PEOPLE'S DEMOCRATIC REPUBLIC OF ALGERIA

THE MINISTRY OF HIGHER EDUCATION AND SCIENTIFIC RESEARCH

UNIVERSITY « DR. MOULAY TAHAR » SAIDA

FACULTY OF SCIENCES

DEPARTEMENT: BIOLOGIE

MEMORY

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In order to obtain

Diploma of **Master's in Biology**

Speciality: **Microbiology**

Bacterial Antibiotic Resistance and *Annona cherimola mill* Seeds Antibacterial Activity Experimental Investigation & Data Annotation and Integration into an Online Accessible Database: http://bioinformaticstools.org/prjs/barid/

Day: 30 September 2019

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2018-2019

ACKNOWLEDGEMENT

First and foremost, praises and thanks to the God, the Almighty, for His showers of blessings throughout my research work to complete the research successfully.

I would like to express my deep and sincere gratitude to my research supervisor, Dr Abdelkerim Rachedi (Ph.D in Structural Biology & Bioinformatics), for giving me the opportunity to do research under the theme of bioinformatics and providing invaluable guidance throughout this research. His dynamism, vision, sincerity and motivation have deeply inspired me. He has taught me the methodology to carry out the research and to present the research works as clearly as possible. It was a great privilege and honor to work and study under his guidance. I am extremely grateful for what he has offered me. I would also like to thank him for his empathy, and great sense of humor.

My sincere thanks also goes to Dr. Mokhtar Benreguieg my Co-supervisor for advising me to do my master project in bioinformatics and leading me into working on diverse exciting projects.

I am extremely grateful to my parents for their love, prayers, caring and sacrifices for educating and preparing me for my future. I am very much thankful to my sisters, brothers too.

I would like to say thanks to my fourth Fatima, Fatima, zohra and safia for their support during my life.

I thank my fellow labmates during the preparation of master memory for the stimulating discussions, for the hard days we were working together and for all the fun we have had in the last four years. Also I thank my new friends in hygiene laboratory; Mona, Nini.

Finally, my thanks go to all the people who have supported me to complete the research work directly or indirectly.

Soumeya kheris

Dedication

To all of you who didn't allow me to give up.

Abstract

Five important bacteria deemed to have developed Antibiotic Resistance; a current medical problem dangerously threatening the health and even human life on global scale, have been selected for subject study of this project. The bacteria namely *Staphylococcus aureus, Clostridium difficile, Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* which are responsible for a myriad of human diseases where growth tested against 14 commonly used antibiotics belonging to a number of classes including Beta-Lactams, Aminoglycosides, Tetracyclines, Macrolides,

Chloramphenicol, and Quinolone and the relatively new group of drugs class Oxazolidinones. Moreover, the database annotated the antibacterial activity of Cherimoya fruit seeds extracts on the listed above bacteria. Cherimoya plant has been used in the study as a model of medicinal plant.

A resource database system named BARID (Bacterial Antibiotic Resistance Investigation Database) has been created for the purposes of data annotation, managing and storing pertaining to mainly empirically data generated through the laboratory experiments done in this project.

In addition to annotating such medically important data, the BARID database offers a number of features based on Datamining and Integration setting the grounds for providing researchers with even deeper insights on the genomic and proteomic causes might be behind the phenomena of antibiotics resistance. More insights could be provided by th 3D-structures, drugs data and scientific literature included in the search outputs of the database.

This database system, through a number of exploration features, presents users with a set of results including which bacteria are resistant to which antibiotic and which bacteria are sensitive to which of the four types of Cherimoya seeds extracts used in the study. The system, as it further develops, may potentially also be used to suggest new natural products, such as the extracts of the Cherimoya seeds as alternatives to antibiotics in the cases where antibiotic resistance is shown.

BARID is available online at the link: http://www.bioinformaticstools.org/barid/

Keywords: Bacterial Antibiotic Resistance, Anti-Bacterial Activity, Disease, Database, Annotation, Datamining, Data Integration, Alternative natural drugs.

Résumé

Disponible en ligne à l'adresse suivante: http://www.bioinformaticstools.org/barid/, BARID (base de données d'enquêtes sur la résistance des antibiotiques bactériens) est une base de données qui annote et stocke des informations relatives principalement à des données empiriques

5 bactéries importantes supposées avoir développé une résistance aux antibiotiques; un problème médical actuel menaçant dangereusement la santé et même la vie humaine à l'échelle mondiale. Les bactéries Staphylococcus aureus, Clostridium difficile, Escherichia coli, Pseudomonas aeruginosa et Klebsiella pneumoniae, responsables d'une myriade de maladies chez les humains, ont été testées contre 14 antibiotiques couramment utilisés appartenant à plusieurs classes, notamment les bêta-lactamines, les aminoglycosides, les tétracyclines, les macrolides. ,

Le chloramphénicol et la quinolone et le groupe relativement nouveau de médicaments de la classe des oxazolidinones. De plus, la base de données a annoté l'activité antibactérienne d'extraits de graines de fruits Cherimoya sur les bactéries énumérées ci-dessus. La plante Cherimoya a été utilisée dans l'étude comme modèle de plante médicinale.

En plus d'annoter de telles données médicalement importantes, la base de données BARID offre un certain nombre de fonctionnalités basées sur Datamining and Integration, ce qui permet aux chercheurs de mieux comprendre les causes génomiques et protéomiques qui pourraient expliquer le phénomène de résistance aux antibiotiques. Les structures 3D, les données sur les médicaments et la littérature scientifique incluses dans les résultats de recherche de la base de données pourraient fournir davantage d'informations.

La base de données BARID, à travers un certain nombre de fonctions d'exploration, présente aux utilisateurs un ensemble de résultats indiquant notamment quelle bactérie est résistante à quel antibiotique et quelle bactérie est sensible à lequel des quatre types d'extraits de graines de Cherimoya utilisés dans l'étude. Le système, au fur et à mesure de son développement, pourrait éventuellement aussi être utilisé pour suggérer de nouveaux produits naturels, tels que les extraits de graines de Cherimoya, en remplacement des antibiotiques dans les cas où la résistance aux antibiotiques est démontrée.

Mots-clés: résistance aux antibiotiques bactériens, activité anti-bactérienne, maladie, base de données, Annotation, datamining, intégration de données.

ملخص

متاح على الإنترنت على الرابط BARID، http://www.bioinformaticstools.org/barid/

(قاعدة بيانات التحقيق في مقاومة المضادات الحيوية البكتيرية) هي قاعدة بيانات تشرح وتخزين المعلومات المتعلقة بشكل أساسي بالبيانات التجريبية التي تمثل التجارب المعملية التي أجريت على

بكتيريا مهمة يعتقد أنها طورت مقاومة للمضادات الحيوية ؛ مشكلة طبية حالية تهدد بشكل خطير الصحة وحتى حياة الإنسان على نطاق عالمي. البكتيريا وهي كلابسيلا بنوموني، اشيريشيا كولي ، ستافيلوكوكوس اوريوس ،بسودوموناس ايروجينوزا ، كلوستريديوم ديفيسيل و والتي تعد مسؤولة عن عدد لا يحصى من الأمراض البشرية حيث تم اختبار النمو ضد 14 من المضادات الحيوية شائعة الاستخدام والتي تنتمي إلى عدد من الفئات بما في ذلك بيتا لاكتام و امينوغليكوسيد الكلورامفينيكول ، والكينولون والمجموعة الجديدة نسبيا من الأدوية من فئة أوكسازوليدينون. علاوة على ذلك ، شرحت على البكتيريا المذكورة أعلاه. تم استخدام في الشيريمويا قاعدة البيانات النشاط المضاد للبكتيريا لمستخلصات بذور فاكهة الدراسة كنموذج للنباتات الطبية الشيريمويا.

