The top pathogenic bacteria resistant to antibiotics :

1.1. MDR and EXD *Mycobacterium tuberculosis* :

Mycobacterium tuberculosis (MTB) is the primary causal agent of human tuberculosis worldwide. Tuberculosis (TB) is one of the oldest diseases known to affect and are also called Koch's tubercle bacillus was discovered by Robert Koch in 1882 , thus identifying TB as an infectious disease **(Koch, 1882).**

M. tuberculosis and seven very closely related mycobacterial species (M. bovis, M. africanum, M. microti, M. caprae, M. pinnipedii, M. canetti and M. mungi) together comprise what is known as the M. tuberculosis complex. Most, but not all, of these species have been found to cause disease in humans except M. caprae and M. mungi **(Giovanni et al., 2013).**

a. Bacteriological study :

- *Mycobacteria* are now known to comprise a large group of acid – fast, alcohol – fast. with a high cell wall content of highmolecular-weight lipids 60% of the cell wall structure **(Daniel et al., 2015).**
- Visible growth takes 3 to 8 weeks on solid media. Growth is slow with a doubling time of 12-24 h under optimal conditions **(Giovanni et al., 2013).**
- *M. tuberculosis* is a tiny thin obligate aerobic (3 μm in length and 0.5 μm in width) and can survive poor nutrient milieu and oxygen deficiency (by becoming anaerobic) **(Myung-Sang Moon et al., 2017).**
- Non – spore forming, non-motile bacilli **(David, 1976)** , with presence of capsule **(Daffe and Draper, 1998)**
- It has no glycocalyx, pili or fimbriae on its cell wall surface for adhesion , It is metabolically catalase-and phenylase-positive **(Myung-Sang Moon et al., 2017).**
- The cell wall of M. tuberculosis is characteristic of the mycobacteria; although classified as Gram-positive, it has a structure similar to that of Gram-negative bacteria with a second ‘outer membrane’ containing the mycolic acids – long-chain, branched fatty acids **(Brennan 2003).**
- It is visualized via the Ziehl–Neelsen acid-fast stain whereby its thick, waxy cell wall retains carbol fuschin stain **(Stephen and Tanya, 2017).**
- Biochemical tests, including positive niacin production and the ability to reduce nitrate **(Stephen and Tanya, 2017).**

b. Pathogenicity and virulence factors :

Mycobacterium tuberculosis is an expert and deadly pathogen, causing the disease tuberculosis (TB) in humans. It has several notable features **:**

- The ability to enter non-replicating states for long periods and cause latent infection.
- A thick, waxy cell wall; slow growth rate in culture **(Stephen and Tanya, 2017).**
- Mycobacteria have unusually impermeable cell walls that are thought to be advantageous in stressful conditions of osmotic shock or desiccation as well as contributing to their considerable resistance to many drugs **(Jarlier and Nikaido, 1990).**
- Mycolic acid compounds, thecord factor in the mycobacterial cell wall is only found in the virulent strains **(Fossati et al., 2003).**
- As a pathogen, M. tuberculosis has a complex relationship with its host, is able to replicate inside macrophages, and expresses diverse immunomodulatory molecules. **(Stephen and Tanya, 2017).**
- The pathogen may become highly contagious (disease spread is rapid) **(Fossati et al., 2003)**

c. **Type of resistance :**

M. tuberculosis currently causes over 1.8 million deaths a year, making it the world's most deadly human pathogen. Global prospects for TB control are challenged by the emergence of drug-resistant strains, especially those that are multidrug resistant (MDR) and extensively drug resistant (XDR) **(Jain and Mondal, 2008).**

Drug resistance in TB is believed to be mediated exclusively by chromosomal mutations, which affect either the drug target itself or bacterial enzymes that activate prodrugs **(Ramaswamy and Musser, 1998).**

1.2. ***Acinetobacter baumannii* :**

Acinetobacter baumannii (AB) has emerged as an important opportunistic pathogen worldwide **(Dijkshoorn et al., 2007).** AB is an emerging human pathogen which causes a broad array of infections (e.g. pneumonia, urinary tract, bloodstream and skin infections) that account for about 10% of all nosocomial infections **(Dijkshoorn et al., 2007; Joly-Guillou, 2005; Peleg et al., 2008).**

a. Bacteriological study :

- AB strains are Gram-negative coccobacilli **(Peleg et all., 2008).**
- Members of the genus Acinetobacter are non-motile, ubiquitous bacteria that can be recovered from a wide range of sources such as soil, water, food products and medical environments **(Bergogne-Berenzin & Towner, 1996).**
- As currently defined, strictly aerobic, non-lactose-fermenting, nonfastidious, catalase-positive and oxidase-negative bacteria **(Antonet al., 2008).**

b. Pathogenicity and virulence factors :

- The K1 Capsular Polysaccharide of Acinetobacter baumannii Strain is a Major Virulence Factor **(Thomas et al., 2010).**
- Strong biofilm formation is a part of virulence pathogenesis strategies of this organisms **(Suresh and Geetanjali, 2013).**
- *A. baumannii* lipopolysaccharide (LPS), a molecule central to the development of Gram-negative sepsis, has also been investigated **(Erridge et al., 2007).**
- AB strains are intrinsically resistant to desiccation and disinfectants and have shown a great capacity to acquire or develop antibiotic resistance **(Wendt et al., 1997; Dijkshoorn et al., 2007).**
- Its ability to adhere to, colonize and invade human epithelial cells **(Lee et al., 2006; Lee et al., 2008).**
- Its repertoire of antibiotic resistance mechanisms that are able to be promptly up-regulated as required **(Smith et al., 2007).**
- its ability to acquire foreign genetic material through lateral gene transfer to promote its own survival under antibiotic and host selection pressures **(Adams et al., 2008).**
- Various mechanisms of resistance: Production of β lactamases, Effl ux pumps, Lower permeability of the outer membrane, Mutations in antibiotic targets (eg, for quinolones) and Production of enzymes inactivating aminoglycosides **(Drosos and Matthew, 2019) .**

c. Type of resistance:

One of the main threats from *A. baumannii* is the high rate of resistance to antibiotics commonly used to treat Gram-negative infections. More than 80% of *Acinetobacter* species are considered to be multidrug resistant (MDR), resulting in infections with poor clinical outcomes, including high rates of morbidity and death, prolonged hospital stays, and substantial health care expenses **(Karageorgopoulos and Falagas, 2008; Livermore, 2009).**

In addition, several strains of extensively drug and even pandrug resistance A. baumannii have been isolated, showing resistance to a wide variety of clinically used antibiotics **(Lee, 2011).**

Today, a substantial proportion of these isolates are carbapenem-resistant *A. baumannii* (CRAB) **(Tacconelli et al., 2018).** CRAB is declared as the top priority pathogen by the World Health Organization for the investment in new drugs **(Tacconelli et al., 2018).**

Resistance of *Acinetobacter* to -lactams is partially intrinsic due to the synthesis of a species-specific cephalosporinase **(Hood and Amyes, 1991; Vila et al., 1993).** However, additional plasmid- or transposon-borne -lactamase genes can be acquired **(Devaud et al., 1982; Goldstein et al., 1983).** Mutations in the gyrA gene have been associated with high-level resistance to fluoroquinolones in this organism **(Bergogne et al., 1996; Vila et al., 1995).**

1.3. ***Pseudomonas aeruginosa* :**

Pseudomonas aeruginosa is a common nosocomial pathogen **(Hidron et al., 2008; Jones et al., 2009; Zhanel et al., 2010)** that causes infections with a high mortality rate **(Mutlu and Wunderink, 2006; Kerr and Snelling, 2009; Mahar et al., 2010; Lambert et al., 2011).**

Pseudomonas aeruginosa is a major opportunistic pathogen causing nosocomial pneumonia, burn wound infections, corneal ulceration in contact lens wearers, septicaemia in the immunocompromised **(Bodey et al., 983)** and is the most prevalent pathogen of cystic fibrosis (CF) **(Fitzsimmons, 1993).**

a. Bacteriological study :

- *P. aeruginosa* is a ubiquitous organism, Gram-negative, aerobic bacillus, with length andwidth ranging from 1.5-3.0 µm and 0.5-0.8 µm respectively **(Bergey, 2005).**
- It is motile by means of a single polar flagella .
- Is an oxidase-positive, non sporulating and non-fermentative species **(Bergey, 2005).**
- Other diagnostic characteristics pigment production - including pyocyanin (bluegreen). (Bergey, 2005).

b. Pathogenicity and virulence factors :

- It is intrinsically high resistant to antimicrobial agents due to low permeability of its cell wall **(Poole, 2002).**

- It has the genetic capacity to express a wide repertoire of resistance mechanisms **(Lambert; 2002).**
- It can become resistant through mutation in chromosomal genes which regulate resistance genes .
- It can acquire additional resistance genes from other organisms via plasmids, transposons and bacteriophages.
- *P. aeruginosa* is a notoriously difficult organism to control with antibiotics or disinfectants **(Lambert; 2002).**
- *P. aeruginosa* possesses a vast array of virulence factors including : lipopolysaccharide, type IV pilli, alginate, exotoxin A, elastase, alkaline protease, phospholipase C **(Salyers and Whitt, 1998).**
- Quorum-sensing (QS) signaling systems in Pseudomonas aeruginosa.
- biofilm formation (the ability to form the biofilm is affected by QS) **(davies et al., 1998).**

c. Type of resistance:

P. aeruginosa isolates generally demonstrate resistance to various antimicrobial agents. Their resistance to multiple antimicrobial agents or MDR is often associated with the production of specific enzymes, such as an inducible AmpC-type cephalosporinase (**Lodge et al., 1990)** , and decreasing permeability (loss of OprD proteins) ; in addition, outer membrane permeability and active efflux systems can play important roles in limiting the access of antimicrobial agents to inner cell **targets (Livermore, 1992; Nikaido, 1994; Nikaido et al., 1991).**

Previous studies comparing genomes of the opportunistic pathogen *Pseudomonas aeruginosa* pointed toward horizontal gene transfer (HGT) as an important factor in its evolution **(Mathee et al., 2008).**

There is a group of carbapenem resistant *P. aeruginosa* (CRPA) strains that is specifically resistant to carbapenems **(Shihomi et al., 2006)**. Thus, carbapenems generally remain one of,the best therapeutic choices for treatment of serious P. aeruginosa infections **(Livermore, 2002).**

1.4 *Enterobacteriaceae* resistant:

according to a recent global report of the World Health Organization (WHO), antibiotic resistance is now defined as a major threat to public health (**World Health Organization, 2014).** Frequent occurrences of resistance to third-generation cephalosporins and carbapenems are reported for *Enterobacteriaceae* especially in *Escherichia coli* and *Klebsiella pneumoniae* **(Babic et al., 2006).**

1.4.1 *Escherichia coli:*

Escherichia coli in humans is a commensal inhabitant of the gastrointestinal tract as well as one of the most frequently isolated bacterial pathogens **(Leclerc et al., 1996).** and important pathogens causing significant morbidity and mortality worldwide **(Kaper et al., 2004).**

a. Bacteriological study :

- *Escherichia coli* is commonly found in the normal microflor in the human gastrointestinal tract and is intricately involved in the lives of humans **(Abigailet al., 2012) .**
- Gram- negative facultative anaerobic bacilli **(Madigan and Martinko, 2005).**
- They are generally motile by peritrichous flagella **(Patricia et al., 1997).**
- Usually produce gas from glucose, non-lactose fermenting, indole negative, urease positive and oxidase negative **(Ali saadi et al., 2017).**
- Non-spore forming **(Madigan and Martinko, 2005).**

b. **Pathogenicity and virulence factors :**

- They possess an endotoxin Lipopolysaccharide LPS (O-antigen), which is the primary contributor to their ability to cause infections**(Abigailet al., 2012) .**
- *E. coli* H where H refers to the flagellar antigen (H-antigen) **(Patricia et al., 1997).**
- The virulence factors that distinguish the various E. coli pathotypes were acquired from numerous sources, including plasmids, bacteriophages, and the genomes of other bacteria **(Reid et al., 2000).**
- One of the most common causes of infantile diarrhea is enteropathogenic *Escherichia coli* (EPEC). **(Clarke et al., 2002 ; www.who.int).**
- Prototypical EAEC infection is characterized by the formation of a biofilm.

1.4.2 *Klebsiella pneumoniae:*

Klebsiella pneumoniae is an opportunistic pathogen that frequently causes nosocomial infections, mainly in immunocompromised patients. *K. pneumoniae* infections range from mild urinary tract infections to severe bacteremia and pneumonia with a high rate of mortality and morbidity **(Bartlett, 1986; Garcıa, 1985; Held, 1992).**

a. Bacteriological study :

- In contrast to many other bacterial pathogens, K. pneumoniae is ubiquitous in nature. The non-clinical habitats include the mucosal surfaces of humans and animals and environmental sources such as vegetation, soil and surface waters **(Bagley, 1985).**
- KP are Gram-negative, nonmotile, bacilli, non-spore forming, capsulated, non-motile and arranged singly **.**
- Are generally facultative anaerobic, and range from to1.0 mm in width and 0.6 to 6.0 mm **(Janda et al. 2007).**
- Positive reaction for catalase test and urease , negative for oxidase, Indole and Methyl red tests **(Mona et al., 2014).**
- Cultivation of isolate on macConkey gives lactose fermenting colonies **(Mona et al., 2014).**

b. Pathogenicity and virulence factors :

- *K. pneumoniae* is an opportunistic pathogen that causes nosocomial infections, such as,pneumonia, urinary tract infections, septic shock, and gastro intestinal disease **(Duyen et al., 2017)** .
- Lipopolysaccharide (LPS), capsular polysaccharide, and fimbriae are recognized major virulence factors of K. pneumoniae and play key roles during early stages of infections **(Duyen et al., 2017)** .
- The prominent polysaccharide capsule expressed by most strains together with the lipopolysaccharide layer protect the bacteria against phagocytosis and the bactericidal activity of serum.
- recent studies have demonstrated the ability of K. pneumoniae to invade cultured epithelial cells **(Fumagalli et al., 1997; Oelschlaeger and Tall, 1997; Sahly et al., 2000; Struve and Krogfelt, 2003).**

c. Type of resistance:

One of the currently most important antibiotic resistance mechanisms in Enterobacteriaceae is based on plasmid-mediated production of enzymes that inactivate b-lactam antibiotics including cephalosporins and monobactams (except carbapenems) by hydrolyzing their b-lactam ring. These so-called extended-spectrum b-lactamases (ESBLs) have been detected in human clinical isolates of *Enterobacteriaceae* since the early 1990s **(Paterson and Bonomo, 2005).** ESBLs arise because of mutations in the TEM-1, TEM-2, or SHV-1 genes, commonly found in the *Enterobacteriaceae* family **(Bradford, 2001 ; Paterson and Bonomo, 2005) .**

The most active is the carbapenems, which are employed in the treatment of infections caused by ESBL, particularly *Escherichia coli* and *Klebsiella pneumonia* **(Peterson, 2006).** Carbapenems have been used for the treatment of infections caused by Enterobacteriaceae **(Vardakas, 2012) .** The percentage of Carbapenem-resistant Enterobactericeae (CRE) has been on the rise **(CDC. Vital Signs, 2012) .** Normally, these MDR infections are hard-to-treat with limited available choices of antibiotics such as tigecycline, colistin, fosfomycin, and aminoglycosides **(Falagas and Kopterides, 2007 ; Falagas et al., 2005).**

One of the successful mechanisms for transmitting (MDR) multiple-druge resistance among bacterial pathogens is horizontal transfer (HGT) **(Munoz, 2009).** The acquired genes are located on the integron structures that reside on mobile genetic elements such as plasmids or transposons **(Walsh *et al.*, 2005).** which have various antibiotic-resistant mechanisms, such as b-lactamases, decreased outer membrane permeability, efflux pumps systems, and target modification **(Jacoby, 2004; Yang, 2004).**

1.5. ***Clostridioides difficile :***

Clostridium difficile is an important nosocomial pathogen associated with potentially fatal disease induced by the use of antibiotics **(Elena-Stella et al., 2018) .** *C. difficile* was first described in 1935 a commensal organism in the fecal flora of healthy newborn infants. Although the severe form of *C. difficile* disease (pseudomembranous colitis; PMC) was first described in 1893, *C. difficile* was not identified as the causative agent of human disease until 1978 **(Bartlett, 1994).** Today, C. difficile is a leading cause of hospital-acquired diarrhoea, ranging from mild cases to severe pseudomembranous colitis, collectively known as *C. difficile* infection (CDI) **(O'Connor et al., 2008).**

a. Bacteriological study :

- *Clostridium difficile*, also known as Clostridioides difficile **(Lawson et al., 2016; Oren and Garrity, 2016)).**
- is a Gram-positive obligately anaerobic bacillus **(Evans and Safdar, 2015).**
- Cells are motile rods, with dimensions of 0.5 -1.9 by 3.0 – 16.9µm, which forms oval sub-terminal spores and show optimum growth on blood agar**.**
- C. difficile are 4 - 6mm in diameter, irregular, raised, opaque, and grey-white after 48hr incubation **(Public Health England, 2016).**
- They ferment sugars but are negative for lecithinase, lipase, oxidase, catalase and indole tests **(Public Health England, 2016).**
- The bacterium are ubiquitous in the environment, and although not part of the normal gut microbiota of humans **(Barbut et al., 2009).**

b. Pathogenicity and virulence factors :

- Toxins A and B (also known as TcdA and TcdB) are exotoxins that belong to the family of large clostridial toxins and are major virulence factors of C. difficile **(Bartlett et al., 1978; Lyerly et al., 1982, 1985; Triadafilopoulos et al., 1987).**
- Both toxins of C. difficile bind to and damage human colonic epithelial cells **(Pothoulakis et al., 1996).**
- *C. difficile* surface proteins mediate adherence to other microbial species, mucus, and intestinal cells within the colon for colonization **(Janoir,2015).**
- The peritrichous flagella produced by C. difficile aid in motility and modulate colonization in an animal model of infection **(Stevenson, 2015).**
- Biofilm formation has been proposed to be another important factor contributing to antimicrobial resistance of C. difficile **(Đapa et al., 2013).**
- *C. difficile* production of capsule suggesting that there might be an antiphagocytic factor on its cell surface **(Dailey et al., 1987).**

c. **Type of resistance:**

C. difficile is the causative agent of hospital-acquired antibiotic-associated diarrhoea in high-risk patients . C. difficile infection (CDI) is 12–14 times more common than other widely publicised infections such as methicillin-resistant Stapholococcus aureus (MRSA) bacteraemia **(Jones et al., 2012).**

The occurrence and spread of *C. difficile* strains that are resistant to multiple antimicrobial drugs (MDR) complicate prevention as well as potential treatment options. antibiotics such as metronidazole, vancomycin and fidaxomicin are therapies of choice for *Clostridium difficile* infection. Several important mechanisms for *C. difficile* antibiotic resistance have been described, including the acquisition of antibiotic resistance genes via the transfer of mobile genetic elements, selective pressure *in vivo* resulting in gene mutations . The multiplicity of mechanisms of resistance, which include ribosomal modification, efflux of the antibiotic and drug inactivation, results in a variety of resistance phenotypes **(Zhong et al., 2017).**

1.6. ***Methicillin-resistant S. aureus (MRSA) :***

Staphylococcus aureus is a common bacterium that can cause a variety of diseases from localized to systemic infections **(Wertheim et al., 2005).** Methicillin-resistant *S. aureus* (MRSA) is a major cause of nosocomial infections worldwide and is becoming increasingly prevalent in community settings **(Boucher and Corey, 2008 ; David and Daum, 2010).**

a. Bacteriological study :

- *Staphylococcus aureus* is a facultative anaerobic Gram positive coccal bacterium about 1 micron in diameter **(Lowy, 1998).**
- *Staphylococcus aureus* is a ubiquitous commensal bacterium on human skins and anterior nares, but frequently causes severe infections in humans **(Kluytmans et al., 1997).**
- *S. aureus*, a coagulase-positive, catalase-positive, produced beta-hemolysis on blood agar **(Cenci-Goga et al., 20003).**
- It does not form spores and it Is not motile. It is frequently found in grape-like clusters or chains **(Murray and Patrick, 2007).**

b. Pathogenicity and virulence factors :

- *Staphylococcus aureus* strains are able to secrete free plasma coagulase, which is an important virulence factor for these bacteria **(Emori and Gaynes, 1993).**
- The capsular polysaccharide or capsule is a cell wall bacterial component that protects bacterium from phagocytic uptake and enhances microbial virulence **(Cocchiaro et al., 2006).**
- *S. aureus* can form biofilms on host and prosthetic surfaces, enabling it to persist by evading host defenses and antimicrobials **(Donlan and Costerton, 2002).**

- *S. aureus* produces numerous enzymes, such as proteases, lipases, and elastases, that enable it to invade and destroy host tissues **(Timmerman et al., 1993).**
- *S. aureus* produces leukocidins that cause leukocyte destruction by the formation of pores in the cell membrane **(Gladstone and Heyningen, 1957).**

c. **Type of resistance:**

The emergence and spread of multidrug-resistant (MDR) MRSA pose a serious problem in the treatment and control of staphylococcal infections, thereby threatening global human health **(Lee et al., 2018 ; Sola et al., 2009) .**

Antimicrobial resistance is genetically based; resistance is mediated by the acquisition of extrachromosomal genetic elements containing genes that confer resistance to certain antibiotics ,which can be transferred between bacteria through horizontal gene transfer **(Jensen and Lyon, 2009).** A defining characteristic of MRSA is its ability to thrive in the presence of penicillin-like antibiotics, which normally prevent bacterial growth by inhibiting synthesis of cell wall material. This is due to a resistance gene, *mecA*, which stops β-lactam antibiotics from inactivating the enzymes (transpeptidases) critical for cell wall synthesis **(Chambers, 2001).**

1.7. ***Resistant Enterococci :***

Enterococci can cause a wide variety of diseases in humans, infecting the urinary tract, bloodstream, endocardium, abdomen, biliary tract, burn wounds, and indwelling foreign devices **(Jett *et al.*, 1994).** Enterococci now rank among the top three nosocomial bacterial pathogens **(Richards *et al.*, 2000; Wisplinghoff *et al.*, 2003),** and strains resistant to currently available antibiotics pose real therapeutic difficulties **(Hunt, 1998).** The two commonly isolated species of Enterococci associated with nosocomial infections are *E. faecalis* and *E. faecium* **(Kristich et al., 2014).**

a. Bacteriological study :

- *Enterococci* inhabit the gastrointestinal tract, the oral cavity,and the vagina in humans as normal commensals **(Jett *et al.*, 1994).**
- *Enterococci* are opportunistic pathogens, gram-positive cocci that are catalase negative, usually facultative, anaerobic bacteria **(Devriese et al., 2002; Devriese et al., 1991).**

- *Enterococci* were formerly classified as group D streptococci **(Lancefield, 1933)**
- *Enterococci*, namely E. faecium and E. faecalis strains, have also been used as probiotics for humans **(Aarestrup et al., 2002 ; Tannock and cook, 2002).**

b. Pathogenicity and virulence factors :

- Aggregation substance (AS) is mediates efficient contact between donor and recipient bacterium, facilitating plasmid exchange during bacterial conjugation. may contribute to the pathogenesis of enterococcal infection through a number of mechanisms **(Mundy et al., 2000).**
- Haemolysin and gelatinase produced by Enterococci have been shown to be virulent in human infections **(Chow et al., 1993 ; Vergis et al., 2002).**
- Capsular Polysaccharide present on the surface of both *E. faecalis* and *E. faecium* **(Huebner et al., 1999).**
- Surface Adhesions [Enterococcal Surface Protein (ESP)] is a cell wall associated protein in *E. faecalis* **(Shankar et al., 1999).**

c. Type of resistance:

The treatment of enterococcal disease in humans has been complicated by the emergence of antimicrobial resistant strains. two species (*Enterococcus faecalis* and *Enterococcus. faecium*) have emerged as multidrug-resistant (MDR) pathogens that are well adapted to spread in hospitals.This has led to an increasing tendency for severe forms of multiple resistance and the resultant reliance on “last line of defence” drugs for therapy, including the glycopeptide antimicrobial vancomycin which is categorized by the World Health Organization as a “highest priority critically important antimicrobial” **(World Health Organization, 2017).** Worldwide, vancomycin-resistant E. faecium (VREfm) has emerged as a major nosocomial pathogen of humans **(Willems et al., 2005).**

They are intrinsically resistant / tolerant to many antibiotics and are able to acquire drug resistance either by chromosome, transfer of plasmid or transposon acquisition containing genetic sequences that confer resistance in other bacteria **(Mollering, 1992).**

1.8. Drug-resistant Streptococcus :

Diseases associated with the streptococci occur chiefly in the respiratory tract, bloodstream, or as skin infections. Human disease is most commonly associated with Group A streptococci. Acute group A streptococcal disease is most often a respiratory infection (pharyngitis or tonsillitis) or a skin infection (pyoderma) **(Bisno, 1991)** . Streptococcus pneumoniae is a major cause of community-acquired pneumonia and meningitis, and it is also found as a commensal, colonizing the human upper respiratory tract of a portion of the human population **(tatiana et al., 2019).**

a. Bacteriological study :

- *Streptococci* are nonmotile, Gram-positive, nonsporeforming bacteria, that live in pairs or chains of varying length **(Patterson, 1996).**
- They are characteristically round or ovoid in shape **(Patterson, 1996).**
- Most *Streptococci* are facultative anaerobes, although some are obligate anaerobes.
- They usually require a complex culture medium in order to grow **(Patterson, 1996).**
- Many streptococci imitate aspects of their host in order to escape detection.
- The capsule of S*treptococcus pyogenes* is chemically similar to that of it's host's connective tissue, and therefore, is nonantigenic **(Patterson, 1996).**
- it's cytoplasmic membrane has antigens similar to human cardiac skeletal and smooth muscle **(Patterson, 1996).**

S pyogenes characteristically is a round-to-ovoid coccus 0.6-1.0 µm in diameter (see annex). They divide in one plane and thus occur in pairs, or (especially in liquid media) in chains of varying lengths.

S *pneumoniae* appears as a 0.5-1.25 µm diplococcus, typically described as lancet-shaped but sometimes difficult to distinguish morphologically from other streptococci.

b. Pathogenicity and virulence factors :

- This pathogen presents a polysaccharide capsule as the most important virulence factor **(Bogaert et al., 2004; Kadioglu et al., 2008; Hyams et al., 2010).**
- M protein and lipoteichoic acid for attachment **(Patterson, 1996).**
- A hyaluronic acid capsule that inhibits phagocytosis **(Patterson, 1996).**

- Other extracellular products, such as pyrogenic (erythrogenic) toxin, which causes the rash of scarlet fever**(Patterson, 1996).**
- Streptokinase, streptodornase (DNase B), and streptolysins. Some strains are nephritogenic. Immune-mediated sequelae do not reflect dissemination of bacteria. Nongroup A strains have no defined virulence factors **(Patterson, 1996).**
- *S pneumoniae* is a normal member of the respiratory tract flora; invasion results in pneumonia. The best defined virulence factor is the polysaccharide capsule, which protects the bacterium against phagocytosis **(larry et al., 1977).**

c. Type of resistance:

Penicillin-non-susceptible Streptococcus pneumoniae (PNSP) were first detected in the 1960s, and are now common worldwide, predominantly through the international spread of a limited number of strains. PNSP were recently listed as one of the most important antimicrobial-resistant threats worldwide **(CDC, 2013; WHO, 2017).**

For more information about the mentioned bacteria (Classification, Form pictures) ; See annex .


Section
03

IV. Medicinal plants :

Medicinal plants are used for healing purposes throughout the human history; even in the current era, there are up to 80% of the world population most of them are living in the developing countries, rely on traditional herbal medicine on their primary health care systems, many of these herbal drugs prescribed in traditional medicine have inadequate knowledge and untested by scientifi c methods **(Ekor 2013 Qazi and Molvi 2016).** On the other side, Modern medicine stands helpless in the front of the growing phenomenon of antimicrobial resistance to antibiotics, which considered as a major health problem and required prompt attention. This crisis encouraged scholars and researchers to develop the current antibiotics, synthesize new antibiotics or to fi nd new alternatives. The latter option is preferable because, in nature, plants arise as one of the largest pharmaceutical factories ever known and plants were the main source of drugs for humankind since antiquity. Many medicinal plants produce diverse groups of secondary metabolites known as phytochemical compounds, which may suppress the microbial growth by different modes of action such as interference with cellular metabolic processes, cellular membrane perturbations or by modulating the signal transduction or gene expression pathways **(Omojate et al., 2014 Mohamed et al. 2017).**

Secondary metabolites :

Bioactive compounds in plants can be defined as secondary plant metabolites eliciting pharmacological or toxicological effects in man and animals. Secondary metabolites are produced within the plants besides the primary biosynthetic and metabolic routes for compounds associated with plant growth and development, and are regarded as products of biochemical “side tracks” in the plant cells and not needed for the daily functioning of the plant. Several of them are found to hold various types of important functions in the living plants such as protection, attraction or signalling. Most species of plants seem to be capable of producing such compounds (**Bernhoft, 2010).**

The main chemical groups of bioactive compounds in plants with their main pharmacological or toxicological effects in man and animal, as well as the main producing plants/plant family are presented here

1- *Saussurea lappa* :

The costus *Saussurea lappa* is a well-known medicinal plant that has been widely used in the traditional medicines in many Asian countries **(Reem et al., 2019).**

Saussurea costus (synonymous with *S. lappa*) is well known in Islamic medicine, which enlisted in the Holy Ahadith said by Prophet Muhammad (Peace be upon him) **(Ahmad et al., 2009).** It is known in Arab countries as "Al-Kost Al-Hindi" and used by traditional healers since the era of the Islamic civilization.

1.1. Geographical distribution :

Saussurea lappa is indigenous to India, Pakistan and China, where it grows in the Himalaya region at 2500 - 3500 m altitude **(Rao et al., 2013)** . It is found in cool temperature and arctic regions of Asia, Europe and North America **.** In India it is found in Kashmir, Jammu, and Western Ghats and is cultivated in Tamil Nadu, Uttar Pradesh and Kashmir to meet commercial demand.

1.2.Taxonomic classification :

Saussurea lappa is member of family asteracea . Asteracea is one of the largest angiosperm families, with more than 1,620 genera and about 23,600 species of plants including herbs, shrubs and trees. The genus *Saussurea* consists of about 300 species **(Kamal et al., 2019).**

Table 2: classification of S. lappa **(Source Zahara K et al., 2014)**

Kingdom	Plantae
Subkingdom	*Virideplantae*
Infrakingdom	*Streptophyta*
Division	*Tracheophyta*
Subdivision	*Spermatophytina*
Infradivision	*Angiospermae*
Class	*Magnoliopsida*
Superorder	*Asteranae*
Order	*Asterales*
Family	*Asteraceae*
Genus	*Saussurea*
Species	*S. lappa*

Figure 5 : *Saussurea lappa* plant

1.3.Morphological Description :

Saussurea lappa is a straight, pubescent, stout, and perennial plant, with 1 to 2m long robust stem. Leaves are membranous, irregularly toothed, and auricled at the base. Basal leaves are 0.5 to 1.25m in length with long petiole, while, upper leaves are sub-sessile and small, having two tiny lobes at the bottom. Flowers; sessile, purplish blue to black, tough, curved, present in form of clusters of 2-5 flowers in leaf axils. Corolla: 0.02 m long, black, tubular. Anther tails fimbriate. Pappus: fluffy, brown. Roots: 0.4m long, robust, grey or brown **(Pandey et al., 2007) .**

Saussurea lappa requires mild-cold and arctic environment for growth. Cultivation is usually through seeds. Optimum temperature for seed germination is 20^0 C **(Kuniyal and Rawat, 2015; Chauhan et al.,1998).** Plants are adaptable to very harsh climates and grow at extremely high altitudes. They require winter rest period of 8 – 9 months and flourish best in humus rich and well-drained loamy soil.

Root (part used):

Roots are 7-15cm long, 1.5-5cm wide. The roots are fusiform (or) conical and tapering, collapse in the centre: thin roots are cylindrical, broad, light and stout, usually contain longitudinal wrinkles with anastamose or ridges running straight (or) Spiral; Fresh roots are dirty grey to light yellow, dried ones are brown or dull red; fractured is short and horny; Odour is strong, sweet and aromatic and taste is bitter, sweetish pungent; fattening , aphrodisiac, alternative **(Mohammad Ali et al., 2006).**

Roots of *Saussurea lappa* are stout about 60cm long with a characteristic penetrating odour . The crop is generally harvested after three years in case of cultivated crop but after 5 years in case of wild plant. The roots are collected in october and cut into pieces of 10cm length and further dried in the sun. The fresh roots are about 0.6m long and 0.3m in width. The dried roots are refined to as "*SAUSSUREA*". It has strong, sweet, aromatic odour and bitter taste. The dried roots are dull brown, stout, cylindrical (or) fusiform about 7- 15cm long and 1-5cm thick; it breaks with short horny fracture **(Vinod D. Rangari et al., 2006).**

1.4. Phytochemicals :

Saussurea lappa contains a variety of phytochemicals of which some are identified and many more are yet to be discovered and isolated. Its main active constituent are terpenes such as costunolide , dihydrocostunolide , 12 methoxydihydro costunolide, dihydrocostus

lactone, dehydrocostus lactone **(Yang et al., 1998),** α-hydroxy dehydrocostus lactone, β-hydroxy dehydrocostus lactone, lappadilactone **(Sun et al., 2003),** mokko lactone, betulinic acid, betulinic acid methyl esters **(Choi et al., 2009),** cynaropicrin, reynosin, santamarine **(Cho et al., 1998)**, saussureamines A-C **(Yoshikawa et al., 199**3), αcyclocostunolide, alantolactone, isoalantolactone **(Zhao et al., 2008),** isodihydrocostunolide, βcyclocostunolide **(Robinson et al., 2008)**, β- hydroxyl arbusculin A **(Choi et al., 2009),** arbusculin B **(Julianti et al., 2011),** saussureal and so on **(Talwar et al., 1992),** which have antitumor and anti-inflammatory properties. It also contains anthraquinones, mainly three compounds aloeemodin-8-o-β-d-glucopyranoside, rhein-8-o-β-d-glucopyranoside and chrysophanol, alkaloids and flavonoids **(Zahara et al., 2014).** Four flavonoids glycosides have antibacterial function **(Rao et al., 2007).** Shikokiols have antitumor activity **(Jung et al., 1998),** whereas chlorogenic acid prevents oxidization.