عددًا من الميزات التي تستند إلى BARID بالإضافة إلى شرح هذه البيانات المهمة طبياً ، توفر قاعدة بيانات والتي تمد الأسس لتزويد الباحثين برؤى أكثر عمقًا حول الأسباب الجينومية Datamining and Integration والبروتينية التي قد تكون وراء ظواهر مقاومة المضادات الحيوية. يمكن توفير المزيد من الأفكار من خلال الهياكل ثلاثية الأبعاد وبيانات الأدوية والمؤلفات العلمية المدرجة في مخرجات البحث في قاعدة البيانات.

، من خلال عدد من ميزات الاستكشاف ، للمستخدمين مجموعة من النتائج بما في ذلك BARID تقدم قاعدة بيانات البكتيريا المقاومة لأي من المضادات الحيوية والبكتيريا الحساسة لأي من الأنواع الأربعة من مستخلصات بذور الشيريمويا المستخدمة في الدراسة. من المحتمل أيضًا أن يستخدم النظام ، مع تطوره ، لاقتراح منتجات طبيعية جديدة ، مثل مقتطفات كبدائل للمضادات الحيوية في الحالات التي تظهر فيها مقاومة المضادات الحيوية بذور الشيريمويا

الكلمات المفتاحية: مقاومة المضادات الحيوية البكتيرية ، النشاط المضاد للبكتيريا ، المرض ، قاعدة البيانات ،

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ABREVIATION LISTE

AFB : Acid Fast Bacilli .

ATCC : American type culture collection.

BAAR : Bacilli Alcol-Acido Resistenti.

BARID : Bacterial Antibiotic Resistance Investigation DATABASE.

BSAC : British Society for Antimicrobial Chemotherapy .

CLS : Clinical and Laboratory Standard Institute .

CSV : Comma Separated Values .

DDBJ : DNA DataBank of Japan .

DNA : deoxyribonucleic acid .

EMBL : European Molecular Biology Laboratory .

EUCAST : European Committee of Antimicrobial Susceptibility Testing .

ExPEC : extra-intestinal pathogenic *E. coli* .

HGT : horizontal gene transfer .

HIV : human immunodéficiency virus .

InPEC : intestinal pathogenic *E. coli* .

KB : The Kirby-Bauer disc diffusion .

MMDB : Molecular Modeling Database .

MIC : Minimum Inhibitory Concentration assay **.**

NCBI : the National Center for Biological Information .

NMR : Nuclear Magnetic Resonance .

OD : optical density .

OMIM : Online Mendelian Inheritance in Man (OMIM).

PBP's : penicillin binding proteins .

PDB : Protein Data Bank .

PHP : Hypertext Preprocessor .

PNLCT : Programme National de Lutte Contre la Tuberculose.

RCSB : Research Collaboratory for Structural Bioinformatics .

rRNA : Ribonucleic Acid.

SQL : Structured Query Language.

XAMPP : X Apache MySQL Perl PHP.

Antibiotic resistance is a form of drug resistant whereby some sub-populations of a microorganism, usually a bacterial species, are able to survive after exposure to one or / more antibiotics. In other word, the term “antibiotic resistance” refers to the ability of a microorganism to withstand the effect of an antibiotic. The use of antibiotics is limited because bacteria have evolved defenses against certain antibiotics. One of the main mechanisms of defense is inactivation of the antibiotic. This is the usual defense against penicillin and chloramphenicol, among others. Another form of defense involves a mutation that changes the bacterial enzyme affected by the drug in such a way that the antibiotic can no longer inhibit it. This is the main mechanism of resistance to the compounds that inhibit protein synthesis, such as the tetracycline

The problem of resistance has been exacerbated by the use of antibiotics as prophylactics, intended to prevent infection before it occurs. Indiscriminate and inappropriate use of antibiotics for the treatment of the common cold and other common viral infections, against which they have no effect, removes antibiotic-sensitive bacteria and allows the development of antibiotic-resistant bacteria **(Witte, 2004)**

The pathogens resistant to multiple antibiotics are considered as Multi-drug resistant pathogens **(D'Costa, 2011)** .Some of the most ten important pathogens types of multiple drug resistant organisms that have been encountered include; *Staphylococcus aureus*, *Klebsilla pneumonia*, *Escherichia coli*, *Pseudomonas aeroginosa*, *Nesseria gonhoreae*, *Clostridium difficile*, *Mycobacterium tuberculosis*, *Burkhoderia cepacia*, *Acenitobacter baumanii*, and *Streptococcus pyogenes*.

In realizing the antibiotic resistance as a major problem, as mentioned above, in the treatment of bacterial infections, there is need to find alternative ways of treating infectious diseases using plants which are abundant in our environment and their extracts may overcome the antibiotic resistance so as to serve as a source of novel drugs for the treatment of these diseases.

Cherimoya, one of the most important fruits of the Annonaceae family, is recognized by some botanists as one of the three best fruits in the world (**National Research Centre, 1989; Gardiazbal and Rosenberg, 1993**). The famous 18th century Czech naturalist Thaddus Haenke considered it "the masterpiece of Nature".

In this age of informatics highly reliance, computers and databases are used to help in solving many problems in biology associated with disease fighting, numerical analysis of complex data generated by the biologists which resulted in the creation of the whole field of science become known as Bioinformatics.

In this project, lots of data have been generated in relation to Antibiotic Resistance and Antibacterial activity experiments carried out in this research. Annotation and data integration and database programming techniques have been implemented to classify, store, analyze the data and also retrieve results and display them in meaningful manner to interested users.

Objective of the study:

The objectives of this research study can be summarized in three points;

a. Confirm the existence of phenomena of **antibiotic resistance** in local strains of bacteria and Explore the range that the bacteria selected in the study may have reached in such resistance, see further below,

b. Investigate of **antibacterial effects** of Cherimoya seed extracts.

c. Create a bioinformatics concepts based application to annotate the results data generated by the project, add value to the data via data integration with other important data and helping in guided conclusion drawing form the data.

Objectives attained:

A bioinformatics application has been created for the purposes of the research topic of this project. The application system in the form an online database which has been generically named as “BARID” for Bacterial Antibiotics Resistance Investigation Database has been successful in annotating the data generated from tests on five selected bacteria from the above listed ten bacteria; *Staphylococcus aureus*, *Klebsilla pneumonia*, *Escherichia coli*, *Pseudomonas aeroginosa*, *Clostridium difficile*. The growth tests of the bacteria were done against a list of commonly used antibiotics. As mentioned above, the investigation aimed at exploring the range of antibiotic resistance that the bacteria may have reached. The tests were also done to investigate antibacterial effects of Cherimoya seeds extract.

Results as explored by the BARID system confirmed the existence of antibiotic resistance of most of the bacteria tested on against many of the antibiotics. Also shown that potential alternative do exist I the seeds of the Cherimoya as the results of tests showed antibacterial activity of the seeds extracts.

Institutions were the Study was carried out:

This study was conducted at Saida University - Dr Moulay Tahar and the Hygiene Laboratory of Saida.

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CHAPTER I : Litterature review

I. The ten most pathogenic bacteria resistant to antibiotics

Ten important pathogens types of multiple drug resistant organisms that have been encountered include; *Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, pseudomonas aeroginosa, Nesseria gonhoreae, Clostridium difficile, Mycobacterium tuberculosis, Burkhoderia cepacia, Acenitobacter baumanii, and Streptococcus pyogenes*. Below is a summary description of all of them in terms of their standard classification, bacteriological study and pathogenicity.

I.1 *Mycobacterium tuberculosis*

I.1.1 Classification

Table 1: Taxonomy of Mycobacterium tuberculosis (Bergey's manual of systematic bacteriology, 1986).