1.5. Costus oil :

The oil extracted from the roots of S. lappa is known as costus oil, which is used in the preparation of hair oil and in high quality perfumes. Costus oil is pale yellow to brownish in color and is also said to be valuable in treating leprosy. In a recent study, 39 components have been identified from the essential oil of S. lappa roots. The chief compounds were dehydrocostus lactone (46.75%), costunolide (9.26%), 8-cedren-13-ol (5.06%) and α-curcumene (4.33%). However, β-costol (13.55%) and δ-elemene (12.69%), α-selinene (5.02%), β-selinene (4.47%), α-costol (4.02%), 4-terpinol (3.38%), elemol (3.21%), α-ionone (3.13%), β-elemene (3.00%), (-)-γelemene (2.08%), p-cymene (1.96%) and 2-β-pinene (1.57%), (-)-α-selinene, (+)-selina-4, 11-diene, (-)-α-transbergamotene, (-)-α-costol, (+)-γ-costol, (-)-elema-1,3,11 (13)-trien-12-ol, (-)-α-costal, (+)-γ-costal, (-)-elema1,3,11(13)-trien-12-al, (-)-(E)-trans-bergamota-2,12-dien14-al, (-)-ar-curcumene, (-)-caryophyllene oxide and 12-m ethoxydihydrodehydrocostuslactone were also reported **(Maurer and Grieder, 1997; Dhillon et al., 1987).**

However, in the other reported studies, the proportion of all these compounds greatly differs. Existing variations in the composition of *S. lappa* essential oil may be due to various factors related to ecotype, phenophases, chemotype and variations in environment conditions such as relative humidity, temperature, photoperiod and irradiance. Furthermore, the chemistry of secondary metabolites of plants may also be affected by genetic background **(Marotti et al.,1994).**

1.6.Uses :

Many herbs and spices contribute appreciably to health despite the minute amount of consumption, as they are full of nutrients such as antioxidant and minerals. Even though the reported work suggests the safety and efficiency of *S. lappa*, the worth of the evidence is inadequate; bioactive components, physiological pathways, bioavailability, and pharmacokinetics are not available in sufficient detail. The extracts of *S. lappa* are reported to possess curative potential. Variety of biological applications reported by the scientists opened the doors for the use of plant in pharmaceuticals. Whole plant extracts have remarkable action against fever, inflammations, microbial infections, and convulsions. Moreover, the elaborative studies can lead to development of the safe activities of *S. lappa* for remedial use in modern medicine and will offer better insight of its pathway of action.

a- General uses :

S. lappa has been used in a wide variety of indigenous medicinal systems all over the globe to cure a number of diseases **(Irshad et al., 2012; Qingnong et al., 2006).** In the southern regions of Punjab, Kashmir, and Himalaya, the root stalks and whole roots are used to cure rheumatism, skin infections, dysentery, toothache, and bronchial asthma. In Ayurvedic medicine systems, the roots of S. lappa are used as skin toner; and to treat vomiting, leucoderma, scabies, epilepsy, itching, blood diseases, and hysteria. In Unani medicinal system, plant extracts are used as aphrodisiac, tonic, carminative, to stimulate CNS, ant-helminthic, and to cure the disorders of liver, blood, and kidney. Plant is also used in the treatment of paralysis, old fever, ophthalmic disorders, and deaf. Roots arre rich in alkaloids and volatile oils and are used as insecticides. Powdered roots are sprayed over crops to kill insects. Ariel parts of plant are used as fodder and fuel. Dried leaf are smoked like tobacco **(Butola and Samant, 2010).** The fragrant oil obtained from S. lappa is very valuable in perfumery. Its odour slightly resembles with the oil of orris. It is also blended with other oriental perfumes in a lasting manner **(Nautiyal et al., 2003).**

b- Pharmacological uses :

Several researchers investigated different extracts of this plant and found the constituents exhibiting anti-inflammatory, anti-bacterial, anti-tumor, hepatoprotective, anti-ulcer and immunomodulatory activities. Till date, different biologically active ingredients of *Saussurea lappa* have been isolated and purified. Among those active compounds sesquiterpene lactones such as costunolide and dehydrocostus lactone has been reported to exhibit medicinal bioactivities.

1.6. Antibacterial Property :

Different type of solvents extracts (e.g. methanolic, ethanolic, aqueous, petroleum ether) have been tested for the in vitro antibacterial activity of the *Saussurea lappa* and it is observed to be effective against variety of resistant pathogens. **(Yang et al., 2005)** studied the in vitro effects of ethanolic extracts on five clinical H. pylori strains. The results showed that, S. lappa extract strongly inhibits all of the strains tested (the MIC was approximately 40 mg/mL). The in vitro antibacterial activity of methanolic extract of Saussurea lappa has shown some degree of antibacterial activity against the tested bacterial strains **(Parekh et al., 2007).** Moreover, it inhibits the expression of hepatitis B surface antigen and corerelated antigens **(Chen et al., 1995)** as well as growth of other microorganisms and pathogens **(Patil, 2009).**

The active ingredients of *S. lappa* inhibit the binding and transfer of R plasmids in Shigella flexneri **(Li et al., 2010).** The in vitro antibacterial activity of different extracts of Saussurea lappa was evaluated against *E. coli, Bacillus thurigenesis* and *Cornybacterium* by disc diffusion method. It was concluded that S. lappa showed significant antibacterial activity against the mentioned organisms at different concentrations of plant extract **(Irshad et al., 2012).** The antimicrobial activity of methanolic and chloroformic extracts of Saussurea lappa roots were tested against S*taphylococcus aureus, Pseudomonas aeruginosa, E. coli, Klebsiella pneumonia, Proteus vulgaris, Candida albicans* and *Aspergillus* through agar well diffusion method. It was found to be significantly effective against all the mentioned organisms **(Thara et al., 2012).** The antimicrobial activity of ethanolic extract of *Saussurea lappa* was tested against multi drug resistant *Staphylococcus aureus, Pseudomonas aeruginosa, E. coli and Klebsiella pneumoniae* through agar well diffusion method. It was found to be effective against all mentioned bacteria with the minimum inhibitory concentration ranges from 2.0µg/µg-12.0µg/µl **(Hassan et al., 2013).** The in vitro antibacterial activity of different solvent extracts of *Saussurea lappa* against *Bacillus subtilis, Staphylococcus aureus, E. coli, Pseudomonas aeruginosa and Klebsiella pneumonia* was studied. It was found that all the extracts showed antibacterial activity against mentioned bacteria but chloroformic extract showed the highest antibacterial activity **(Alaagib et al., 2015)** .

2. *Ajuga iva* :

The species *Ajuga iva* is a common plant in North Africa, known as “Chendgoura” in Algeria, is an herbaceous plant widely used as an aromatic plant and in phytotherapy.

2.1. Geographical distribution :

This aromatic plant, A. iva, develops in deep soil of 2700m heightIt grows in period from spring to late summer. The flowering period is between May and June **(Batanouny et al. 1999; Halimi, 2004).** It is widely distributed in the Mediterranean region: southern Europe and northern Africa, particularly in Algeria, Morocco, Tunisia, and Egypt **(Halimi, 2004).**

2.2. Taxonomic classification :

The genus *Ajuga* (Lamiaceae) comprises 40-50 species, which grow in different parts of the world **(Tsurng-Juhn et al., 2009).**

Table 3 : classification of *Ajuga iva* **(El. Hilaly, 2007)**

Kingdom	Plantae
Division	*Angiospermae*
Subdivision	*Spermatophytina*
Class	*Dicotylédones*
Order	*Lamiales*
Family	*Lamiaceae*
Genus	*Ajuga*
Species	*Ajuga iva*

Figure 6: *Ajuga iva* plant.

2.3. Morphological Description :

A. iva is a small aromatic perennial bitter taste of 5 to 10cm, with green stems creeping and hairy. Leaves are narrowly oblong to linear, pubescent, 14-35 mm long. A. iva grows in rocky slopes up to 2700 m of altitude **(Gordon et al. 1997; Batanouny et al., 1999).** The flowers are purple, pink, or yellow, 20 mm of long, the upper lip of the corolla is absent or reduced and the lower lip is divided into three lobes hairy. The side lobes are small, while the central lobe is relatively larger, decorated in base with a central yellowish spots with the same color of the flower, usually in purple. Within the flower there are four stamens related to four carpels black. The seeds are brown and have the size of the seeds of Nigella sativa **(Halimi, 2004).**

2.4.Phytochemicals :

Ajuga genus contains many important bioactive compounds like anthocyanins, diterpenoids, sterols, ionones, iridoids, phenylethanol and flavonoid glycosides **(Maria et al, 1997, Chen et al., 1996; Shimomura et al., 1987; Terahara et al., 2001; Akbay et al., 2003).**

Ajuga iva L. was found to contain a large number of compounds. such as 8-O-acetyl harpagide, ajugarine, apigenin-7-O neohesperidoside, barpagide, caffeine, clorogenes, cyasterone, diglycerides, 14,15-dihydroajugapitin, ecdysones, ecdysterones, flavonoids, iridoides, makisterone A, neohesperidoside, phenylcarboxylic acids and tanninpolyphenols **(Khafagy et al., 1979; Jannet et al., 1997).** It was also reported that *A. iva* contain tannins, phytoecdysteroids, polyhydroxylated-sterols, few essential **oil (Wessner et, 1992; Ghedira et al 1991). Bondi et al (2000)** reported that *A. iva* includes a large amounts of three major ecdysteroids (makisterone A, 20-hydroxyecdysone and cyasterone) with several minor compounds including 24,28-dehydromakisterone A and two new phytoecdysteroids (22-oxocyasterone and 24,25-dehydroprecyasterone). In addition it contains polypodine B and 2-deoxy-20-hydroxyecdysone. The occurrence of the antifeedant 14, 15-dihydroajugapitin in the aerial parts of *A. iva* from Algeria was also shown **(Bondi et al., 2000).**

2.6. use:

Several *Ajuga* species have been used in African and Asian folk medicine **(El Hilaly et al., 2004).** In Algeria it has been used in phytomedicine for a variety of diseases and disorders **(Taleb-Senouci et al., 2009).**

a- General uses :

In traditional medicine, *Ajuga iva* is used to treat diabetes and hypertension **(Meyre-Silva C et al., 2005)** as well as gastrointestinal disorders and stomach ulcers **(Sahpaz S et al., 2002).** The ivory is effective against fever, diarrhea, gas, headache and toothache. It is used as an infusion for gastrointestinal disorders (infuse 5 grams of the dried plant in a teaspoon of boiling water for 15 minutes, and filter; take 3 cups of herbal tea per day) **(Stulzer H et al., 2006).** For external use, it is often used in local applications against rheumatism, as an antiseptic and healing on wounds. On the other hand, maceration or infusion would be useful in ridding the scalp of parasites **(Adjadj, 2009).**

b- Pharmacological uses :

Ajuga iva L. is one of the plants widely used to treat diabetes and other disorders **(Ziyyat *et al*, 1997; Bnouham et al., 2002; El-Hilaly et al., 2003; Tahraoui et al., 2007).** It is ingested for its useful action against stomach and intestinal pains, enteritis, fever, sinusitis and headache **(Ghedira et al 1991).** Pharmacological studies have shown that *A. iva* has anti-ulcerous **(Habib et al, 1990)** and anti-inflammatory activities **(Hilaly et al, 2002).** It has been used to treat dysuria and painful joints of the limbs. *Ajuga iva* extract decreases plasma cholesterol and triglycerides **(El-Hilaly *et al*., 2006).**The Powder of dried plant or its infusion taken after meals against diabetes and hypertension, in addition the infusion of flowering branches is considered as antidiarrheic, depurative, and very effective for feminine sterility **(Ghedira et al 1991).** In addition Ajuga *iva* induce an Inhibition of calcium oxalate monohydrate crystal growth **(Beghalia et al, 2008)** and no apparent toxicity was observed for this plant **(El-Hilaly et al., 2004).**

Other reported biological effects of *A. iva* include hypoglycemic (**El Hilaly and Lyoussi, 2002),** vasorelaxan**t (El Hilaly et al., 2004),** hypolipidemic **(El Hilaly et al., 2006),** hypotensive, antioxidant **(Chenni et al., 2007),** antifungal, antimicrobial, antipyretic, antihelmintic, and vulnerary activity **(El Hilaly and Lyoussi, 2002; El Hilaly et al., 2004, 2006).**

Antibacterial Property :

The antimicrobial compounds from ajug iva may inhibit bacterial growth by different mechanisms than those presently used. Antimicrobials therefore, may have a significant clinical value in treatment of resistant microbial strains. In particular, the antimicrobial activity of plant oils and extracts have formed the basis of many applications including raw and processed food preservation, pharmaceuticals, alternative medicine, and natural therapies **(Sarac and Ugur, 2007).**

This activitie is related to there contents in active compounds, they contains a wide range of compound such as ajugapyrin A, bracteonin A and lupulin C and Iridoids which had a wide range of biological and pharmacological activity**.** The plant contains also, a class of secondary metabolites which are produced by plants primarily as a defence against infection by microorganisms **(Suomi et al., 2000 ; Israili and Lyoussi, 2009).**

3- Rosmarinus officinalis :

Rosmarinus officinalis L. (family, Lamiaceae), commonly known as rosemary, is one of the most popular perennial culinary herbs cultivated all over the world **(Joana et al., 2017).**

3.1. Geographical distribution :

Rosemary is one of the oldest known medicinal plants in Algeria. is a Mediterranean region plant, and it has been cultivated as a common household plant around the world **(Dong et al., 2012).** The main producers are Italy, Dalmatia, Spain, Greece, Turkey, Egypt, France, Portugal and North Africa, but is now grown worldwide **(Svoboda and Deans,2012).**

3.2. Taxonomic classification :

Rosemary belongs to the Lamiaceae family, which is one of the largest and most distinguished families of flowerin plants (Angiosperms), including about 236 genera and 6900–7200 species worldwide **(Naghibi et al., 2005; Raja, 1998).**

Table 4 : classification of rosemary.

Kingdom	Plantae
Subkingdom	*Tracheobionta*
Division	*Magnoliophyta*
Superdivision	*Spermatophyta*
Class	*Magnoliopsida*
Sub class	*Asteridae*
Order	*Lamiales*
Family	*Lamiaceae*
Genus	*Rosmarinus*
Species	*Rosmarinus officinalis*

Figure 7: *Rosmarinus officinalis* plant.

3.3. Morphological Description :

Rosmarinus officinalis is a woody, perennial herb with fragrant evergreen needle-like leaves. Forms range from upright to trailing; the upright forms can reach 1.5 m, rarely 2 m. The leaves are evergreen green above, and white below with dense short woolly hair. Flowering, very common in a mature and healthy specimen, usually appears in winter or spring and is variable in color, being white, pink, purple, or blue **(Pintore, 2002).**

Leaves (part used) :

Leaves are leathery, opposite, strongly recurved, fringed margins and with prominent midrib. Size of the leaf is 1.0-2.5 cm long and 4 cm width. The upper surface of the leaf is green coloured and the lowered surface is grey somewhat wooly due to numerous trichomes. The margins are entire and strongly revolute with obtuse apex, tapering and non petiolate base **(European Pharmacopoeia 2007).**

3.4.Phytochemicals :

Rosemary is one of the most important medicinal species of Lamiaceae family. It contains volatile oils, flavonoids, diterpenes, phenolic compounds and rosmarinic acid **(Troncosoet al., 2005).**

When the aqueous extract of rosemary was analyzed to identify the active principles, its chemical composition revealed the presence of many substances, namely rosmarinic acid (RA), caffeic acid (CA), chlorogenic acid, carnosolic acid, rosmanol, carnosol and different diterpenes **(Hoefler et al., 1987),** rosmari-dipheno , rosmariquinone **(Houlihan and chang, 1982)** and many other natural antioxidants, ursolic acid, glucocolic acid and the alkaloid rosmaricine **(Kotb, 1985).**

3.5.Rosemary oil:

The essential oil of rosemary contains mainly monoterpenes **(Angioni *et al.*, 2004; Diaz-Maroto *et al.*, 2007).** The principal volatile compounds in rosemary are camphor and 1,8-cineole (eucalyptol), followed by borneol, verbenone, α-pinene and camphene **(Pino *et al.*, 1998; Zaouali *et al.*, 2005; Diaz-Maroto *et al.*, 2007; Calin-Sanchez *et al.*, 2011; Apostolides *et al.*, 2013).** The volatile compounds from rosemary samples could be grouped in chemical families; therefore, the predominant group was monoterpenoids **(Bozin *et al.*, 2007; Szumny *et al.*, 2010; Calin-Sanchez *et al.*, 2011).**

The chemical composition of essential oils of *R. officinalis* in the world is highly variable **.** The major components, also having significant variability, are α-pinene **(4.2–61.2%),** camphene **(0–13.8%),** eucalyptol **(0–61.4%),** camphor **(0–24%)** and borneol **(0–15.6%).** According to **(Napoli *et al.*, 2010),** rosemary essential oil can be classified into three chemotypes from a chemical point of view: cineoliferum (high content in 1,8-cineol); camphoriferum **(camphor > 20%);** and verbenoniferum **(verbenone > 15%).**

3.6.Use :

Rosemary is a common household plant grown in many parts of the world. It is used to flavor food, in cosmetics, and in traditional medicine for its choreretic, hepatoprotective, and antitumorigenic activities **(Slamenov et al., 2002).** Rosemary is also known to exhibit antioxidant and antimicrobial activities. Although its antioxidant properties have been reported extensively **(Bicchi et al., 2000; Elgayyaret al., 2003),** only a few studies have evaluated the potential role of rosemary essential oil as an antimicrobial agent **(Pintore, 2002).**

a- General uses :

Rosmarinus officinalis L. (Labiatae/Lamiaceae), "Rosemary," is an aromatic, restorative herb that relaxes spasms, relieves pain, and increases the perspiration rate. It also stimulates the liver and gall bladder, improves digestion, and controls many pathogenic organisms. Used parts of it include the leaves, flowering tops and essential oil. For culinary purposes, fresh or dried leaves are used to flavor meat (especially lamb and kid), soups and stews; they have a bitter, resinous taste and a tough texture, so should be used either finely chopped or in sprigs that can be removed before serving. Very small amounts (usually as powder) are added to biscuits and jams. Fresh sprigs steeped in vinegar, wine, or olive oil, flavor sauces and dressings **(Panizzi et al., 1993; Mangena et al., 1999).** In the latter case, the European Union has approved rosemary extract (E392) as a safe and effective natural antioxidant for food preservation **(Food Standards Agency, 2016).**

b- Pharmacological uses :

Medicinally, internally it is used for depression, apathy, nervous exhaustion, headaches and migraines, associated with nervous tension or feeling cold, poor circulation, and digestive problems associated with anxiety. Excess consumption causes abortion in pregnant women and convulsions. Externally it is used for rheumatism, arthritis, neuralgia, muscular injuries, wounds, dandruff and scurf **(Panizzi et al., 1993; Mangena et al., 1999).**

It has been used as an antispasmodic in renal colic and dysmenorrhoea and in relieving respiratory disorders. It has also been used as an analgesic, antirheumatic, carminative, cholagogue, diuretic, expectorant, antiepileptic and for effects on human fertility . Other uses are as a general tonic in case of excessive physical or intellectual works and in heart diseases; and also as an insecticide and herbicide. Externally, it is a rubefacient, and is used to stimulate

the growth of hair and treatment of eczema of the scalp, boils and wounds. Recently Rosemary plant has have a role inAlzheimer's disease treatment **(Habtemariam, 2016)**

3.7. Antibacterial Property :

Rosmarinus is one of the oldest known medicinal plants in Algeria. It is used as an antispasmolytic and Most plants produce antimicrobial secondary metabolites, either fromits normal course of growth and development, or in response to stress or pathogen attack. The use of essential oils represents a new way to reduce the proliferation of microorganisms **(Kacaniova et al., 2014).** *Rosmarinus officinalis* L. is widely used today as a food preservative and known for its powerful antibacterial activity **(Wang et al., 2012).**

The increasing use of antibiotics in medicine, agriculture and livestock has largely contributed to the increase of multiple drug resistant microorganisms . Antimicrobial resistance is a global public health concern, and researchers have been increasingly engaging in this area in demand for new effective antimicrobial bioactives **(Qabaha, 2013; Petrolini et al., 2013).** as a flavor and fragrance ingredient in the food.

Since the 1990s until 2014, the essential oil of rosemary has demonstrated the highest antimicrobial activity, with 65% of anti-infectious activity studies. The antimicrobial activity of the essential oil was superior, when compared with the single compounds 1,8-cineole and α-pinene **(Jiang et al., 2011).**

Experimental *in vitro* studies, concerning MICs, minimal bactericidal concentration and time-kill dynamic processes, have reported that there is a possible synergistic effect between the antimicrobial compounds in essential oil **(Luqman et al., 2007 ; Swamy et al., 2016).** These studies were performed testing carnosic acid, carnosol, rosmarinic acid, oleanolic acid, ursolic acid and essential oil, against Gram-positive bacteria and Gram-negative bacteria.


Section
04

1. Introduction to Databases and their Bioinformatics implemetations:

An organization must have accurate and reliable data for effective decision making. To this end, the organization maintains records on the various facets maintaining relationships among them. Such related data are called a **database**. A **database system** is an integrated collection of related files, along with details of the interpretation of the data contained therein. Basically, database system is nothing more than a computer-based record keeping system i.e. a system whose overall purpose is to record and maintain information/data.

A **database management system (DBMS)** is a software system that allows access to data contained in a database. The objective of the DBMS is to provide a convenient and effective method of defining, storing and retrieving the information contained in the database. The DBMS interfaces with the application programs, so that the data contained in the database can be used by multiple applications and users. In addition, the DBMS exerts centralized control of the database, prevents fraudulent or unauthorized users from accessing the data, and ensures the privacy of the data.

Generally a database is an organized collection of related information. The organized information or database serves as a base from which desired information can be retrieved or decision made by further recognizing or processing the data. People use several databases in their day-to-day life. Dictionary, Telephone directory, Library catalog, etc are example for databases where the entries are arranged according to alphabetical or classified order.

1.1 Definition :

The word 'DATA' means a fact or more specially a value of attribute of an entity. An entity in general, may be an object, idea, event, condition or situation. A set of attributes describes an entity. Information in a form which can be processed by a raw computer is called data. Data are raw material of information. The term 'BASE' means the support, foundation or key ingredient of anything. Therefore base supports data.

A 'DATABASE' can be conceived as a system whose base, whose key concept, is simply a particular way of handling data. In other words, a database is nothing more than a computer-based record keeping. The objective of database is to record and maintain information. The primary function of the database is the service and support of information system which satisfies cost.

1. Prakash Naveen : "Database is a mechanized shared formally defined and central collection of data used in an organization".

2. J.M.Martin : " Database is a collection of inter-related data stored together without harmful or unnecessary redundancy to serve multiple application".

3. Mac-Millan dictionary of Information Technology : defines a database as a " a collection of inter-related data stored so that it may be accessed by authorized users with simple user-friendly dialogues".

A Formal Definition : A database is an ordered collection of related data elements intended to meet the information needs of an organization and designed to be shared by multiple users. A database is typically available to a community of users, with possibly varying requirements.

2. Bioinformatics Tools

Bioinformatics tools are software programs that are designed for extracting the meaningful information from the mass of molecular biology / biological databases & to carry out sequence or structural analysis. There are data-mining software that retrieves data from genomic sequence databases and also visualization tools to analyze and retrieve information from proteomic databases. These can be classified as homology and similarity tools, protein functional analysis tools, sequence analysis tools and miscellaneous tools.

The National Center for Biotechnology Information **(NCBI, 2001)** defines bioinformatics as: “Bioinformatics is the field of science in which biology, computer science, and information technology merge into a single discipline. There are three important sub-disciplines within bioinformatics: the development of new algorithms and statistics which assess relationships among members of large data sets, the analysis and interpretation of various types of data including nucleotide and amino acid sequences, protein domains, and protein structures; and the development and implementation of tools that enable efficient access and management of different types of information.”

2.1 Biological Databases :

Relational database concepts of computer science and Information retrieval concepts of digital libraries are important for understanding biological databases. Biological database design, development, and long-term management are a core area of the discipline of bioinformatics . Data contents include gene sequences, textual descriptions, attributes and ontology classifications, citations, and tabular data. These are often described as semi-structured data,

and can be represented as tables, key delimited records, and XML structures . Cross-references among databases are common, using database accession numbers **(Liu Z et al., 2009) .**

Despite having highly different functions, these databases are all architecturally similar. Each consists of three tiers of software (FIG. 8). At the bottom is a database management system (DBMS) that manages a collection of facts.At the top is the web browser that transmits requests for data to the database and renders the responses as web pages. In the middle is a software layer that mediates between the DBMS and the web browser to turn data requests into database queries, and to transform the query responses into hypertext mark-up language (HTML) **(Lincoln, 2003).**

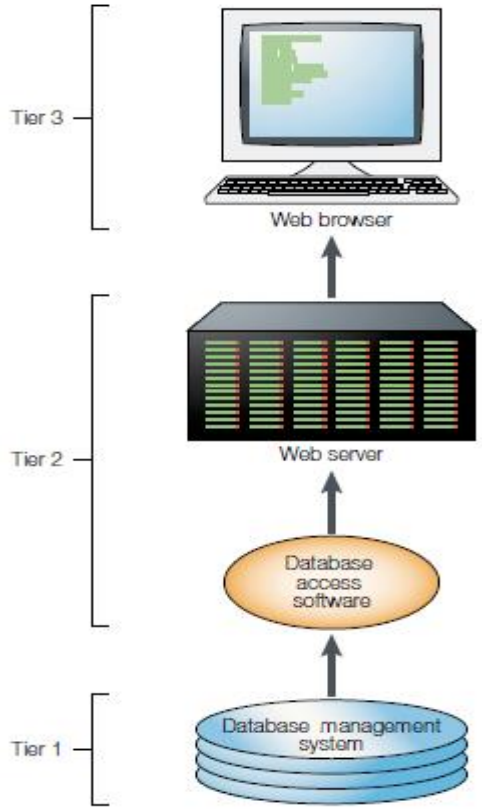


Figure 8: Biological database architecture. Most biological databases use a three-tier architecture that consists of a database management system, a middleware layer and a web interface.

A biological database is a collection of data that is organized so that its contents can easily be accessed, managed, and updated. The activity of preparing a database can be divided into:

- Collection of data in a form which can be easily accessed
- Making it available to a multi-user system

2.2 Type of biological databases :

Databases in general can be classified in to primary, secondary and composite databases. A **primary database** contains information of the sequence or structure alone. Examples of these include Swiss-Prot & PIR for protein sequences, GenBank & DDBJ for Genome sequences and the Protein Databank for protein structures **(Singh et al., 2010).**

A **secondary database** contains derived information from the primary database. A secondary sequence database contains information like the conserved sequence, signature sequence and active site residues of the protein families arrived by multiple sequence alignment of a set of related proteins . A secondary structure database contains entries of the PDB in an organized

way. These contain entries that are classified according to their structure like all alpha proteins, all beta proteins, turns, helices . These also contain information on conserved secondary structure motifs of a particular protein. Some of the secondary databases created and hosted by various researchers at their individual laboratories include SCOP, developed at Cambridge University; CATH developed at University College of London, PROSITE of Swiss Institute of Bioinformatics, eMOTIF at Stanford (Vaseeharan and Valli, 2011).

2.3 Databases Used in Bioinformatics:

According to a report of 2014 Molecular Biology Database Collection in the journal Nucleic Acids Research, there are a sum of 1552 databases that are publicly accessible online **(Fernandez-Suarez et al., 2014).** It should be noted, however, that such count of publicly accessible databases is conservative. In fact, there are some databases providing online services without publication in peer-reviewed journal (e.g., **The RNA Modification Database at http://mods.rna.albany.edu)** or being developed by commercial companies (e.g., **Ingenuity Pathway Analysis at http:// www.ingenuity.com/products/ipa),** making them underrepresented in the scientific community. Considering the continuously proliferating number of biological databases, it becomes increasingly daunting and time-consuming to navigate in the huge volume of databases of interest. The completion of the Human Genome Project in 2003 holds significant benefits for many fields from human evolution to personalized healthcare and precision medicine. In this report, we present a collection of biological databases relevant to human research and provide a mini-review by classifying them into different categories.

Table 4: List of URL for major biological databases **(Dong Zou et al., 2015).**

Name	URL	Brief description	Category
1000 Genomes, AFND, dbSNP, DEG, EGA	http://www.1000genomes.org http://www.allelefrequencies.net http://www.ncbi.nlm.nih.gov/snp http://www.essentialgene.org http://www.ebi.ac.uk/ega	▪ A deep catalog of human genetic variation ▪ Allele Frequency Net Database ▪ Database of single nucleotide polymorphisms ▪ Database of Essential Genes ▪ European Genome–phenome Archive	**DNA**
ChIPBase, DARNED,	http://deepbase.sysu.edu.cn/chipbase http://darned.ucc.ie	▪ Database of transcriptional regulation of lncRNA and miRNA genes.	**RNA**

DIANA-LncBase, GENCODE, H-DBAS	http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=lncBase/index http://www.gencodegenes.org http://www.h-invitational.jp/h-dbas	▪ DAtabase of RNa EDiting in humans. ▪ miRNA targets on lncRNAs. ▪ Encyclopedia of genes and gene variants. ▪ Human-transcriptome DataBase for Alternative Splicing.	
CATH, CPLM, DIP, EKPD, HPRD, Swiss-Prot/TrEMBL	http://cath.biochem.ucl.ac.uk http://cplm.biocuckoo.org http://dip.doe-mbi.ucla.edu http://ekpd.biocuckoo.org http://www.hprd.org http://www.expasy.org/sprot/	▪ Protein structure classification . ▪ Compendium of Protein Lysine Modifications . ▪ Database of Interacting Proteins . ▪ Eukaryotic Kinase and Phosphatase Database . ▪ Human Protein Reference Database ▪ Description of the function of a protein, its domains structure, post-translational modifications etc .	**Protein**
BioGPS, Expression Atlas, Human Protein Atlas, MOPED	http://biogps.org http://www.ebi.ac.uk/gxa http://www.proteinatlas.org https://www.proteinspire.org	▪ Portal for querying and organizing gene annotation resources. ▪ Differential and baseline expression . ▪ Tissue-based map of the human proteome . ▪ Multi-Omics Profiling Expression Database .	**Expression**
CPDB, HMDB, KEGG PATHWAY, MetaCyc	http://consensuspathdb.org http://www.hmdb.ca http://www.genome.jp/kegg/pathway. http://metacyc.org	▪ Database of human interaction networks ▪ Human Metabolome Database ▪ KEGG pathway maps ▪ Metabolic pathway database	**Pathway**
AlzBase, CADgene, COSMIC, DiseaseMeth, DisGeNET	http://alz.big.ac.cn/alzBase http://www.bioguo.org/CADgene http://cancer.sanger.ac.uk http://bioinfo.hrbmu.edu.cn/diseasemeth http://www.disgenet.org/web/DisGeNET/v2.1	▪ Database for gene dysregulation in Alzheimer's disease . ▪ Coronary Artery Disease gene database . ▪ Catalog Of Somatic Mutations In Cancer . ▪ Human disease methylation database . ▪ DisGeNET Gene–disease associations .	**Disease**
GO, HGNC	http://geneontology.org http://www.genenames.org	▪ Gene ontology . ▪ Database of human gene names .	**Standard and ontology**
Europe PMC, PubMed, PubMed Central	http://europepmc.org http://www.ncbi.nlm.nih.gov/pubmed http://www.ncbi.nlm.nih.gov/pmc	▪ Literature database in Europe . ▪ Database of biomedical literature from ▪ MEDLINE Free full-text literature archive .	**Literature**

3. PHP & MySQL : MySQL is the most popular database system used with PHP ;

3.1. **MySQL** is a RDBMS (Relational Database Management System) which has more then 11 million installations. The program runs as a server providing multi-user access to a number of databases. MySQL is owned and sponsored by a single for-profit firm based in Sweden. The project's source code is available under terms of the GNU General Public License, as well as under a variety of proprietary agreements. MySQL is a popular Open Source Software relational database management system which uses a subset of ANSI (American National Standard Institute) SQL (Structured Query Language). For more information, see www.musql.com.

- **MySQL database server offers several advantages:**
  - The data in a MySQL database are stored in tables which consists of columns and rows.
  - MySQL is a database system that runs on a server.
  - MySQL is ideal for both small and large applications.
  - MySQL is very fast, reliable, and easy to use database system.
  - MySQL compiles on a number of platforms.
  - MySQL is easy to use, yet extremely powerful, fast, secure, and scalable.
  - MySQL runs on a wide range of operating systems, including UNIX or Linux, Microsoft Windows, Apple Mac OS X, and others.
  - MySQL supports standard SQL (Structured Query Language).
  - MySQL is ideal database solution for both small and large applications.
  - MySQL is developed, and distributed by Oracle Corporation.
  - MySQL includes data security layers that protect sensitive data from intruders.

MySQL database stores data into tables like other relational database. A table is a collection of related data, and it is divided into rows and columns. An important aspect of a table is that each must have a primary key column so that each row (or record) has a unique field to identify it.

Each row in a table represents a data record that are inherently connected to each other such as information related to a particular person, whereas each column represents a specific field such as *id*, *first_name*, *last_name*, *email*, etc. The structure of a simple MySQL table that contains person's general information may look something like this:

id	first_name	last_name	email
1	Peter	Parker	peterparker@mail.com
2	John	Rambo	johnrambo@mail.com
3	Clark	Kent	clarkkent@mail.com
4	John	Carter	johncarter@mail.com

Websites like **Facebook**, **Twitter**, **Wikipedia** uses MySQL for their storage need. So you can easily understand what MySQL is capable of.

3.2. PHP (Hypertext Preprocessor) :

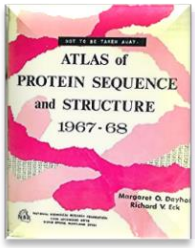
PHP is a server-side scripting language for creating dynamic Web pages that helps create web pages. When a visitor opens the page, the server processes the PHP commands and then sends the results to the visitor's browser. PHP is Open Source and cross-platform that runs on Windows NT and many Unix versions, and it can be built as an Apache module and as a binary that can run as a CGI (Common Gateway Interface). When built as an Apache module, PHP is especially lightweight and speedy. Designed to operate on the web, many applications are open-source and thus less expensive to develop.

3.3. HTML (HyperText Markup Language)

It's the authoring language used to create documents on the World Wide Web. HTML defines the structure and layout of a Web document by using a variety of tags and attributes. HyperText is the method by which you move around on the web by clicking on special text called HyperLinks which bring you to the next page. Markup is what HTML tags do to the text inside them. They mark it as a certain type of text (italicised text, for example).

4. Importance of Databases

- Databases act as a store house of information.
- Databases are used to store and organize data in such a way that information can be retrieved easily via a variety of search criteria.
- It allows knowledge discovery, which refers to the identification of connections between pieces of information that were not known when the information was first entered. This facilitates the discovery of new biological insights from raw data.
- Secondary databases have become the molecular biologist's reference library over the past decade or so, providing a wealth of information on just about any gene or gene product that has been investigated by the research community.
- It helps to solve cases where many users want to access the same entries of data.
- Allows the indexing of data.
- It helps to remove redundancy of data.



First biological database

- Atlas of protein sequence and structure (1965)
- Margaret Oakley Dayhoff (Columbia University, US)
- First release: 65 protein sequences .


Material &
Methods

I. Material :

I. 1Plant Material:

The dry roots of *S. costus* were purchased from a herbal market in Mecheria city (naama), Algeria. The herbal seller showed the trademark of the package and it has been confirmed that it was exported from India.

Two medicinal plants herbs, rosemary and chendgoura were collected from Mecheria in December of 2019. The taxonomic identity of these plants was confirmed by Dr. Terras in the laboratory ecology, Ain El Hdjar of Saida University.

Figure 9: Mecheria, Algeria Geographic map **(Latlong.net 2020).**

1.1 Sample Collection :

The aerial part of *Ajuga iva*, leaves of *Rosmarinus officinalis* and the root of *Saussurea lappa* were selected, dried, and used in this study because of their use in traditional medicine.

a. **Drying :** The plant is dried in the shade in a dry place at room temperature and ventilated for one month .
b. **Grinding:** The dried out plants were then milled into a fine powder using an electric grinder (Figure 10) The powdered material was put in sealed glass bottle and stored in cold room until use.