Phylum :	Actinobacteria
Class :	Actinobacteria
Order:	Actinomycetales
Family :	*Mycobacteriaceae*
Genus:	*Mycobacterium*
Species:	*Tuberculosis*

I.1.2 Bacteriological study

- *M. tuberculosis* is an immobile red bacillus with 0.2 to 0.3 micron wide by 3 to 5 microns long, slightly curved, with rounded ends **(Emane, 2016)**.
- M. tuberculosis can appear either Gram-negative or Gram-positive **(Emane, 2016).**
- *M. tuberculosis* species are without capsule and spore **(Nguyen et *al.*, 2018)**.
- *M. tuberculosis* is aerobic strict. It is positive catalase, positive nitrate **(Emane, 2016)**.
- *M. tuberculosis* is a strict parasite of the human species. Human-to-human transmission is usually direct and is done by air. The pets of the man can occasionally be contaminated **(Woods et *al.*, 1987)**.

I.1.3 Pathogenicity

The penetration of the bacillus in the body leads to disease in only 10% of cases on average. In 90% of cases, multiplication of bacilli stops quickly. It is the simple primary

infection that results in the development of tuberculin hypersensitivity and superinfection immunity **(Getahun et *al*., 2015)**.

Two types of localization can be observed. The pulmonary locations are the most frequent (about 90% of cases) and the most dangerous epidemiologically because it is they (including caves) that allow the transmission of bacilli. Extra-pulmonary locations are generally poor in bacilli but disabling (osteoarthritis) or very serious (meningitis) **(Flynn, 1993)**.

The anatomical substratum of the lesions is the same; it is granuloma and especially caseification.

In subjects infected with the AIDS virus (HIV), BK infection very frequently leads to tuberculosis, which is often generalized and is reflected in nearly 50% of cases by multiple pulmonary and extrapulmonary localizations **(Selwyn et *al.*, 1989)**.

I.2 *Klebsiella pneumoniae*

I.2.1 Classification

Table 2 : Taxonomy of *Klebsiella pneumoniae* **(Diancourt et *al*., 2005)**

Phylum :	Proteobacteria
Class :	Gammaproteobacteria
Order :	Enterobacteriales
Family :	*Enterobacteriaceae*
Genus :	*Klebsiella*
Species :	*Klebsiellapneumoniae*

I.2.2 Bacteriological study

- *Klebsiella pneumoniae* is an immobile bacillus, Gram negative, Oxidase negative, nitrate reductase positive and glucose fermenting bacillus **(Paterson et *al*., 2000).**
- rod-shaped species. It appears as a mucoid lactose fermenter on MacConkey agar.
- *Klebsiella pneumoniae* can be found in digestive tract of human and warm-blooded animals: may be found also in water, water soil and dust **(Munoz-Price et *al*., 2013)**

I.2.3 Pathogenicity

Klebsiella pneumoniae is opportunistic germ implicated in nosocomial infections, usually urinary tract infections, pneumonitis and sepsis. And it can be both, commensal of

the body, and a pathogen responsible for various infections. In addition, *Klebsiella pneumoniae* causes community-acquired respiratory infections, mainly in fragile individuals (elderly, diabetic or alcoholic) **(Diancourt et *al*., 2005)**

K. pneumoniae is naturally resistant to penicillins (amoxicillin, ticarcillin) by producing a beta-lactamase class A species (chromosomal) called K2, inhibited by clavulanic acid (example: Klebsiella pneumoniae 1189) **(Kitchel et *al*., 2009)**.

I.3 *Escherichia coli*

I.3.1 Classification

The genus *Escherichia* belongs, as well as *Salmonella* or *Shigella*, to the family Enterobacteriaceae. This genus includes 5 species, however, *Escherichia coli* one is the most important **(Kahlouche, 2014)**.

Table 3 : Taxonomy of *Escherichia coli* **[(Miszczycha, 2013) ; (Balière, 2016)]**

Phylum:	Proteobacteria
Class:	Gammaproteobacteria
Order:	Enterobacteriales
Family:	Enterobacteriaceae
Genus:	Escherichia
Species:	Escherichia coli

I.3.2 Bacteriological study

- Gram-negative, non-sporulating, immotile bacillus with a peritrichous flagellar structure, varies from 2 to 6 µm and from 1.1 to 1.5 µm **(Miszczycha, 2013).**
- Its optimum growth temperature is 37 ° C. able to grow on ordinary media. E. coli is able to ferment lactose and produce indole **(Miszczycha, 2013).**
- *Escherichia coli* are optional aerobic anaerobes that can ferment nitrate, catalase positive, oxidase negative and non-halophilic **(Balière, 2016)**.
- *E. coli* is found commensally in the intestinal and faecal flora, both in humans and in some animals. The intestinal flora is colonized shortly after birth. The bacteria and the host coexist without impacting their respective health. This coexistence brings mutual benefits **(Zekri, 2017)**.

I.3.3 Pathogenicity

E. coli can not only be a commensal, but also a pathogen. The pathogenesis of these bacteria is done in stages. First, they colonize a mucous membrane. Then they multiply and cause damage to the host while trying to escape his defenses. Pathogenic *E. coli* can be separated into two categories, intestinal pathogenic *E. coli* (InPEC) and extra-intestinal pathogenic *E. coli* (ExPEC) **[(Kahlouche, 2014) ; (Zekri, 2017)]**.

I.4 *Pseudomonas aeruginosa*

I.4.1 Classification

Table 4 : Taxonomy of *Pseudomonas aeruginosa* **(Elmeskini, 2011)**

Phylum :	Proteobacteria
Class :	Gammaproteobacteria
Order :	Pseudomonadales
Family :	*Pseudomonadaceae*
Genus :	*Pseudomonas*
Species :	*Pseudomonas aeruginosa*

I.4.2 Bacteriological study

- Gram-negative bacilli, 1 to 5 μm and 0.5 to 1 μm, aerobic strict, mobile thanks to a monotrichic polar type ciliature, does not form spores or spheroplasts, oxidative, non-fermentative, catalase positive and oxidase positive. Mesophilic able to multiply from 4 to 45 ° C. Optimum growth temperature between 30 and 37 ° C **(Elmeskini, 2011).**
- Ubiquitous, present especially in the soil and in aquatic environments **(Kahlouche, 2014).**

I.4.3 Pathogenicity

Pseudomonas aeruginosa, the type species, Frequently isolated on the skin and mucous membranes of humans or animals, it is also particularly resistant to antibiotics and even antiseptics. In hospitals, it causes super infections and local or deep suppurations, isolated mainly in patients with local or general immunodeficiency (burns, cancer, etc.), and very frequently involved in nosocomial infections (pulmonary infections, skin ...). It is also phytopathogenic with many other species of the same genus **[(Ben Haj Khalifa, 2010) ; (Kahlouche, 2014)]**.

It is responsible for 10% of all nosocomial infections, ranking third after *E. coli* and *S. aureus*, but ranking first for low-grade lung infections and third for urinary tract infections **(Zekri, 2017)**.

I.5 *Burkholderia cepacia*

I.5.1 Classification

Table 5 : Taxonomy of *Burkholderiacepacia* **(Coenye et *al*., 2001).**

Phylum :	Proteobacteria
Class :	Beta Proteobacteria
Order :	Burkholderiales
Family :	*Burkholderiaceae*
Genus :	*Burkholderia*
Species :	*Burkholderiacepacia*

I.5.2 Bacteriological study

- Gram negative, mobile, straight bacillus, strict aerobics, catalase positive, difficult to highlight oxydase, Do not ferment glucose **(Coenye et *al*., 2001).**
- Opportunistic pathogen for both human and animal, it can be found in soils, sludge, various waters (including distilled water and tap water), sediments, rhizosphere, plants, also in hospital environment: survives in antiseptic solutions (quaternary ammonium, chlorhexidine, polyvidone iodine) and in injectable solutions. May contaminate aerosols, irrigation fluids and tubing of respiratory ventilation systems **(Mahenthiralingam et *al*., 2000).**

I.5.3 Pathogenicity

Variation in pathogenicity between strains of the environment, clinical strains in asymptomatic patients and those responsible for severe infections. It causes pulmonary infections: Respiratory infections correlated with contamination of contamination material or in patients with cystic fibrosis, necrotizing pneumonia in cystic fibrosis patients associated with bacteremia in cepacia syndrome. **(Govan et *al*., 1996).**

The transmission mode is by direct contact with contaminated water, aerosols or aspirations, with respiratory secretions from infected individuals or via contaminated solutions or poorly sterilized medical equipment. Transmission taking place from person to person within the cystic fibrosis population **(Govan et *al*., 1996).**

I.6 *Acinetobacter baumannii*

I.6.1. Classification:

Table 6: Taxonomy of *Acinetobacter baumannii* **(Peleg et *al*., 2008).**

Phylum :	Proteobacteria
Class:	Gammaproteobacteria
Order:	Pseudomonadales
Family:	*Moraxellaceae*
Genus:	*Acinetobacter*
Species:	*Acinetobacterbaumannii*

I.6.2 Bacteriological study

- Gram negative Bacillus, immobile, the bacteria of the genus *Acinetobacter* are strictly aerobic and oxidase negative, the latter character to differentiate bacteria belonging to the genus *Pseudomonas* **(Eliopoulos et *al*., 2008).**
- Bacteria often found in aqueous media and in wet, watery conditions that can survive to wetness and survive for up to 8 days, naturally resistant to many antibiotics **(Eliopoulos et *al*., 2008).**

I.6.3 Pathogenicity

A. baumannii is an opportunistic pathogen with no particular tropism in humans, affecting people with compromised immune systems, and is becoming increasingly important as a hospital-derived (nosocomial) infection. Plus, responsible for different types of infections, mostly pulmonary or bacteremic **(Fournier et *al*., 2006).**

The transmission to patients is from inert surfaces or from the hands of health care workers who may be transiently colonized by this species. Note that transmission can also be done by airborne contamination from a colonized or infected patient **(Eliopoulos et *al*., 2008).**

I.7 *Neisseria gonorrhoeae*

I.7.1 Classification

Table 7 : Taxonomy of *Neisseria gonorrhoeae* **(Wi et al., 2017).**

Phylum :	Proteobacteria
Class :	Betaproteobacteria
Order :	Neisseriales
Family :	*Neisseriaceae*
Genus :	*Neisseria*
Species :	*Neisseriagonorrhoeae*

I.7.2 Bacteriological study

- *Neisseria gonorrhoeae* Gram-negative reniform cocci, usually grouped in diplococci, appear classically in more or less large clusters inside altered polynuclear cells.
- *Neisseria gonorrhoea*e is aerobic strict, oxidase positive, glucose positive but maltose negative **(Cámara et *al.*, 2012)**.

I.7.3 Pathogenicity

They are the agent of one of the venereal diseases or sexually transmitted diseases, The symptomatology is most often noisy in humans, in the form of urethritis acute ; in women, the infection is asymptomatic in more than half of cases ; when symptomatic, the clinical signs are usually nonspecific. Carrying infection in the anorectal and oropharyngeal region is most often asymptomatic, in men and women **(Wi et *al.*, 2017)**

In pregnant women, gonococcal infection may affect the course of pregnancy. It can also contaminate the newborn, during the passage of the die genital tract, which is most often manifested by purulent ophthalmia and Bilateral whose risk is blindness **(Landig et *al.*, 2019)**.

N. gonorrhoeae infection can promote HIV transmission, but also its reception **(Sewankambo et al., 1997).**

I.8 *Staphylococcus aureus*

I.8.1 Classification

Table 8 : Taxonomy of Staphylococcus aureus**(Lays, 2012) .**

Phylum :	Firmicutes
Class :	Bacilli
Order :	Bacillales
Family :	*Staphylococcaceae*
Genus :	*Staphylococcus*
Species :	*Staphylococcus aureus*

I.8.2 Bacteriological study

- Gram-positive, spherical in shape, with a diameter of 0.5 to 1 μm. They are grouped into diplococci or small regular or irregular clusters (bunch of grapes) **(Lays, 2012)**.
- Immobile, asporulate, usually without capsule **(Lays, 2012)**.
- Optional anaerobic, positive catalase and negative oxidase. Many strains of S. aureus produce a golden yellow pigment **(Kahlouche, 2014)**.
- Opportunistic, commensal of the skin and mucous membranes (nasal cavity, perineum, gastrointestinal tract and pharynx) of man and many animal species **(Zekri, 2017)**.

I.8.3 Pathogenicity

Staphylococcus aureus is responsible for many nosocomial and community-acquired infections. Its pathogenicity results from the secretion of enzymes (catalase, coagulase, deoxyribonucleases, etc.) and toxins (haemolysins, leucocidines, enterotoxins, etc.) which give it respectively its invasiveness and toxinogenicity **[(Lays, 2012) ; (Kahlouche, 2014) ; (Zekri, 2017)].**

I.9 *Streptococcus pyogenes*

I.9.1 Classification

Table 9 : Taxonomy of *Streptococcus pyogenes* **(Efstratiou, 2017).**

Phylum :	Firmicutes
Class :	Bacilli
Order:	Lactobacillales
Family :	*Streptococcaceae*
Genus:	*Streptococcus*
Species:	*Streptococcus pyogenes*

I.9.2 Bacteriological study

- *Streptococcus pyogenes* are Gram-positive cocci, catalase negative, with anaerobic metabolism. Some are parasites of the human species, other are commensals of the oral mucosa **(Efstratiou, 2017).**

I.9.3 Pathogenicity

S*treptococcus pyogenes* are the most pathogenic which is responsible for the majority of streptococcal diseases. The immunological reactions of the host infected with *S.pyogenes* are much more complex than those observed when infected with *S. aureus* and may lead to the formation of high level specific antibodies **[(Zhu et *al*., 2017) ; (Westman et *al*., 2018)]**.

Acute infections: cutaneous, mucous or septicemic. Some are local, or on infections of wounds and burns. Bacteremia is often secondary to a local infection. This is the case of puerperal fever that follows a genital infection of the postpartum. We must also mention acute endocarditis, meningitis **(Watson et *al*., 2016).**

I.10 *Clostridium difficile*

I.10.1 Classification

Table 10 : Taxonomy of *Clostridium difficile* **(McFarland et *al*., 1989).**

Phylum :	Firmicutes
Class :	Clostridia
Order :	Clostridiales
Family :	*Peptostreptococcaceae*
Genus :	*Clostridioides*
Species :	*C. difficile*

I.10.2 Bacteriological study

Gram positive bacillus anaerobic, sporulated, motile bacteria, ubiquitous in nature, and especially prevalent in soil. *C. difficile* is catalase- and superoxide dismutase-negative, and produces two types of toxins: enterotoxin A and cytotoxin B. *C. difficile* may become established in the human colon ; it is present in 2–5% of the adult population **(Wilcox, 2017).**

I.10.3 Pathogenicity

Its pathogenicity related to the production of two related pathogenicity at the production of two toxins A and B both of which may produce diarrhea and inflammation in infected patients. Additional virulence factors include an adhesin factor that mediates the binding to human colonic cells and a hyaluronidase **[(Lessa et *al*., 2015) ; (Leffler et *al*., 2015)]**.