FIGURE 10. PLANT MATERIAL BEFORE & AFTER GRINDING; A. ROOTS OF *SAUSSUREA LAPPA*, B. AERIAL PART OF *AJUGA IVA* AND C. LEAVES OF *ROSMARINUS OFFICINALIS*).

I. 2Bacterial Material :

Seven bacterial strains reported antibiotic resistance were available for use in this project: *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 25923**),** *Enterococcus faecalis* (ATCC 49452) and *Bacillus cereus* (ATCC 11778) according to the American type culture collection (ATCC). The bacterial strains were obtained from the Department of cellular and molecular biology, Faculty of Science, University of Telemcen.

Clostridium difficile is isolated locally in standard hygienic conditions at the microbiology laboratory of Saida University.

I.3. Antibiotics products :

The antibiotics used were chosen to be from the list of the commonly and usually given by local doctors to treat patients with illnesses cases thought caused by the five bacteria mentioned above.

The antibiotics used included the following: Oxaciline, Ampiciline, Amoxiciline, Cefazolin, Ceftazidime, Gentamicine, Amikacin, Kanamycin, Tetracycline, Speramicine, Linezolid, Chloramphenecole, Levofloxin, Nitroxoline.

II. Methods :

II.1. Extraction methods :

Extraction is the separation of medicinally active portions of plant using selective solvents through standard procedures . The purpose of all extraction is to separate the soluble plant metabolites, leaving behind the insoluble cellular marc (residue).

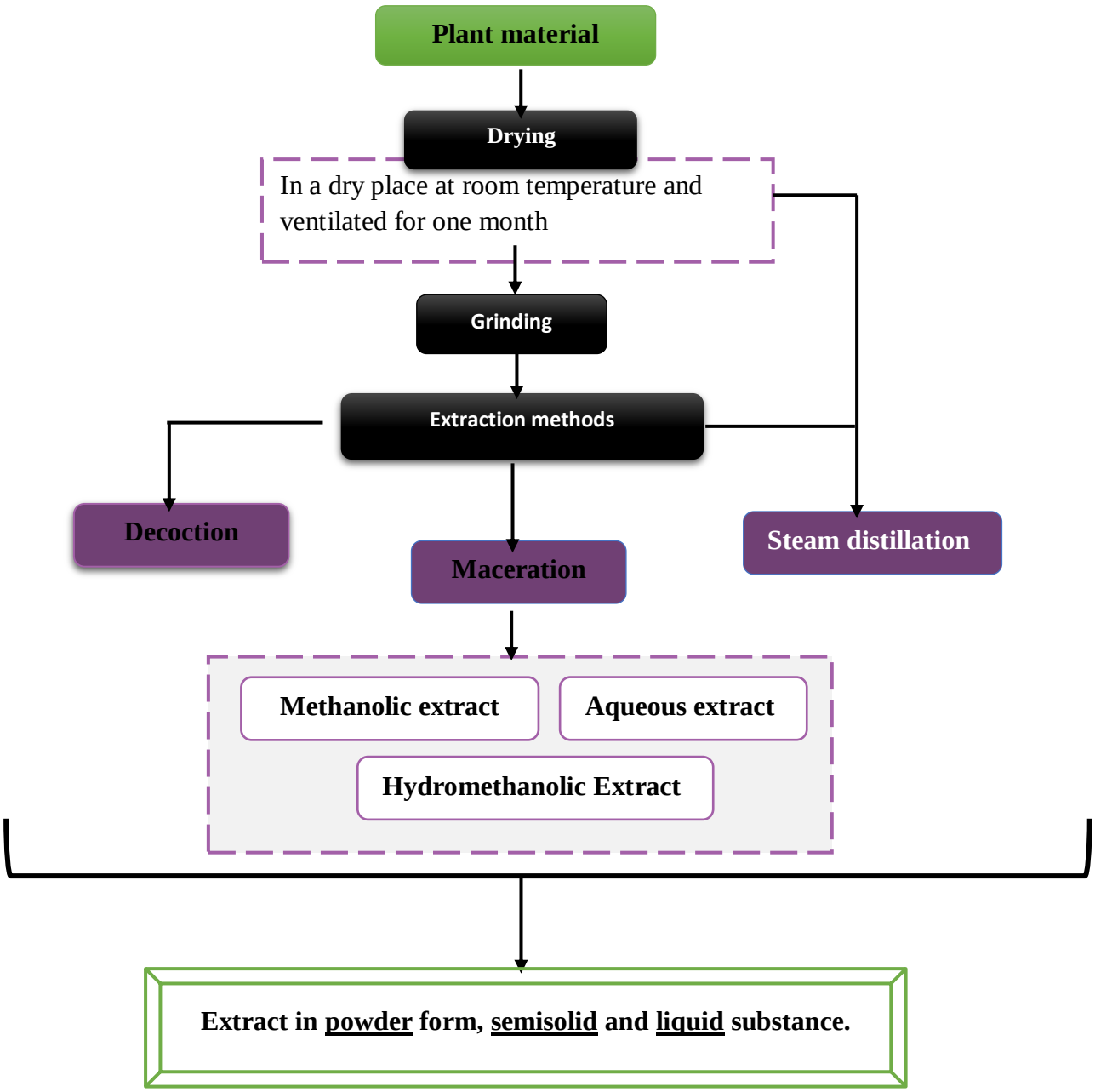


Figure 11. Diagram illustrating the experimental approach followed in this study.

1.1. Maceration

Maceration is a technique use in wine making and has been adopted and widely used in medicinal plants research. Maceration involved soaking plant materials (powdered) in a stoppered container with a solvent and allowed to stand at room temperature for a period of minimum 3 days with frequent agitation.

The processed intended to soften and break the plant's cell wall to release the soluble phytochemicals. After 3 days, the mixture is pressed or strained by filtration (Figure 12).

a. **Methanolic extract :**

   50g of extracts powder were macerated with 300 ml methanol at room temperature for 24 hours filtered with suction filtration. methanol was then removed by using rotary evaporator at 70°C.

   Methanol was used because of its broad spectrum and relative nonselective property of extraction.

b. **Hydromethanolic Extract :**

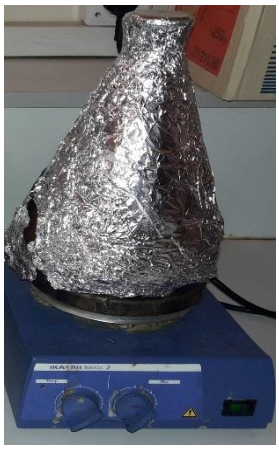
   50 g of the extracts powder was weighed and extracted with 80% methanol and distilled water (240 ml methanol / 60 ml water). The extract was filtered and concentrated using a rotary vacuum evaporator then stored and kept in the laboratory refrigerator till usage.

c. Aqueous extract :

   About 50g of the powdered material was leached in 300 ml of distilled water for 24 h at room temperature with occasional shaking. The mixture was then filtered and was evaporated to dryness to obtained a brown semisolid substance.

1.2. Decoction :

The dried form of powder was mixed with sterile distilled water (50 g powder in 300 ml water) and was heated till boiling for 30 minutes. After cooling, the extract was filtered by using Whatman No.1 filter paper. The filtrate was collected and dried in container to obtain concentrated aqueous extract in powder form



Maceration

Filtration

Evaporation

Drying

Figure 12.: Extraction by maceration method

1.3. Steam distillation :

Plants previously dried (1 kg) put in a can on which we pass water vapor which entrains, by distillation, the molecules of essential oil (Figure 12 a).

- The vapor charged with essential oil is condensed by cooling .
- A siphon (angled return tube forming a triangle) allows the condensed water to return to the container, which avoids clogging in the upper part and also recycling of the water used (Figure 12b).
- The oil condenses along with the water and, as it is lighter, floats above the siphon water. It thus accumulates during the operation which lasts 2 to 3 hours in general.
- The oil is recovered using the tap placed at the bottom of the siphon, taking care to remove the hydrosol first.

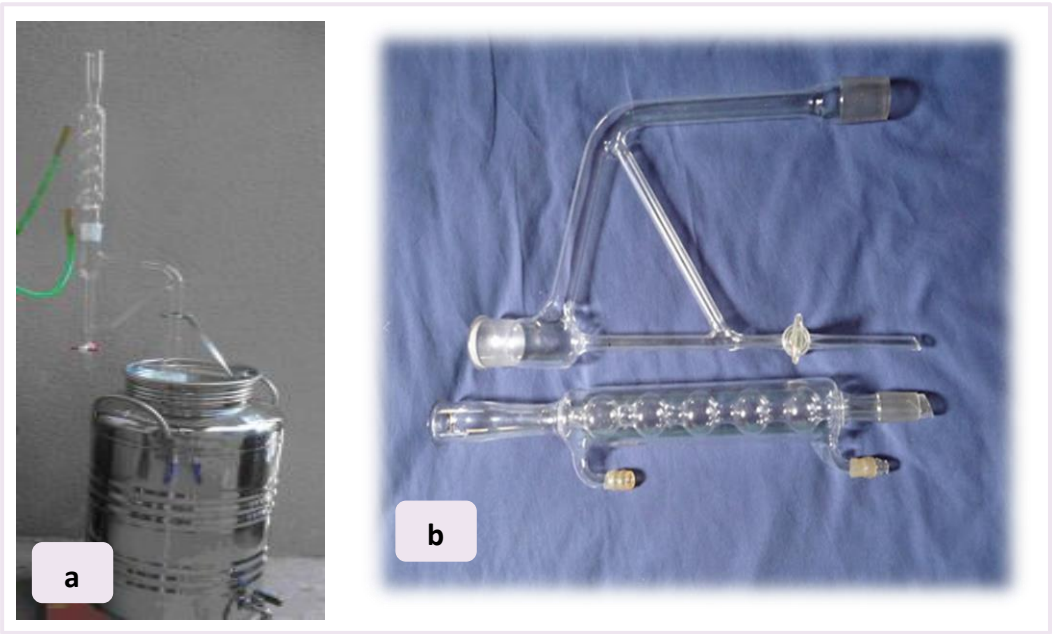


Figure 13 : extraction of the essential oils by steam training.

II.2. The extraction yield :

The yield in percentage (%), is defined as being the ratio between the mass of extract and the dry plant powder **(Bouchouka, 2016)**, It is calculated by the following formula :

$$\text{Extract yield } \% = (v / w) \times 100$$

- V: Net weight of powder in grams after extraction (g).
- W: Total weight of wood powder in grams taken for extraction (g).

II.3. Phytochemical screening :

It refers to the extraction, screening and identification of the medicinally active substances found in plants. Some of the bioactive substances that can be derived from plants are flavonoids, alkaloids, quinones, tannin, anthocyanins and phenolic compounds.

a. **Detection of Alkaloids**:

1g of methanolic extract were dissolved individually in 3ml dilute hydrochloric acid and filtered. The filtrates were used to test the presence of alkaloids.

- **Mayer's test**: Filtrates were treated with 4 drops of Mayer's reagent. Formation of a yellow cream precipitate indicates the presence of alkaloids.
- **Wagner's test**: Filtrates were treated with 4 drops of wagner's reagent. Formation of brown/ reddish brown precipitate indicates the presence of alkaloids.
- **Dragendorff 's test**: Filtrates were treated with 4 drops of Dragendorff 's reagent. Formation of orange / reddish orange precipitate indicates the presence of alkaloids.

b. Detection of Tannins:

A small quantity of methanolic extract was mixed with methanol and heated on water bath. The mixture was filtered and ferric chloride was added to the filtrate. A dark green color formation indicates the presence of tannins.

c. Detection of Anthocyanins :

The presence of anthocyanins has been demonstrated by adding 2 mL of the methanolic extract with 2 mL of 2 N HCl. The appearance of a pink-red color that turns purplish blue after addition of ammonia indicates the presence anthocyanins.

d. **Detection of Flavonoids :**

1 g of the methanolic extract was mixed with 5 mL hydrochloric alcohol in a test tube. Then, two to three magnesium turnings were added. The addition of three drops of isoamyl alcohol intensifies a pink-orange or violet, which shows the presence of flavonoids.

e. Detection of Quinones :

2g of methanolic extract triturated in 5 mL of HCl diluted 1/5 and then brought the solution to the boiling water bath for 30 min in a test tube. After cooling under a stream of cold water, the hydrolyzate was extracted with 20 mL of chloroform in a test tube. The chloroform layer was then collected in another test tube and then, 0.5 mL of ammonia diluted twice was added thereto. The appearance of a color ranging from red to purple characterizes the presence of quinones.

f. Volatile oil :

0.5 mL of diluted sodium hydroxide and 0.5 mL of diluted hydrochloric acid were added to 2 mL of methanolic extract and mixed well. The formation of a white precipitate was observed for a positive test.

II.4. Purification and isolation of bacterial strains :

4.1. Isolation of clostridium from municipal sewage water sample :

a. Collection of Sample :

The water sample was collected aseptically from Municipal sewage water of " city daoudi moussa " (figure 14). Sample was collected in a sterile bottle and transported to the Saida University laboratory for identification. Identification was done through the Serial dilution method and Pour Plate culturing technique, see next.

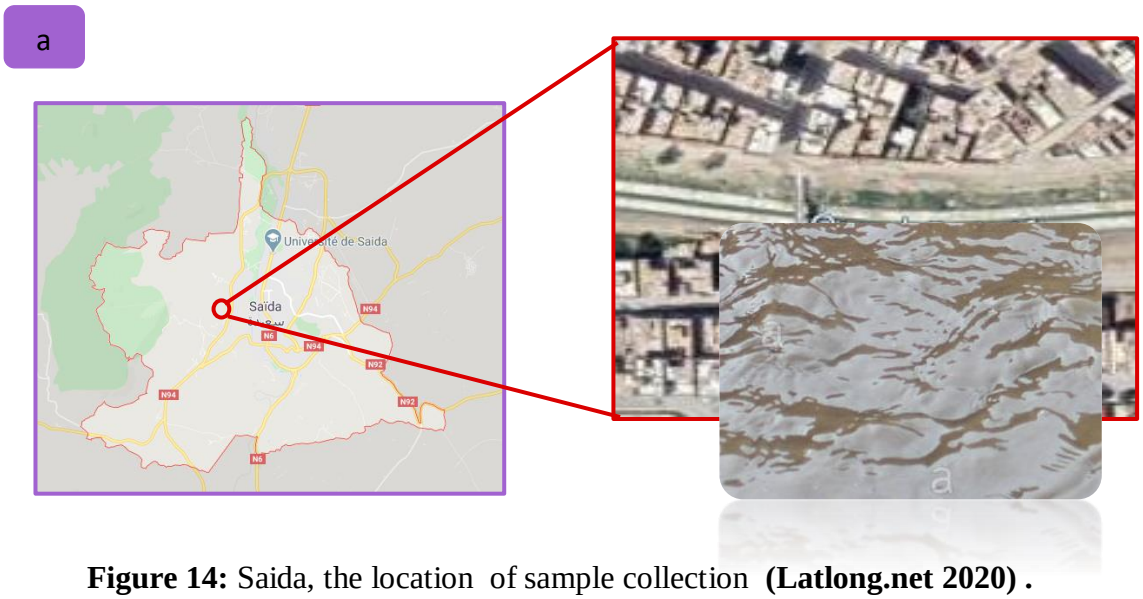


Figure 14: Saida, the location of sample collection **(Latlong.net 2020) .**

b. Serial dilution :

A serial dilution is a series of sequential dilutions used to reduce a dense culture of cells to a more usable concentration. Each dilution will reduce the concentration of bacteria by a specific amount

- The collected sample has been serially diluted with sterile physiological water; 1mL of the initial suspension was added to 9 mL of physiological water .
- Mix well and transfer 1 mL from tube 1 into tube 2 using a pipetter and sterile 1 mL tips.
- Repeat Step 2 for the remaining tubes, each time transferring 1 mL from the most recently used tube to the next tube.

c. Pour Plate:

Colonies were identified in liver-meat agar media in which sodium sulfide 10% and 4 drops of iron salt 5% were added. Each prepared dilution was poured in petri dish media.

- The inoculum (serially diluted from the original specimen) is added to the sterile Petri dishes containing the melted and cooled (40-45 °C) medium and thoroughly mixed by rotating the dishes which were then allowed to solidify.
- This procedure is repeated for all dilutions to be plated.
- After incubation, presence of sulfate reducing germs should show up in black colonies.

d. McIntosh Fildes Jar :

McIntosh and Fildes' anaerobic jar is an instrument used in Microbiology laboratory, for the generation of anaerobic condition (anaerobiosis) to culture obligates anaerobes such as *Clostridium* spp (Figure 15).

- The culture media are placed inside the jar, stacked up one on the other in an inverted position. .
- Add gaz bag envelope to the jar.
- Incubated in anaerobic jar for 24hours at 37 °C .