Antibiotic treatment of this species infections may be difficult, due both to antibiotic resistance and physiological factors of the bacterium (spore formation, protective effects of the pseudomembrane) **(Nelson et *al*., 2017)**.

II. Antibiotics and Antibiotics Resistance

II.1 The brief history and benefits of antibiotics

II.1.1 The early days

Most people, even without a background within life science, have heard the intriguing story of how Alexander Fleming by coincidence contaminated his agar plates with mould and discovered penicillin back in 1929 **(Mitscher, 2005**). Of less common knowledge is the pioneering work of Alexander Ehrlich and SahachiroHata, which led to the discovery of salvarsan, in 1909, a novel drug for treating the sexual transmitted disease syphilis that is caused by the spirochete Treponemapallidium**.(Linder et al., 2002)**Salvarsan and its derivative neosalvarsan, were the most prescribed drugs until they were replaced by penicillin in the 1940s **(Emmerson ,2003)**. The large-scale screening method used by Ehrlich and Hata in the discovery of salvarsan, became the gold standard for identifying novel drugs and led to the discovery of the first sulfa drug in 1934, sulfonamidochrysoidine, a precursor of the active compound sulfanilamide, which inhibits folic acid synthesis in bacteria **(Emmerson ,2003)(Aldred et al.,2014)**.

II.1.2 The golden age of antibiotics

The discovery of the sulfa drugs and the release of penicillin for clinical use kick-started a period of 30 years known as the golden age of antibiotics (1940-1970), in which almost all of the antibiotic drug classes used in the clinic today were discovered **(see Figure 1)(Werner et al.,2011)(Dalhoff,2012)**. Most of the antibiotics discovered in this period were isolated from natural extracts from different microorganisms. Followingthe isolation of streptomycin, in 1944, from the soil growing filamentous bacteria Streptomyces griseus. Soil samples were collected from around the world and in 1952 the vancomycin producing Streptomyces orientalis was isolated from a soil sample from Borneo, leading to the release of vancomycin for clinical use in 1958 (**Werner et al.,2011).**

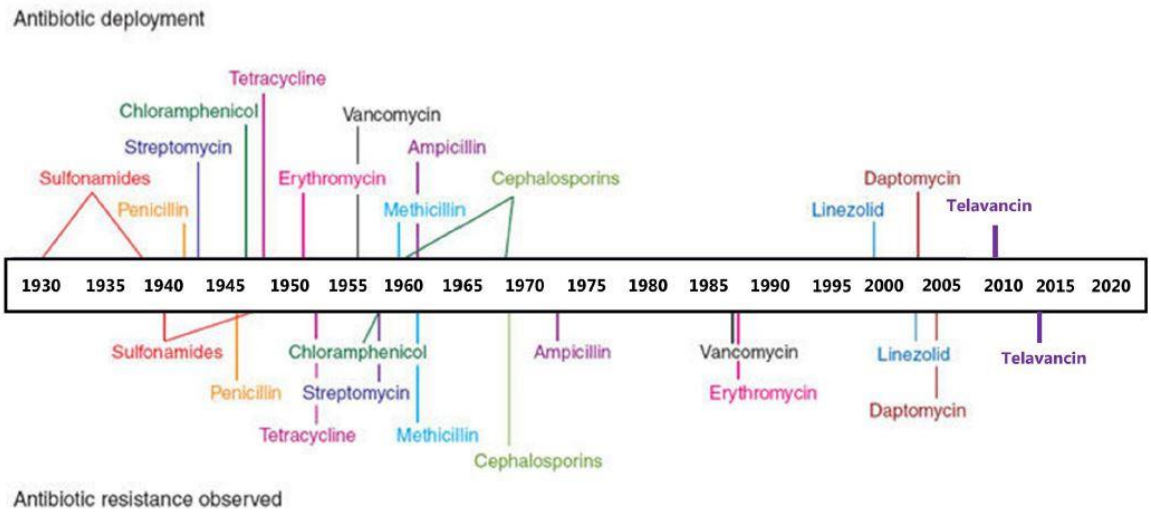


Figure 1: The top panel indicates the time at which different antibiotics and classes of antibiotics were discovered. The bottom panel, indicates when resistance was observed for the given antibiotics. Modified from (**Clatworthy et al., 2007)**.

Despite of its name it was also in the golden age that it became evident that clinical antibiotic resistance would become a problem. In 1945, Alexander Fleming, during his Nobel lecture, warned that underdosing of penicillin could potentially lead to the development of resistance **(White et al., 2004)**. In the decade following Flemings warning, it became apparent that antibiotic resistance was a problem. To overcome resistance scientists started to make derivatives of already know drugs, this led to the development of antibiotics that were impervious to the resistance mechanisms and in some cases improved the pharmacodynamics and pharmacokinetics of the drugs. However, it was also the start of a race between the evolution of antibiotic resistance and the development and discovery of antibiotics. A race that currently seems to be led by the bacteria(**Werner et al.,2011)**.

II.1.3 The present and future of antibiotics

In the last 40 years, the only truly novel class of antibiotics that has been introduced into the clinic are the oxazolidinones, initially represented by the synthetic compound linezolid that was released in 2000 **(Rodriguez et al., 2016)** Due to its synthetic nature it was anticipated that linezolid resistance would evolve slowly.This presumption unfortunately turned out to be wrong, as soon after its release, linezolid resistance was

identified in clinical isolates of Staphylococcus aureus and several enterococcus species **(Baba et al.,2006)**

A list of 48 antibiotics have reached phase I to III clinical trials as estimated in December 2017.

Most of these antibiotics are derivatives of known antibiotics, almost half do not target pathogens listed as being a critical threat by the World Health Organisation (WHO) and even fewer are expected to display activity against the multi-drug resistant group of Gram negative ESKAPE pathogens **(McClure at al., 2013)**.

Considering that on average only one third of these antibiotics will make it through the clinical trials and become a marketable product, the current antibiotic pipeline is not robust enough to support the current and future clinical need **(Avasthi et al.,2011)**.In addition, a report commissioned by the government of the United Kingdom in 2014, estimated that the annual number of deaths attributable to antimicrobial resistance would be 10 million by 2050 and that it will generate a loss of 100 trillion dollars globally **(Cirz RT et al., 2005).** Even though these numbers are only estimates, there is no doubt; antibiotic resistance is a major global health care problem and it will only become more evident with time, if proper action is not taken.

II.1.4 Benefits of Antibiotics

Antibiotics have not only saved patients' lives, they have played a pivotal role in achieving major advances in medicine and surgery **(Gould et al.,2013)**.They have successfully prevented or treated infections that can occur in patients who are receiving chemotherapy treatments; who have chronic diseases such as diabetes, end-stage renal disease, or rheumatoid arthritis; or who have had complex surgeries such as organ transplants, joint replacements, or cardiac surgery **(Wright ,2014).**

Antibiotics have also helped to extend expected life spans by changing the outcome of bacterial infections**.(Piddock , 2012),(Rossolini , 2014)** In 1920, people in the U.S. were expected to live to be only 56.4 years old; now, however, the average U.S. life span is nearly 80 years **(Congressional research service report).** Antibiotics have had similar beneficial effects worldwide. In developing countries where sanitation is still poor,

antibiotics decrease the morbidity and mortality caused by food-borne and other poverty-related infections **(Rossolini , 2014)**.