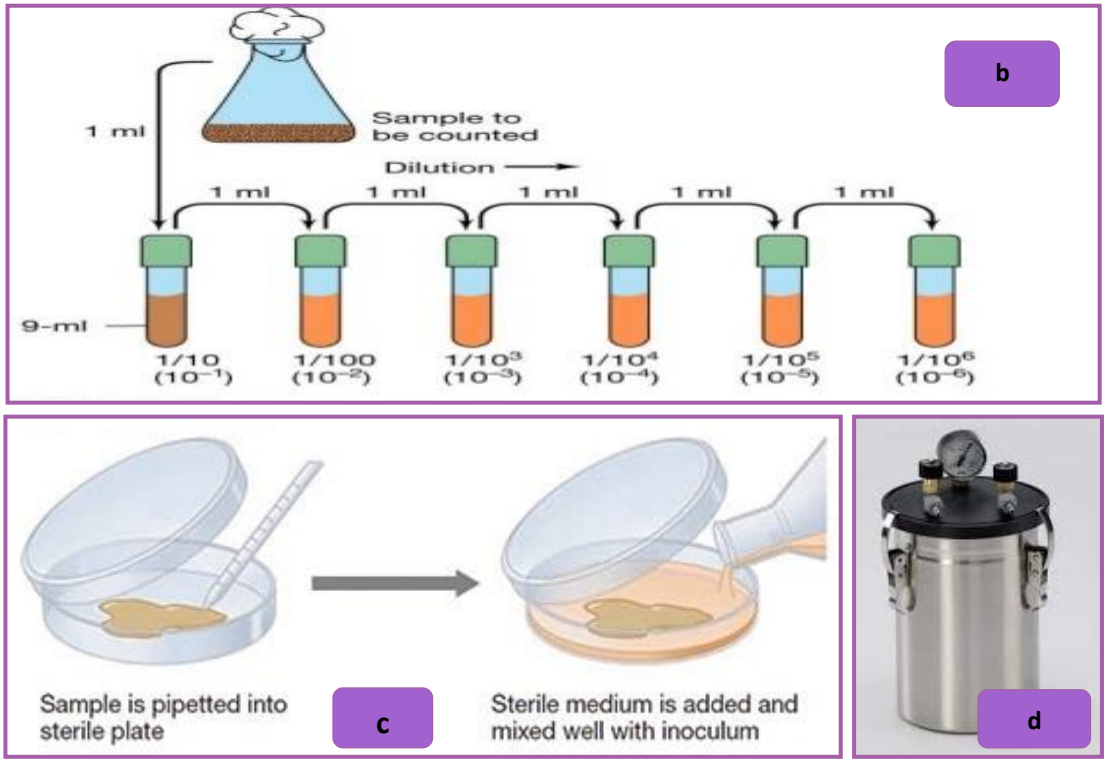


Figure 15 : The main step for isolated of clostridium from sewage water .

4.2. Confirmation of bacterial :

4.2.1. Characterization of bacterial culture :

- **Morphological studies :**

In order to identify the unknown bacteria, examination of seven characteristics ofthe unknown bacteria is necessary. These seven characteristics are:

- colony morphology,
- cell morphology,
- Gram stain reaction,
- oxygen requirements for growth,
- carbon source utilization ;
- Motility ;
- finally comparison was performed based on Bergy's manual .

a. Colony morphology :

Bacteria grow on solid media as colonies. A colony is defined as a visible mass of microorganisms all originating from a single mother cell, therefore a colony constitutes a clone of bacteria all genetically alike.

In the identification of bacteria much weight is placed on how the organism grows in or on media. This exercise will help you identify the cultural characteristics of a bacterium on an agar plate - called colony morphology. Although one might not necessarily see the importance of colonial morphology at first, it really can be important when identifying the bacterium. Features of the colonies may help to pinpoint the identity of the bacterium. Different species of bacteria can produce very different colonies.

- **Whole shape of colony** : Varies from round to irregular to filamentous .
- **Size of colony :** Can vary from large colonies to tiny colonies less than 1mm .
- **Edge /Margin of colony :** Magnified edge shape (use a dissecting microscope to see the margin edge well)
- **Chromogenesis :** Color of colonies, pigmentation: white, bleu, red, purple, etc.
- Elevation of coloney :How much does the colony rise above the agar .
- **Surface of colony** : Smooth, glistening, rough, dull (opposite of glistening), rugose (wrinkled).
- **Texture :** Butyrous (buttery), viscid (sticks to loop, hard to get off), brittle/friable (dry, breaks apart), mucoid (sticky, mucus-like).

Media used :

Bacterial isolation can be done using a general medium, wherein various bacteria can grow, and selective media that allows growth of specific genera.

Nutrient Agar is a general purpose, nutrient medium used for the cultivation of microbes supporting growth of a wide range of organisms.

Hektoen enteric agar (HEK) is a selective and differential agar primarily used to recover enteric pathogens such as *Klebsiella* or *Escherichia* and *Salmonella* .

King Agar A and B enhances the elaboration of phycocyanin; P. aeruginosa build colonies surrounded by a blue-green zone due to phycocyanin production.

Mannitol salt agar is a commonly used selective and differential growth medium in microbiology , It contains a high of salt (NaCl), making it selective for *Staphylococcus aureus* .

Liver Meat Agar is used for cultivation of fastidious anaerobic microorganisms.

b. Gram staining :

The Gram stain characterizes bacteria based on the structural characteristics of their cell walls. By combining morphology and Gram-staining, most bacteria can be classified as belonging to one of 4 groups (Gram-positive cocci, Gram-positive bacilli, Gram-negative cocci, and Gram-negative bacilli).

This technique is used to stain a slide such as a fecal smear to observe the bacterial microflora present based on their gram stain reaction :

- **Fixation of bacterial smear :** Take a clean dry slide and place one drop of distilled water of the center ; prepare bacterial smear by taking small inoculum from the bacterial culture ; heat fixing of bacterial smear.
- **Application of the primary stain :** Flood the fixed smear with crystal violet solution and allow to remain for 1 minute. ; Rinse off the crystal violet with distilled or tap water.
- **Application of iodine solution :** Flood the slide with iodine solution . Allow to remain for one minute ; Rinse off the iodine solution with distilled or tap water.

- **Decolorization step**: The decolorization step distinguishes gram-positive from gram-negative cells by using alcohol.
- **Application of counterstain :** fushin is applied so that the decolorized gram-negative cells will take on a contrasting color; the gram-positive cells now appear purple.

c. **Biochemical tests :**

Catalase test :

(This test demonstrate the presence of catalase), Aerobic and facultative anaerobic organisms produce two toxins during normal metabolism, hydrogen peroxide (H2O2) and superoxide radical (O2−). These bacteria have two enzymes that detoxify the products of normal metabolism. One of these enzymes, catalase, is capable of converting hydrogen peroxide to water and oxygen.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O2 \text{ (gas bubbles).}$$

The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide, and the rapid elaboration of oxygen bubbles occurs (Positive) . The lack of catalase is evident by a lack of or weak bubble production (Negative). The culture should not be more than 24 hours old.

Oxidase test :

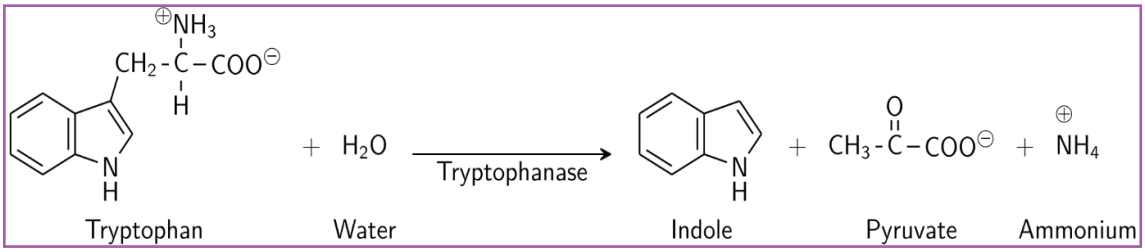
This is a test to see if an organism is an aerobe. It is a check for the presence of the electron transport chain that is the final phase of aerobic respiration. Normally, oxygen is the final electron acceptor for this system. In the oxidase test, identifies organisms that produce the enzyme cytochrome oxidase (transferring electrons from a donor molecule to oxygen).

Oxidase test can be performed by several methods The dry filter paper method is performed by impregnating strips of filter paper with oxidase reagent. The paper is smeared with the bacterial colonie to be tested by a glass rod. the Kovac's oxidase reagent contains a chromogenic reducing agent, which is a compound that changes color when it becomes oxidized. If the test organism produces cytochrome oxidase, the oxidase reagent will turn blue or purple within 15 seconds.

Urea indole test :

The Urea Indole medium allows the detection of urease. The bacteria with a urease transform the urea into ammonium carbonate resulting in an alkalization which causes a purplish red coloration of the medium in the presence of phenol red .

It is also used to determine the ability of an organism to split amino acid tryptophan to form the compound indole. Tryptophan is hydrolysed by tryptophanase to produce three possible end products one of which is indole.



Indole production is detected by Kovac's reagent, this reacts with indole to produce a red coloured compound . Indole test helps to differentiate *Enterobacteriaceae* and other genera.

Incubating at 37°C for 24hours. Then, 5 drops of Kovac's reagent (code 55313) added to the inoculated medium. Positive test is indicated by formation of a red ring at the surface of the medium. No color change indicates a negative test.

Mannitol motility :

Mannitol Motility Test Medium is designed to differentiate bacteria on the basis of their motility and ability to ferment mannitol. Semisolid nature of the medium due to 0.3% agar helps to detect motility. Motile bacteria produce diffused growth throughout the medium while non-motile bacteria grow only along the line of inoculation. Fermentation of mannitol produces acidity in the medium. Phenol red is the pH indicator, which detects acidity by exhibiting a visible colour change from red to yellow.

Inoculate tubes with a pure culture (young colony 18 h -24h) by stabbing the center of the column of medium to greater than half the depth. Incubate tubes for 24-48 hours at 37°C in an aerobic atmosphere.

Simmons Citrate agar :

Simmons Citrate Agar is an agar medium used for the differentiation of Enterobacteriaceae based on the utilization of citrate as the sole source of carbon. Bacteria that can grow on this medium produce an enzyme, citrate-permease, capable of converting citrate to pyruvate. Pyruvate can then enter the organism's metabolic cycle for the production of energy. Growth is indicative of utilization of citrate, an intermediate metabolite in the Krebs cycle.

When the bacteria metabolize citrate, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the bromthymol blue indicator in the medium from green to blue above pH 7.6.

Inoculate Simmons citrate agar lightly on the slant by touching the tip of a needle to a colony that is 18 to 24 hours old. Incubate at 37°C for 18 to 24 hours. Observe the development of blue color; denoting alkalinization (Citrate positive).

Voges Proskauer (VP) Test:

Voges–Proskauer or VP is a test used to detect acetoin in a bacterial broth culture. The test is performed by adding alpha-naphthol and potassium hydroxide to the Voges-Proskauer broth (formulated by Clark and Lubs) which has been inoculated with bacteria.

The principle of Voges–Proskauer Test is to check for microorganism's ability to produce acetylmethyl carbinol from the fermentation of glucose .

1. Inoculate VP broth with a pure culture of the organism ; Incubate at 35°-37°C for a minimum of 24 hours in ambient air ;
2. Add 6 drops of VP reagent I (alpha napthol) and 2 drops of VP reagent II(40% KOH) (Note: It is essential that the reagents be added in this order.)
3. Observe for the color change in the broth medium.

A cherry red color indicates a positive result, while a yellow-brown color indicates a negative result .

Kligler's Iron Agar (KIA) :

The Kligler's Iron Agar test employs a medium for the identification of Enterobacteriaceae by demonstrating hydrogen sulfide production and the fermentation of dextrose and lactose. In 1918, Kligler described a medium for detection of H_2S and differentiation of Salmonella spp. Bailey and Lacey further modified the medium by substituting phenol red indicator for Andrade indicator. This medium became known as KIA. It is recommended for determination of H2S production by enteric gram-negative bacilli and for detection of H2S produced by some strains of *Pseudomonas*.

With a straight inoculating needle, inoculate KIA by stabbing through the center of the medium to the bottom of the tube and then streaking the slant while withdrawing the needle.Incubate tubes aerobically with at 37ºC for 18-24 hours and examine the reaction of the medium.

Carbohydrate fermentation :

For slant :

- **Positive test:** yellow (acid)
- **Negative test:** red (alkaline)

For butt :

- **Positive test:** yellow
- **Negative test:** red

Red slant/yellow butt: dextrose positive, lactose negative .

Yellow slant/yellow butt: dextrose positive, lactose positive .

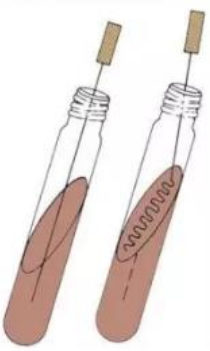
Red slant/red butt: dextrose negative, lactose negative .

H_2S Production:

- **Positive Test:** Black precipitate or color throughout the medium or at the junction between slant and butt
- **Negative Test:** No black color development

Gas production :

- **Positive test:** the presence of bubbles or cracks in the medium
- **Negative test:** the absence of bubbles or cracks in the medium



Inoculation of KIA

II.5. Antibiotic sensitivity test method (The Kirby-Bauer disc diffusion):

Antibiotic testing (KB testing or disk diffusion antibiotic sensitivity testing) is a test which uses antibiotic-impregnated paper disks to test whether particular bacteria are susceptible to specific antibiotics. A known quantity of bacteria are grown on Mueller-Hinton agar plates in the presence of thin filter paper discs containing relevant antibiotics. If the bacteria are susceptible to a particular antibiotic, an area of cleaning surrounds the wafer where bacteria are not capable of growing.(called zone of inhibition). The Kirby Bauer Agar disk diffusion method provides qualitative interpretive category results of susceptible, intermediate and resistant bacterial isolates.

This method was used to test antibacterial resistance. A total of 14 antibacterial agents belonging to 7 antibacterial classes (Table 5).

Table 5 : Details of antibiotics used for antibiogram profile

Classes	Antibiotics (µg)	Cod/concentration
Beta-lactam	Oxaciline	Ox (5µg)/disc
	Ampiciline	Am (10µg)/disc
	Amoxiciline	Ax (25µg)/disc
	Cefazolin	Kz (30µg)/disc
	Ceftazidime	Caz (30µg)/disc
Aminoglycosides	Amikacin	Ak (30µg)/disc
	Kanamycin	K (30UI)/disc
Tetracyclines	Tetracycline	Te (30UI)/disc
Macrolides	Speramicine	Sp (100µg)/disc
Oxazolidinones	Linezolid	Lnz (30µg)/disc
Chloramphenicol	chloramphenecole	C (30µg)/disc
Quinolone	Levofloxin	Lev (µg)/disc
	Nitroxoline	ctx/ntx (30µg)/disc

5.1. Procedure for Modified Kirby Bauer method :

1. Prepare the inoculum from the primary culture plate by touching with a loop the tops of each of 3 – 5 colonies, of similar appearance, of the organism to be tested and transfer this growth to a tube of saline. If the inoculum has to be made from a pure culture, suspend a loopful of the confluent growth similarly.

- All bacteria for which the evaluation of antibacterial activity are shown in the below table (Table 6).

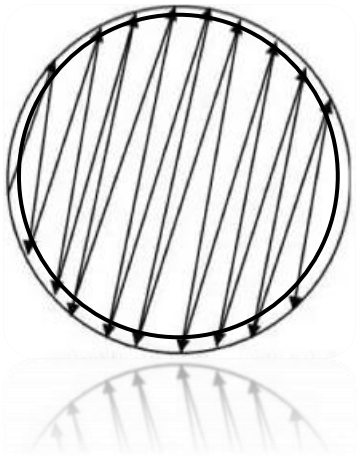
Table 6 : Bacterial strains used to evaluate antibacterial activity.

Bacterial strains used		**References**
Gram (+)	*Staphylococcus aureus*	(ATCC 25923)
	Bacillus cereus	(ATCC 11778)
	Enterococcus faecalis	(ATCC 49452)
	Clostridium difficile	isolated
Gram (-)	*Escherichia coli*	(ATCC 25922)
	Pseudomonas aeruginosa	(ATCC 27853)
	Klebsiella pneumonia	(ATCC 25923**)**

2. Proper adjustment of the turbidity or the inoculum is essential to ensure that the resulting lawn of growth is confluent or almost confluent .

Compare the tube with the **0.5 McFarland turbidity standard (**approx cell density 1.5 x10^6 CFU/ml**)** and adjust the density of the test suspension to that of the standard by adding more bacteria or more sterile saline using the spectrophotometer to reach the final concentration .

3. Inoculate the plates by dipping a sterile swab into the inoculum.
4. Pass the swab round the edge of the agar surface. The swab should follow as it is drawn across the plate (as shown in figure).



5. Leave the inoculum to dry for a few minutes (at least 3 to 5 minutes, but no more than 15 minutes) at room temperature with the lid closed.
6. Place the appropriate antimicrobial-impregnated disks on the surface of the agar using a pair of sterile forceps .
7. The plates should be placed in an incubator for 24h at 37 °C.
8. After overnight incubation, the diameter of each zone (including the diameter of the disc) should be measured and recorded in mm.

9. Results :

Results can be read after 18-24 hours of incubation. Following incubation, measure the zone sizes to the nearest millimeter (mm) using a ruler or caliper; include the diameter of the disk in the measurement :

- Diameters less than 7 mm: no antimicrobial activity (-)
- Diameters from 7 to 9.9 mm: low antimicrobial activity (+)
- Diameters from 10 to 11.9 mm: modest antimicrobial activity (+ +)
- Diameters from 12 to 15 mm: high antimicrobial activity (+ + +)
- Diameters greater than 15 mm: strong antimicrobial activity (+ + + +)

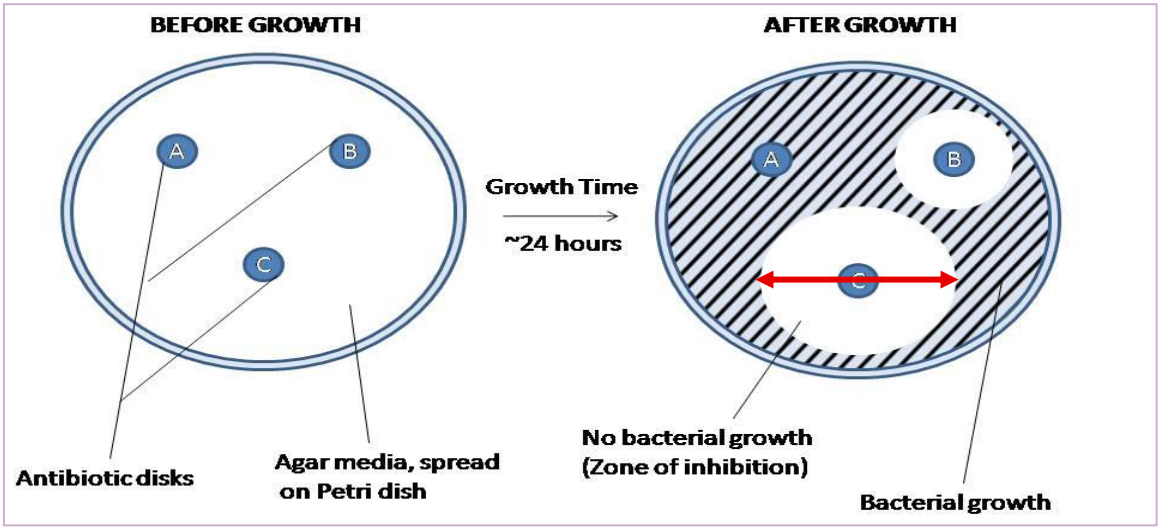


Figure 16 : Principle of the disc diffusion method.

5.2. Preparation of solutions:

a. Discs preparation

The disc was prepared by using Whatmann No.1 filter paper. Then, the filter paper disc of 6mm diameter were sterilized and soaked in 50 µl of each extract in the different concentrations (methanolic , hydromethanolic and aqueous).

b. Essential oils:

We prepared a solution (3ml) in a 5ml test tube from 1ml (1000µl) of the essential oils and 0.5ml Tween 80. This volume was supplemented by dimethylsulfoxide DMSO up to 3ml (3000 µl). Then we homogenized the solution using a vortex shaker and aseptically filtered with a sterile millipore filter.

c. Inoculum preparation

The bacteria were maintained on nutrient agar plates and were revived for bioassay by subculturing in fresh nutrient agar for 24 h before being used.

cultures were transferred into 5 ml of freshly prepared nutrient broth and standardised to 0.5 McFarland turbidity standards using the spectrophotometer to obtain the desired cell density of 10^8(cells/ml).

5.3. Screening of Antimicrobial activity

1. 10ml of sterilized Mueller-Hinton agar medium were poured into the each sterile petridish.
2. After solidification, the sterile cotton swab was dipped into the culture or broth of *Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, Enterococcus faecalis, Klebsiella pneumonia, Staphylococcus aureus* and *Clostridium dificile*.
3. The entire agar surface of each plate was inoculated with this swab first in a horizontal direction and then in a vertical direction, which ensure the even distribution of organism over the agar surface.
4. The filter paper disc soaked in extract were placed on the surface of the bacteria seeded agar plate and then the plate were incubated at 37◦ C for 24 hours. The antibacterial activity was recorded by measuring the width of the clear inhibition zone around each disc.

5.4. Minimum Inhibitory Concentration assay (MIC)

Minimum Inhibitory Concentration (MIC) assays determine the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism.

Assay was performed in 96-wells microliters late total volume of assay system in each well was kept 100 µl

1. Column 11 contained 50 µl of standardised inoculum and Column 12 contained 100 µl of the medium broth (as a control to monitor sterility),
2. pipette was then used to transfer and mix each dissolved seeds extract (1g extact in 2 ml muller hinton broth) from column 1–10, resulting in 50 µl extract per well.
3. The tested concentrations of the different extracts achieved through serial dilutions from columns 10–1.
4. 50 µl of the adjusted OD of 0.06 at λ =625nm for bacterial suspension was then added to all wells containing extracts except the control wells, The time taken to prepare and dispense the OD adjusted bacteria did not exceed 15 min.
5. After incubation for 24 h at 37 °C, microplates read with spectrophotometer ELISA.

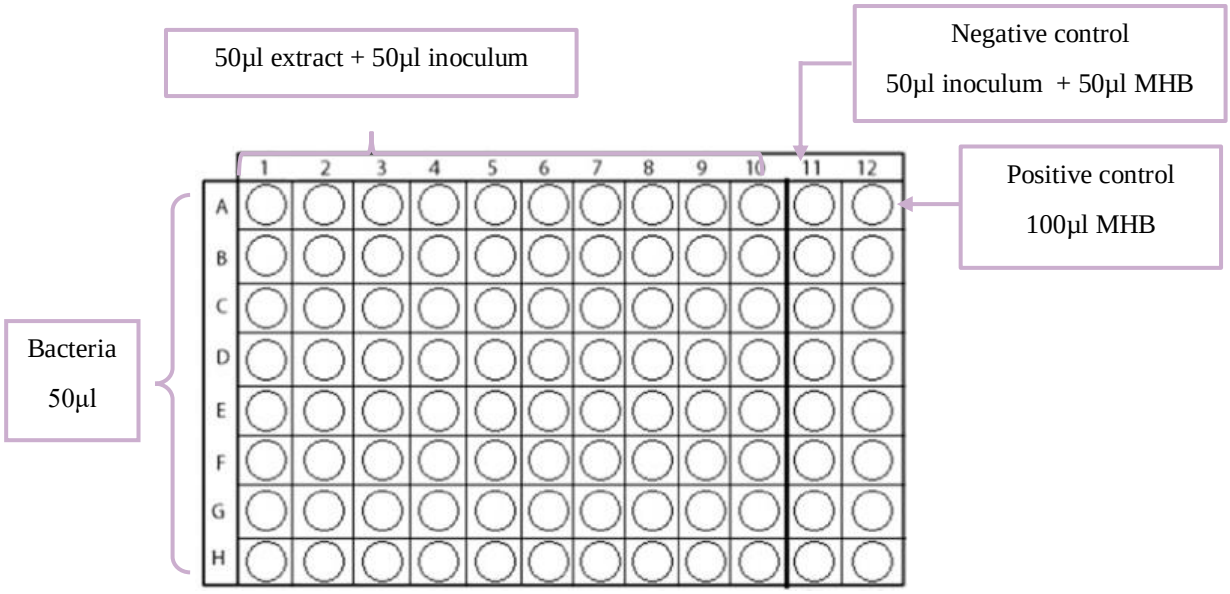


Figure 17 : Minimum Inhibitory Concentration (micro dilution method).

III. Bioinformatics tools and Methods:

A limited initial training into bioinformatics tools and methods had been undergoing for the appropriately deal with types of data and results generated by this projects. Unfortunately, the training was put at halt due to the COVID-19 pandemic preventive measures and the lenthy delays incured globally by everyone at almost all levels of life.

However, the data and results collected during this project pertaining to the selected bacteria, tested anibiotics, medicinal plants and extacted antobacterial active compounds, would be valorazied then annotated and stored (by the project's suppervisor) into the online dabatase BARID (**Kheris et al., 2019**) and made available for querying by the scientific community via the web-address: http://www.bioinformaticstools.org/prjs/barid/. This is since BARID is the online database created, last year, for the larger project Antibiotics Resistance medically outstanding problem and Antibacterial effects of compounds from plant origins (**Kheris, 2019**) to which this project belong.

Results

This chapter concerns the review of detailed lay out of the various results obtained during the research work of this project.

The first part is related to the wet-laboratory research work which includes Bacteriology, Anti-bacterial activity of the three plant extracts experiments. This part was not completed due to the Covid 19 quarantine .

I.1 Extraction yield

The yields obtained for different extracts are shown in Table 7 and Figure 21**.**

Table 7 : different yields extraction results.

	ME	**HME**	**AQE**	**OIL**
P1	11.63%	7.94 %	9.71 %	1.25 %
P2	13.78 %	8.82 %	12.06 %	0 %
P3	11.26 %	15 %	13.86 %	3.1 %

P1 : *Saussurea lappa* .

P2 : *Ajuga iva* .

P3 : *Rosmarinus officinalis* .

ME : methanolic extract – (methanol).

HME : hydromethanolic extract (methanol - water).

AQE : aqueous extract (distilled water).

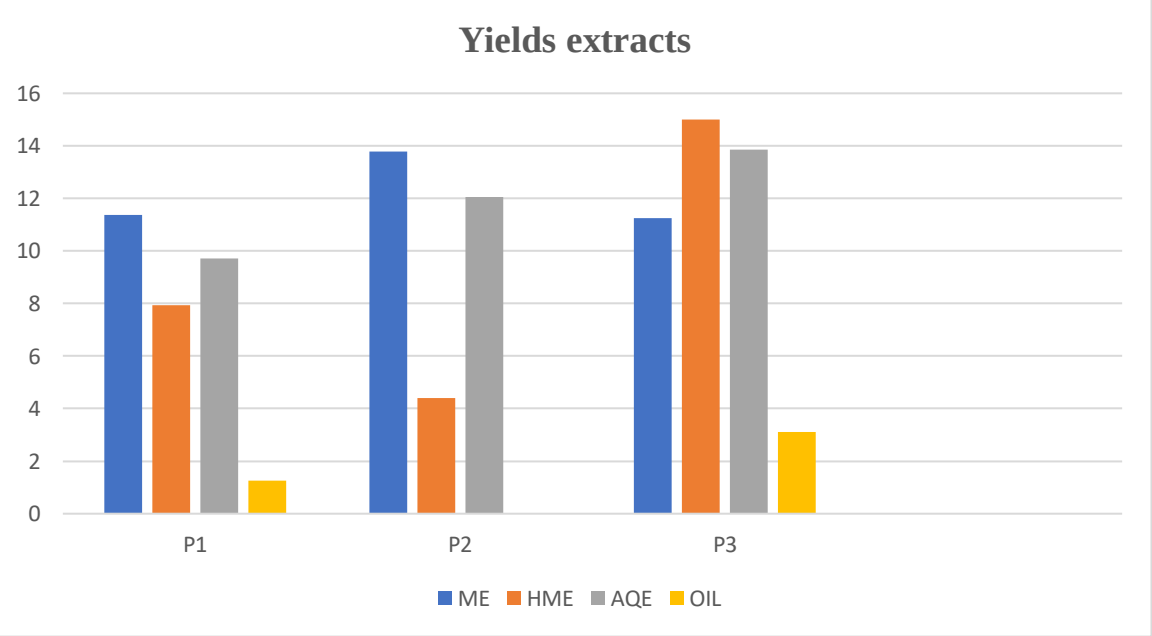


Figure 18: The yield amounts variation resulted from the different processes of extraction.

The highest yield was obtained from hydrometanolic extract (15%) and aqueous extract (13.86 %) of : *Rosmarinus officinalis*, followed by the methanolic extract (13.78 %) of : *Ajuga iva.*), and finally the extrac methanolic extract (11.36 %) of : *Saussurea lappa*. However, the lowest yield was obtained from essential oils of three plants.

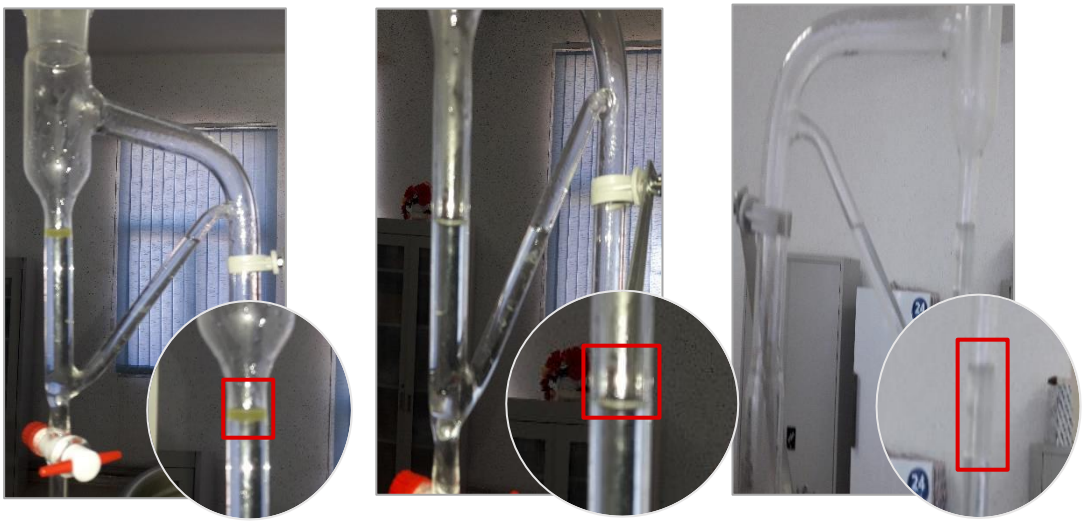


Figure 19: The yield amounts variation resulted from the different processes of extraction.

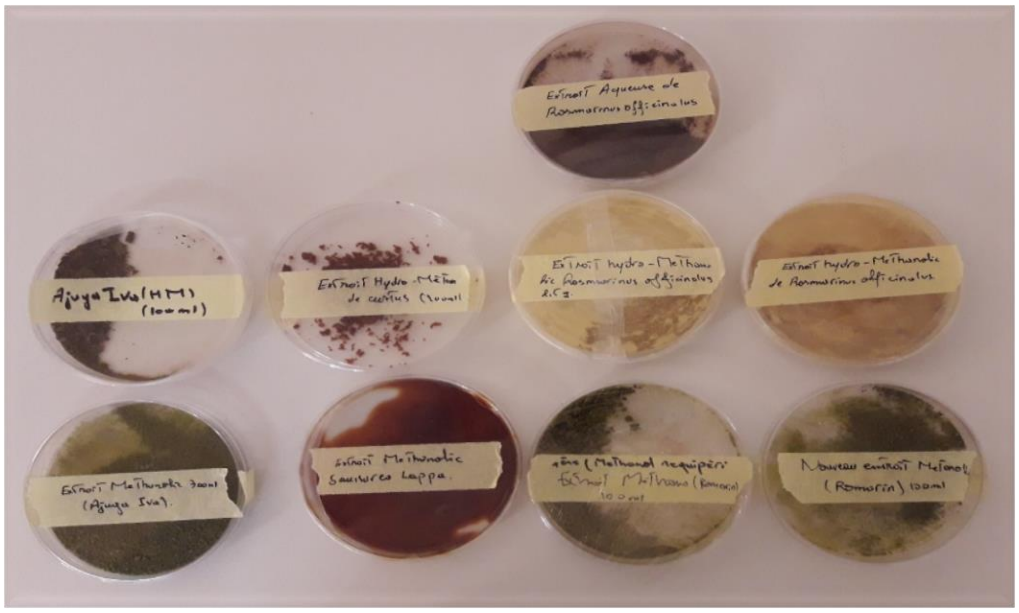


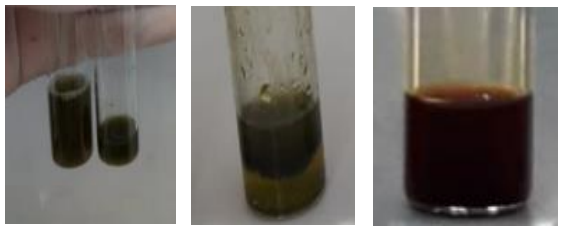
Figure 20 : The variation resulted from the different processes of extraction.

All plant extracts used have different properties (color and aspect) from each other .It depends on the type and polarity of secondary metabolites ; Part used : root, leaf, flower, stem ; phytochmicals composition .

I.2 Phytochemicals screening :

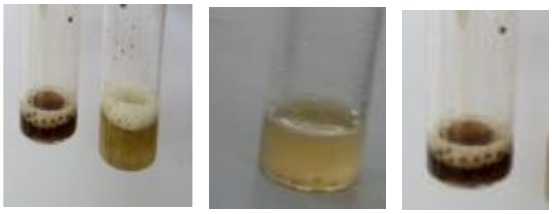
The phytochemical screening of three plant metanolic extract revealed the presence of secondary metabolite such as alkaloids, tannins, anthocyanins, flavonoids and quinones shown in (**Table 8)**.

	P1	P2	P3
Alkaloids	+	-	-
Tannins	+	+	+
Anthocyanins	-	+	-
Flavonoids	+	+	+
Quinones	+	-	+
Oil	+	-	+

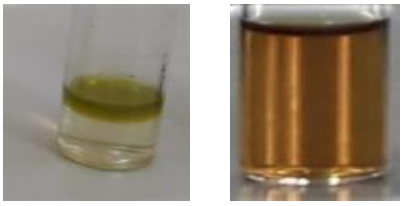


Alkaloids

Tanins



Flavonoids



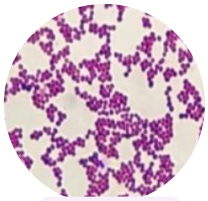
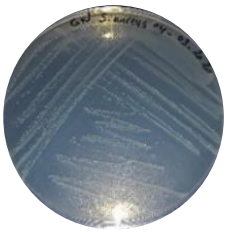
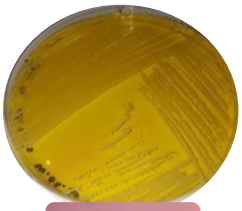
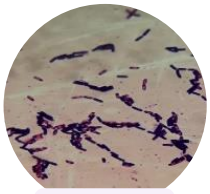
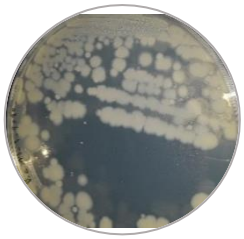
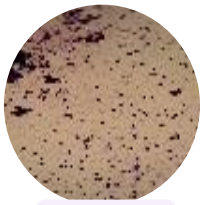
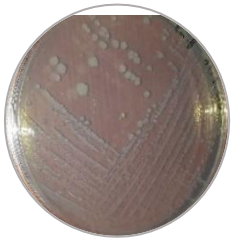
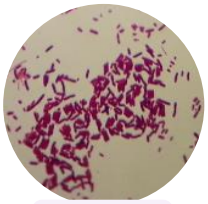
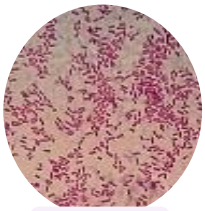
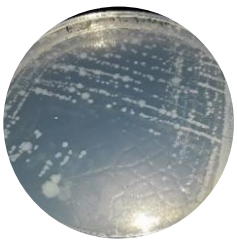
Anthocyanins

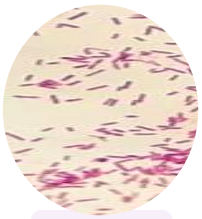
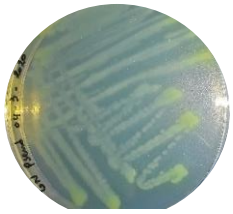
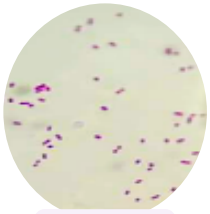
Quinones

I.3. Purification and isolation of bacterial strains :

I.3.1. Confirmation of bacterial strains purity

Table 9 : Purification and confirmation of bacterial strains used :

Microorganisms	Characteristics of colonies	Gram staining	Inoculated by streak-plate (GN)	streak-plate selective medium
Staphylococcus aureus	▪ Cocci clusters or chains ▪ The use of mannitol results in acidification of the medium (yellow). ▪ Small, creamy and round colony.	 **Gram +**		 **chapman**
Bacillus cereus	▪ Large bacillus ; sporulated ▪ Circular and irregular colonies with a creamy and grainy appearance.	 **Gram +**		—
Enterococcus faecalis	▪ diplococcus ▪ Small white, flat colonies with a smooth appearance.	 **Gram +**		—
Clostridium difficile	▪ anaerobic, bacillus ; sporulated bacteria and occur in pairs or short chains. ▪ Are irregular, raised, opaque, forming black colonies on LMA .	 **Gram +**	—	 **Liver meat agar**
Escherichia coli	▪ Bacillus bacteria ▪ Round, flat and regular colonies.	 **Gram -**		—

Pseudomonas aeruginosa	▪ Bacillus bacteria ▪ Isolated or diplobacillus ▪ Develop a green pigmentation on king b and blue on king a .	 **Gram -**		 King A-B
Klebsiella pneumonia	▪ Bacillus bacteria ▪ Large, bulging, mucous and translucent colonies.	 **Gram -**		—

Due to the COVID-19 pandemic delaying problems, the Biochemical and Kligler Iron Agar tests on the bacterial strains used were not followed up and hence not results were obtained.