II.2 Development of Antibiotic Resistance

Bacteria develop antibiotic resistance primarily by: point mutations and horizontal gene transfer (HGT). The development of resistance by point mutation occurs only by chance i.e., bacteria develop mutations at a rate faster than other organisms, as they have shorter generation times and the rapid rate of DNA replication may produce errors leading to mutations **(Walsh, 2003).** Some of these mutations give selective advantage for their survival, such as protection against antibiotics in their surroundings. Such resistance mutation can remain in the bacterial population and it can be passed on to successive generations through vertical transmission **(Walsh, 2003)**. For instance, M. tuberculosis developed multidrug resistance exclusively by spontaneous mutation. Streptomycin is the antibiotic most commonly used against M. tuberculosis but streptomycin resistant strains are creating problems in patients with compromised immune system and resulting in high morbidity and mortality rates **(Shah et al., 2007; Sotgiu et al., 2009; Velayati et al., 2009).** Mutations in the ribosomal protein S12 or within the 530 loop of 16S rRNA are responsible for streptomycin resistance in M. tuberculosis. A single amino acid change from lysine to either arginine or threonine on S12 and 16S rRNA is enough for M. tuberculosis to become resistant to streptomycin .Thus, mutation plays an essential role in the development of drug resistance in bacteria. However, bacteria can also gain drug resistance by other means **(Figure 2) (Fajardo, et al., 2009; Freifelder, 1987; Phornphisutthimas et al., 2007).**

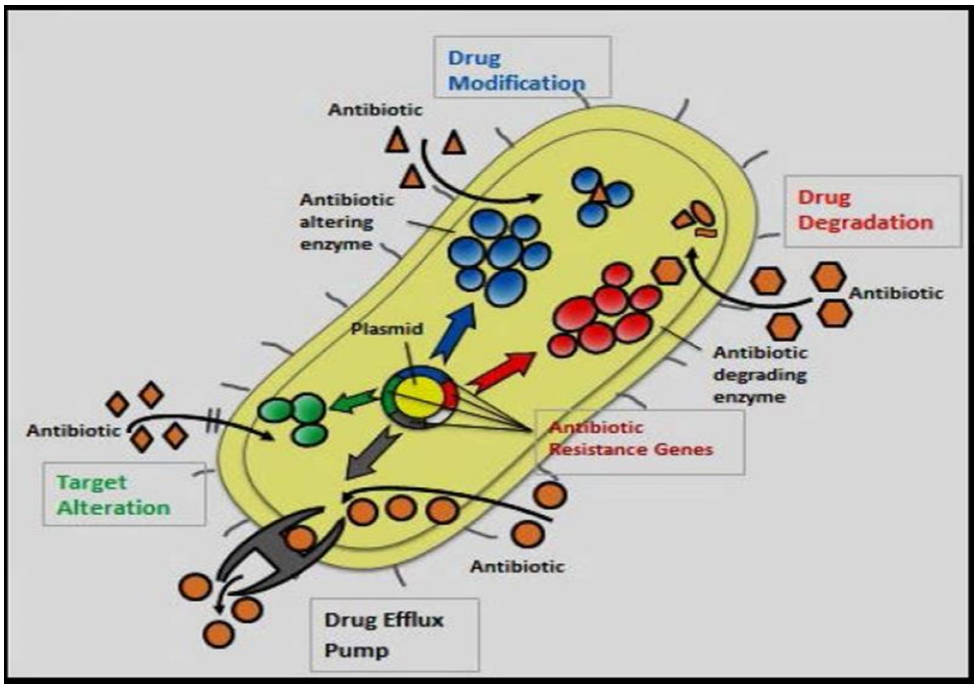


Figure 2: Mechanism of antibiotic resistance. The four mechanisms involved in antibiotic resistance are: 1. drug inactivation, 2. drug modification, 3. drug efflux, and 4. target alteration.

II.3 Mechanisms of antibiotic action and resistance

There are several major classes of antibiotics that can be categorized based on their mode of antibacterial action. In general, antibiotics can be defined as those that inhibit cell wall synthesis, those that inhibit protein synthesis, and those that inhibit nucleic acid synthesis**.(see figure 3)**The selective toxicity of antibiotics lies in the differences in cellular structures between eukaryotic and prokaryotic cells. However, differences in cellular structure among bacterial species can lead to resistance to certain antibiotics.

The definition of bacteria as resistant or susceptible is critical for clinicians. It is also very important to note the difference between intrinsic and acquired resistance to an antibiotic. Intrinsic resistance can best be described as resistance of an entire species to an antibiotic, based on inherent (and inherited) characteristics requiring no genetic alteration. This is usually due to the absence of a target for the actio0n of a given antibiotic or the inability of a specific drug to reach its target. For example, mycoplasmas are always resistant to β -lactam antibiotics since they lack peptidoglycan (which the β-lactams act upon). Similarly, the outer membrane of gram negative cells can prevent an antibiotic

from reaching its target. For example, *Pseudomonas aeruginosa* exhibits high intrinsic resistance to many antibiotics due to its drug efflux pumps and restricted outer membrane permeability.

Acquired resistance can arise either through mutation or horizontal gene transfer. Presence of the antibiotic in question leads to selection for resistant organisms, thereby shifting the population towards resistance. The major mechanisms of acquired resistance are the ability of the microorganisms to destroy or modify the drug, alter the drug target, reduce uptake or increase efflux of the drug, and replace the metabolic step targeted by the drug.

II.3.1 Inhibitors of Cell Wall Synthesis:

There are two major groups of cell wall synthesis inhibitors, the β-lactams and the glycopeptides. As bacterial cell walls are wholly unlike the membranes of eukaryotes, they are an obvious target for selectively toxic antibiotics.

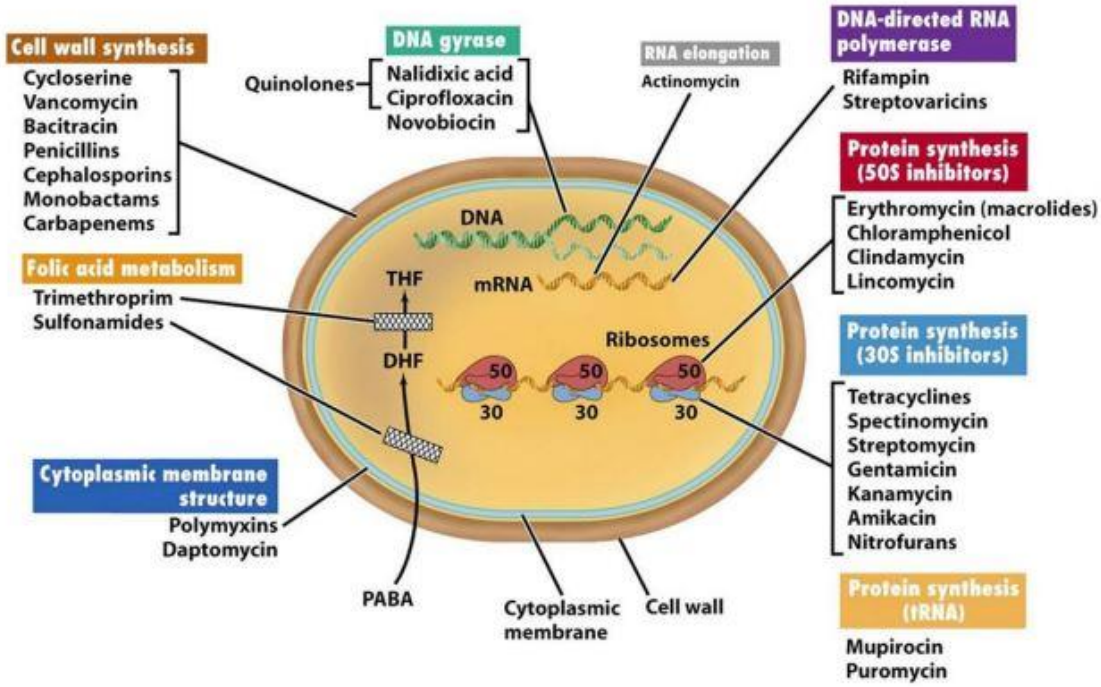


Figure 3 : Antibiotic target sites (Madigan and Martinko, 2006).

II.3.1.1 The β-lactams

The β-lactamsinclude the penicillins, cephalosporins, and the carbapenems. These agents bind to the penicillin binding proteins (PBP's) that cross-link strands of peptidoglycan in the cell wall. In gram negative cells, this leads to the formation of fragile spheroplasts that are easily ruptured. In gram positive cells, autolysis is triggered by the release of lipoteichoic acid (**Greenwood ,2000)**

The mechanism of β-lactams resistance is via the action of the β-lactams. These enzymes catalyze hydrolysis of the β-lactams ring and, thereby, inactivate these antibiotics. Many bacteria contain chromosomally encoded β-lactams necessary for cell wall production and it is only through over-production of these enzymes that resistance occurs (**Greenwood ,2000)** β-lactamases encoded on plasmids or other transmissible elements can lead to such overproduction and, therefore, to resistance **(Normark, 2002)** There are also some bacteria that possess altered PBP's that result in reduced penicillin binding (**Greenwood ,2000).**

Since the discovery of penicillin and resistant bacteria, various new versions of the β-lactams have been used that have different spectrums of activity and different susceptibility to β -lactamases. Since the 1970s, several compounds, such as clavulanic acid, have beendiscovered that have the ability to bind irreversibly to N-lactamases and, thereby, inhibit their action. Combinations of these compounds with N-lactam drugs have been very successful in treatment of disease **(Bryan,1984).**

II.3.1.2 The glycopeptides

The glycopeptides are a group of antibiotics that include vancomycin, avoparcin, and others that bind to acyl-D-alanyl-D-alanine. Binding of this compound prevents the addition of new subunits to the growing peptidoglycan cell wall. These drugs are large molecules that are excluded from gram negative cells by the outer membrane, thus limiting their action to gram positive organisms.

Glycopeptide resistance was long thought to be rare, but has recently been shown to be quite common **(Bryan,1984).** Resistance in enterococci has developed through newly discovered enzymes that use D-alanyl-D-lactate in place of acyl-D-alanyl-Dalanine,

allowing cell wall synthesis to continue. Other mechanisms of resistance involve the over-production of peptidoglycan precursors which overwhelm the drug (**Greenwood ,2000).**

II.3.2 Inhibitors of protein synthesis:

There are many types of antibiotics that inhibit bacterial protein synthesis. These drugs take advantage of structural differences between bacterial ribosomes and eukaryotic ribosomes.

II.3.2.1 The aminoglycoside

The aminoglycoside antibiotics are a group whose mechanism of action is not completely understood. The three major groups of aminoglycosides are the streptomycins, neomycins, and kanamycins. These drugs enter bacterial cells by an active transport that involves quinones that are absent in anaerobes and streptococci, thus excluding these organisms from the spectrum of action. Streptomycins act by binding to the 30S ribosomal subunit. Kanamycins and neomycins bind to both the 50S subunit and to a site on the 30S subunit different from that of streptomycin (**Greenwood ,2000)**Activity involving initiation complexes and cell membrane proteins that contribute to cell death plays a role in the action of these antibiotics, but this is poorly understood **(Bryan,1984)**(**Greenwood ,2000).**

There are three mechanisms of aminoglycoside resistance that have been identified to date. The first involves only streptomycin. Since streptomycin binds to one particular protein on the ribosome, alteration of this protein, even by a single amino acid in its structure, confers high-level resistance to the drug **(Bryan,1984).**

The other mechanisms involve decreased uptake of the antibiotic and in one of these the cell membrane is altered, preventing active transport of the drug. In the other, one of many enzymes alters the antibiotic as it enters the cell, causing a block in further active transport **(Bryan,1984).**

II.3.2.2 Chloramphenicol

Chloramphenicol is a broad-spectrum antibiotic that, although naturally occurring, is produced by chemical synthesis. Chloramphenicol inhibits peptide bond formation on 70S ribosomes **(Bryan,1984).**This drug is especially useful in that it can penetrate eukaryotic

cells and cerebrospinal fluid, making it a drug of choice for treatment of meningitis and intracellular bacterial infections such as those caused by chlamydia. It is not in widespread use, however, because of potentially fatal side-effects, namely, aplastic anemia (**Greenwood ,2000).**Resistance to chloramphenicol is conferred by the enzyme chloramphenicol acetyl-transferase. A number of these enzymes have been discovered, each altering the chloramphenicol molecule to prevent binding to the bacterial ribosome. Chloramphenicol resistance in gram negative cells can also arise from alteration in outer membrane permeability that prevents the drug from entering the cell **(Bryan,1984).**

II.3.2.3 The tetracyclines

The tetracyclines are another group of broad-spectrum antibiotics that inhibit bacterial protein synthesis **(figure 4)**. They are brought into the cell by active transport and, once there, bind to the 30S subunit to prevent binding of aminoacyl tRNA**(Roberts,1996)**

Resistance to the tetracyclines occurs via three mechanisms. First, production of a membrane efflux pump removes the drug as rapidly as it enters and there are several genes encoding these pumps. Second, several ribosome protection proteins act to prevent tetracycline from binding to the ribosome, thus conferring resistance. Third, a protein found only in Bacteroides spp., enzymatically inactivates tetracycline **(Roberts,1996).**Interestingly, efflux pump inhibitors have recently been discovered that may allow combinations of these inhibitors and tetracyclines to be used against previously resistant strains **(Chopra,2002)**

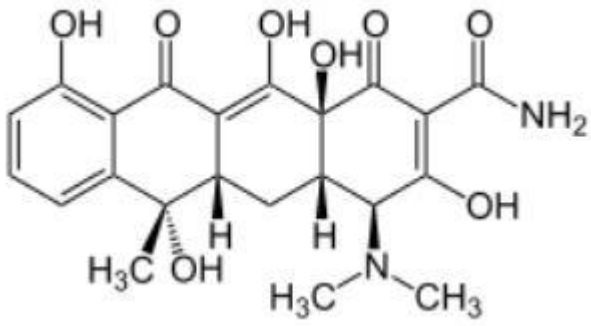


Figure 4 : Structure of Tetracycline **(Chopra and Roberts, 2001).**

II.3.2.4 The macrolides

The macrolides are a group of antibiotics commonly used to treat gram positive and intracellular bacterial pathogens **(Figure 5).** Erythromycin was the first of these, and several other important macrolides have been discovered since, including clarithromycin and azithromycin. Azithromycin has a longer plasma half-life which allows treatment with a single dose for some pathogens or a once daily dose for others. Clarithromycin has enhanced absorption and causes less gastrointestinal discomfort **(Gaynoret al.,2003)**It was originally believed that erythromycin inhibited protein synthesis by competing with amino acids for ribosomal binding sites, but newer research shows several mechanisms are involved **(Garrod,1971).** The macrolides are now believed to promote dissociation of tRNA from the ribosome,inhibit peptide bond formation, inhibit ribosome assembly, and prevent amino acid chain elongation **(Gaynoret al.,2003).**