Same situation has been casted over the tests of the selected Antibiotics on the studied bacteria.

Discussion :

a. **Extraction yield :**

A comparative study of extraction yield was carried out in order to test optimum condition of extraction, by usig maceration and steam distilation extraction methods using consecutive application of solvents with increasing polarity such as methanol, and Water. The results of extract yield (expressed as weight of extract relative to the weight of the starting plant material) are given in **Table 8** . It can be seen that the yield of extraction using extraction method increased with increasing polarity of solvents, ranged from 1 ± 0.25 to 15 ± 0.00%. In which water extracts gave the highest yield from all plants, while methanol and acetone gave lower yields, the yields of steam extraction were remarkably lower compared to maceration extracts.

The extraction yield of plants is significantly influenced by the methods and solvents of extraction , But no significant change in extraction yield from plant sites was recorded. These results showed that the solvent polarity is of great importance **(Clara et al., 2010).** In fact, since Water and methanol are a polar solvent, the polar components from three plants be extracted. Thus, the variation in the yields of various extracts can be attributed to the polarities of the different compounds. Such differences have been reported in the literature **(Khlifi et al., 2011).**

These findings were in agreement with **(El Hilaly et al., 2004)** in *rosmarinus officinalis* extract . The close finding is reported for other lamiaceae such as T. vulgaris, Salvia officinalis and Origanum majorana **(Roby et al., 2013),** Thymus numidicus **(Ben El Hadj Ali et al., 2014).**

Although many reports are available on the *ajuga iva* oils and their characteristics , However, it was not extracted in this study possibly because it was not picked up in flowering season **(Besma et al., 2013).**

The results were also similar in approximate proportions in the *Saussurea lappa* according to Pharmaceutical research **(Imad et al., 2017).**

About the process of decoction stopped at the last stage (dried in a container). for this reason, the final aqueous extract was not obtained.

b. **Phytochemicals screening :**

From all the results obtained, we were able to get a general idea of the chemical composition of the three plant studied. Indeed, we can conclude that *Saussurea lappa* is more rich in secondary metabolites, particularly the methanolic extract which revealed the presence of the chemical families; alkaloids, tannins, flavonoids and quinones except anthocyanins .

We noted the presence of tannins, flavonoids and quinones in the methanolic extract of *Rosmarinus officinalis*, also we have marked the presence of polyphenols (tannins and anthocyanins) and flavonoids in the methanolic extract of *ajuga iva*

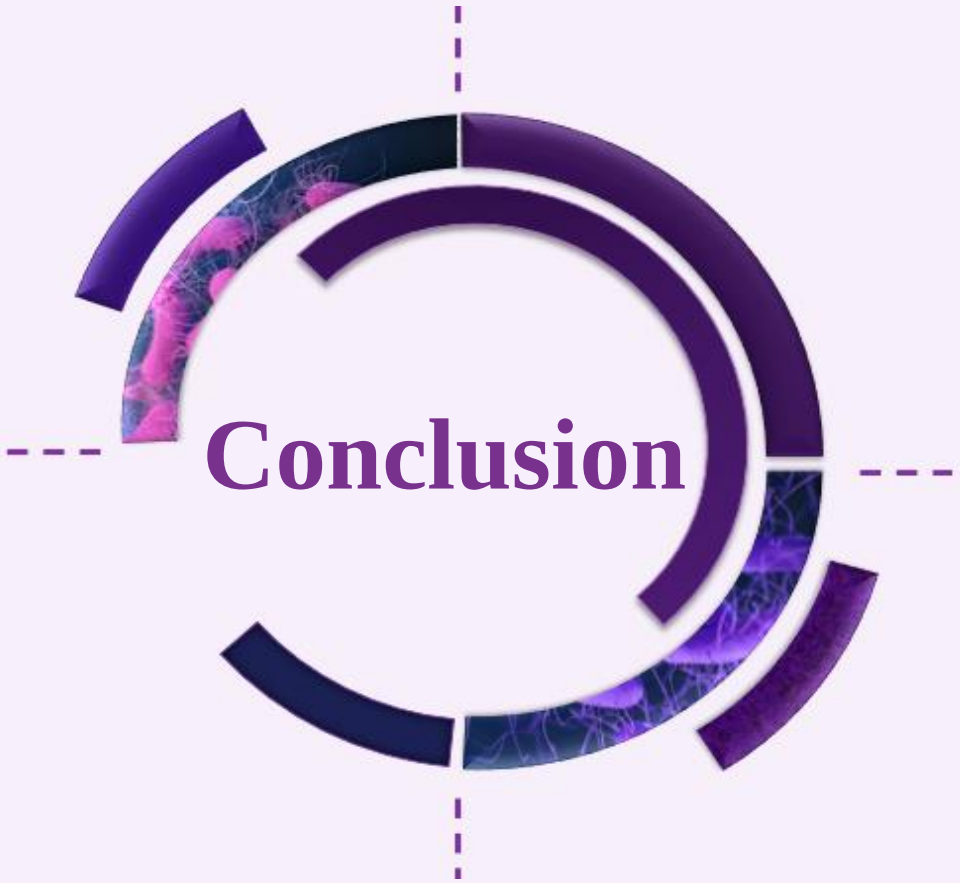
c. **Purification and isolation of bacterial strains :**

We confirmed the identification of all strains tested by macroscopic examination by selective media and as well as by microscopic examination by gram stain and biochemical test .

Except *clostridium difficille* because it is contaminate and the conditions did not allow its purification .

d. **Data annotation, Storing and Querying:**

The data and results collected during this project pertaining to the selected bacteria, tested anibiotics, medicinal plants and extacted antobacterial active compounds, would be verified and valorized then annotated and stored (by the project's suppervisor) into the online dabatase BARID (**Kheris et al., 2019**) and made available for querying by the scientific community via the web-address: http://www.bioinformaticstools.org/prjs/barid/. It is worthwhile to note that BARID is the online database created, last year, for the larger project under the theme Antibiotics Resistance medically outstanding problem and Antibacterial effects of compounds from plant origins (**Kheris, 2019**) to which this project belong.



Conclusion

Conclusion

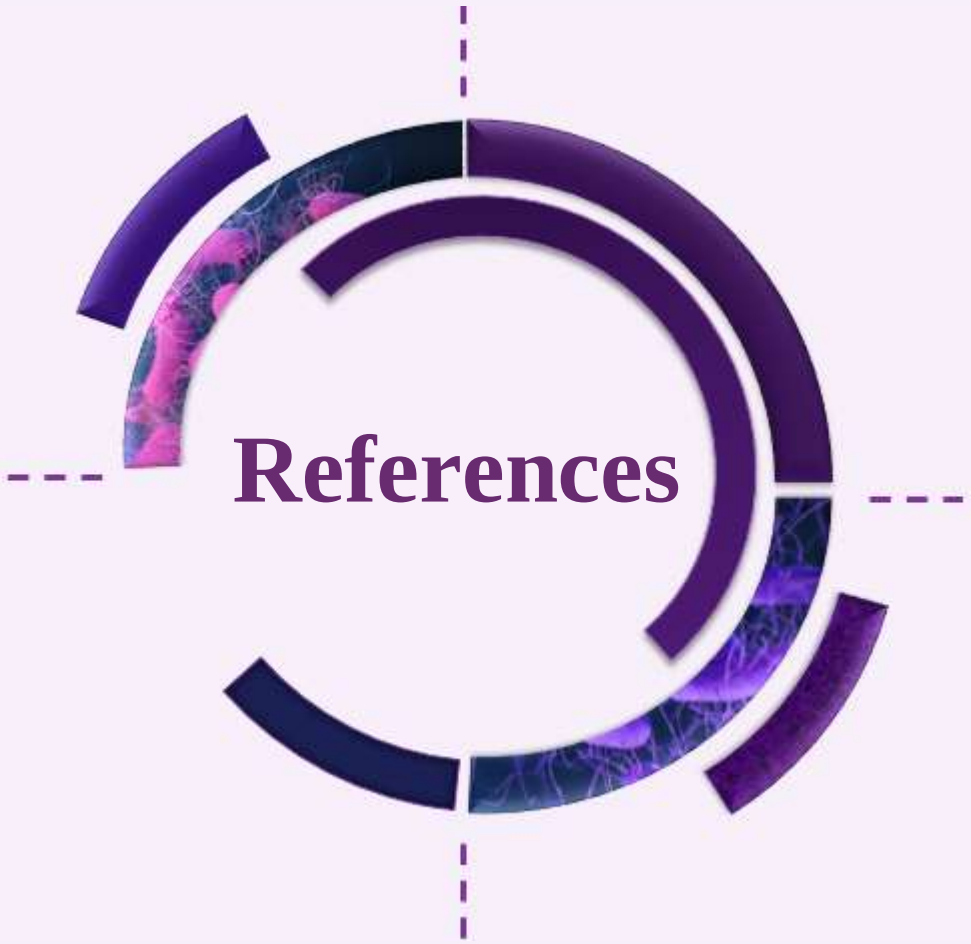
The pharmacological benefits of medicinally important plants are primarily due to bioactive phyto-chemicals produced in the plant tissues secondary metabolites.

According to bibliographical research, the three plants studied (saussurea lappa, ajuga iva and rosmarinus officinalis) can be used as a potent source of natural antimicrobial agents by replacing commercially available synthetic drug that may have a large number of side effects.

This study proved the presence of alkaloids, tannins, flavonoids and quinones and essential oils in saussurea lappa (asteracea). Several polyphenols (tannins and anthocyanins) and flavonoids in the aerial part of ajuga iva (Lamiaceae) have been detected . rosmarinus officinalis (Lamiaceae), contains tannins, flavonoids, quinones and essential oils . These phytochemical constituents are important for the use of health care.

In conclusion, there is an urgent need to continue research models to support the development of botanicals to counter drug resistant microbes, as well as regulatory reforms of clinical development programs. The use of botanical medicines is accelerating and improving the channel of drug development. There are several reasons to use herbal medicines of which two may play pivotal roles.

- First, herbal treatment provides other mechanisms of action, encompassing in many cases a single drug to treat a single disease.
- Second, the utilization of unique traditional knowledge of herbal medicine has great potential to generate biocompatible, cost effective solutions and will hasten the discovery of new medicines.



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Annex

DRUG-RESISTANT

TUBERCULOSIS (TB)

CARBAPENEM-RESISTANT

ACINETOBACTER

MULTIDRUG-RESISTANT

PSEUDOMONAS AERUGINOSA

CARBAPENEM-RESISTANT

ENTEROBACTERIACEAE

RESISTANT

CLOSTRIDIOIDES DIFFICILE

METHICILLIN-RESISTANT

STAPHYLOCOCCUS AUREUS

VANCOMYCIN-RESISTANT

ENTEROCOCCI (VRE)

RESISTANT

GROUP A STREPTOCOCCUS

DRUG-RESISTANT

STREPTOCOCCUS PNEUMONIAE

Classification of *Mycobacterium tuberculosis*:

Reign	Bacteria
Branching	Actinobacteria
Order	Actinomycetales
Suborder	Corynebacterineaes
family	Mycobacteriaceae
Genus	Mycobacterium
Species	*Mycobacterium tuberculosis*

Classification of *Acinetobacter baumannii*:

Reign	Bacteria
Branching	γ-Proteobacteria
Class	Gammaproteobacteria
Order	Pseudomonadales
family	Moraxellaceae
Genus	Acinetobacter
Species	*Acinetobacter baumannii*

Classification of *Pseudomonas aeruginosa*:

Reign	Bacteria
Division	Proteobacteria
Class	Gammaproteobacteria
Order	Pseudomonadales
family	Pseudomonadaceae
Genus	Pseudomonas
Species	*Pseudomonas aeruginosa*

Classification of *Clostridioides difficile*:

Reign	Bacteria
Division	Firmicutes
Class	Clostridia
Order	Clostridial
family	Peptostreptococcaceae
Genus	Clostridioides
Species	*Clostridioides difficile*

Classification of *Staphylococcus aureus*:

Reign	Bacteria
Division	Firmicutes
Class	Bacilli
Order	Bacillales
family	Staphylococcaceae
Genus	Staphylococcus
Species	*Staphylococcus aureus*

Classification of *Escherichia coli* :

Reign	Bacteria
Phylum	Proteobacteria
Class	Gamma Proteobacteria
Order	Enterobacteriales
family	Enterobacteriaceae
Genus	Escherichia
Species	*Escherichia coli*

Classification of *Enterococcus faecalis* :

Reign	Bacteria
Division	Firmicutes
Class	Bacilli
Order	Lactobacillales
family	Enterococcaceae
Genus	Enterococcus
Species	*Enterococcus faecalis*

Classification of *Streptococcus pneumoniae*:

Reign	Bacteria
Division	Firmicutes
Class	Cocci
Order	Lactobacillales
family	Streptococcaceae
Genus	Staphylococcus
Species	*Streptococcus pneumoniae*

(Bergey's manual of systematic bacteriology, 2004)

Composition of Nutrient Agar :

- Agar …….15.0 gm
- Peptone…….……...5.0 gm
- Beef extract ……...….3.0 gm
- Sodium chloride …….5.0 gm
- Final pH…….....6.9

Composition of king A Agar :

- peptone dite "A" : ……20,0 g
- glycerol: …… ..10.0 g
- potassium sulphate: …… ... 10.0 g
- magnesium chloride: …… 1.4 g
- purified agar: ……..… .12.0 g
- pH = …….…… ... 7.2

Composition of king B Agar :

- peptone dite "B" ……20,0 g
- glycerol ……. 10.0 g
- potassium hydrogen phosphate……... 1.5 g
- magnesium sulfate heptahydrate……. 1.5 g
- purified agar …….…… 12.0 g
- pH = ……7,2

Composition of Mannitol salt agar :

- Peptone: ..... 10.0 g
- Beef extract: ..... 1.0 g
- Sodium chloride: ..... 75.0 g
- Mannitol: ..... 10.0 g
- Phenol red: ... . 0.025 g
- Agar-agar: ..... 15.0 g
- pH = ……7,4

Composition of Liver Meat Agar :

- Peptone viande-foie……30 g
- Sulfite de sodium……...2,5 g
- Glucose…….2 g
- Citrate ferrique ammoniacal …….0,5 g
- Amidon soluble……2 g
- Agar……11 g
- pH = ……7,4

Composition of MR/VP broth :

- Polypeptone ……7 g
- Glucose ……...5 g
- Dipotassium phosphate……...5 g
- Distilled water ……...1 L
- Final pH…….....6.9

Composition of Kligler's Iron Agar :

- Beef extract:…….…… 3 gm
- Yeast extract: ……...3 gm
- Peptone: …….15 gm
- Proteose peptone: ……5 gm
- Lactose ……..10 gm
- Glucose: ……..1 gm
- Ferrous sulfate: ……0.2 gm
- Sodium chloride: ……5 gm
- Sodium thiosulfate: …….….0.3 gm
- Agar:……. 12 gm
- Phenol red: ……..0.024 gm
- Distilled water to equal…… 1 L
- Final pH : …….7.4

Composition of Simmons Citrate agar:

- Sodium Chloride (NaCl)…….5.0 gm
- Sodium Citrate (dehydrate)…….2.0 gm
- Ammonium Dihydrogen Phosphate……1.0 gm
- Dipotassium Phosphate……1.0 gm
- Magnesium Sulfate (heptahydrate)……..0.2 gm
- Bromothymol Blue……0.08 gm
- Agar……...15.0 gm
- Distilled water to equal……...… 1 L
- Final pH : …….7.4

Composition of Mannitol motility agar:

- Agar - ……..3.0 g
- Peptone - ……20.0 g
- Mannitol - ……2.0 g

  Potassium nitrate - ……...1.0 g
- 1% Phenol red solution ……4 ml
- Distilled water to equal…..…… 1 L
- Final pH : ……...……..7.6