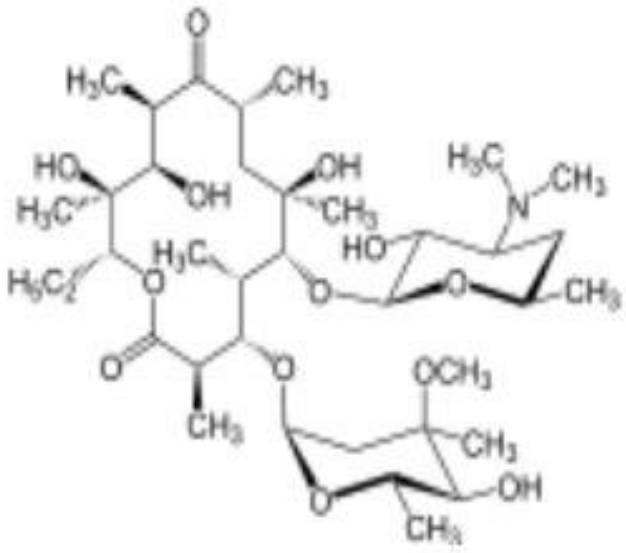


Figure 5 : Structure of Macrolide **(Hamilton-Miller, 1973).**

There are two major mechanisms of macrolide resistance. First, an efflux pump has been found that removes the drug from the cell. Second, modification of the ribosome can confer resistance. Mutations at several sites of the ribosome can allosterically prevent macrolide binding and a common alteration is dimethylationof one nucleotide on the 23S rRNA. This dimethylation not only prevents macrolide binding, but also confers resistance to lincosamide and streptogramin antibiotics **(Gaynoret al.,2003).**

II.3.2.5 The streptogramins

The streptogramins are another class of antibiotic that inhibits bacterial protein synthesis, mostly in gram positive organisms (due to decreased permeability of the gram negative outer membrane). These antibiotics are actually combinations of structurally different drugs, types A and B, that act synergistically. These compounds bind to separate sites on the 50S subunit. Type A drugs block attachment of substrates at two sites on the 50S subunit, whereas type B drugs cause release of incomplete protein chains. The synergistic effect arises from a conformational change induced by the binding of a type A drug which significantly increases affinity of type B drugs **(Johnston ,2002).** Streptogramins currently in use include virginiamycin, pristinamycin, and quinupristin/dalfopristin.

Resistance to streptogramin antibiotics can be found in several forms. Efflux pumps for both type A and B streptogramins have been identified. Type A streptogramins can be inactivated by one of the virginiamycin acetyl-transferases, and several enzymes have been identified that can inactivate type B streptogramins. Alteration of bacterial ribosomal proteins or RNA can also confer resistance. A common mutation is the dimethylation of one nucleotide on the 23S rRNA, mentioned previously, that gives rise to resistance to type B drugs, as well as macrolides and lincosamides **(Johnston ,2002).**

II.3.3 Inhibitors of nucleic acid synthesis:

II.3.3.1 The sulfonamides and the diaminopyrimidines

The sulfonamides and the diaminopyrimidines should be discussed together, in that both only indirectly inhibit nucleic acid synthesis by inhibiting folate synthesis. Folate is a coenzyme necessary for the synthesis of purines and pyrimidines. Although both types of drugs are useful on their own, they exhibit a synergistic effect when combined. Sulfonamides are currently not used commonly in medicine, but the combination drug trimethoprim-sulfamethoxazole is sometimes used in the treatment of urinary tract infections. Sulfonamides serve as an analog of p-aminobenzoic acid. Therefore, they competitively inhibit an early step in folate synthesis. Diaminopyrimidines, of which trimethoprim is the most common, inhibit dihydrofolatereductase, the enzyme that catalyzes the final step in folate synthesis (**Greenwood ,2000).**

There are several resistance mechanisms microorganisms employ against each of the anti-folate drugs. For example, sulfonamides are rendered ineffective by over-production

of p-aminobenzoic acid or production of an altered dihydropteroatesynthetase. The substrate fordihydropteroatesynthetase is p-aminobenzoic acid, and the altered form has a much lower affinity for sulfonamides than for p-aminobenzoic acid **(Then, 1982)**.Trimethoprim resistance can also result from several mechanisms, e.g., over-production of dihydrofolatereductase or production of an altered, drug-resistant form can lead to resistance **(Bryan,1984) .**In addition, both drugs can be enzymatically inactivated, resulting in resistance **(Then, 1982)**.

II.3.3.2 The quinolones

The quinolones are a chemically varied class of broad-spectrum antibiotics widely used to treat many diseases, including gonorrhea and anthrax. Drugs in this class include nalidixic acid, norfloxacin, and ciprofloxacin. These drugs are commonly used and, worldwide, more ciprofloxacin is consumed than any other antibacterial agent . Quinolones inhibit bacterial growth by acting on DNA gyrase and topoisomerase IV, which are necessary for correct functioning of supercoiled DNA (**Greenwood ,2000)** Although quinolones target both enzymes, in gram negative organisms the primary target is DNA gyrase and, in gram positive
organisms, the primary target is topoisomerase IV **(Ruiz,1996).**

There are three main mechanisms of resistance to quinolones. Resistance to some quinolones occurs with decreased expression of membrane porins. Cross-resistance to other drugs requiring these porins for activity also results from these changes. A second mechanism of resistance is expression of efflux pumps in both gram negative and gram positive organisms (**Normark,2002)** and the third is alteration of the target enzymes. Several mutations have been described in both quinolone target proteins that result in reduced binding affinities **(Ruiz,1996).**It is believed that high-level quinolone resistance is brought about by a series of successive mutations in the target genes, rather than a single mutation (**Normark,2002).**

II.4 Cross-Resistance

Microorganisms resistant to a certain drug may also be resistant to other drugs that share a mechanism of action. Such relationships exist mainly between agents that are closely related chemically (eg, different aminoglycosides) or that have a similar mode of

binding or action (eg, macrolides and lincomycins). In certain classes of drugs, the active nucleus of the chemical is so similar among many congeners (eg, tetracyclines) that extensive cross-resistance is to be expected.

II.5 Limitation of Drug Resistance

Emergence of drug resistance in infections may be minimized in the following ways: (1) by maintaining sufficiently high levels of the drug in the tissues to inhibit both the original population and first-step mutants; (2) by simultaneouslyadministering two drugs that do not give cross-resistance, each of which delays the emergence of mutants resistant to the other drug (eg, rifampin and isoniazid [INH] in the treatment of tuberculosis); and (3) by avoiding exposure of microorganisms to a particularly valuable drug by limiting its use, especially in hospitals **(Jawertz et al.,).**

III. Antibacterial activity of cherimoya seeds

Since many of the commonly used antibiotics are becoming almost useless in the fight against many virulent bacteria, see the previous, a trend of medical and clinical oriented research has developed project looking for alternative plant based products to be included the fight endeavors against the mounting problem of antibiotics-resistance. Below is a review about one of the promising medicinal plants; Cherimoya.

III.1. Classification

III.1.1 Family annonaceae

Cherimoya (*Annona cherimola Mill*) belongs to the family Annonaceae, evolutionary, ecologically as well as economically an important plant family. Annonaceae are with about 2,500 species in 140 genera, the biggest family **(Mabberley, 1990)** within the order of Magnoliales **(Cronquist, 1981**), an order of rather primitive angiosperms. Annonaceae are distributed (Figure 1.8) throughout the tropical areas of America (900 species), Africa (450 species) and Australasia (1,200 species). Two clear subfamilies are distinguished: Annonoidae with free spirally arranged carpels and Monodoroideae with united cyclically arranged carpels. Genus Annona (ca 150 spp.) is, together with Guatteria (265 spp.) and Duguetia (100 spp.), one of the largest genera of the family and has its main distribution area in the Neotropics, but occurs in tropical Africa as well **(Chatrou, 1999**).

Commercially, the most significant Annonaceae are genera Annona and Rollinia **(Sanewski, 1991).**

III.1.2 Genus Annona

The genus Annona contains approximately 100 species and can be found in tropical America and Africa **(Mabberley, 1990).** The name of the genus is derived from the Latin 'annona' (literally 'yearly produce') and indicates their typical annual producing cycle. The characteristic feature of the genus Annona is their fruit that is a syncarpium, formed by amalgation of many pistils and the fleshy receptacle. Annona trees are small trees to 7 m tall, with simple, entire and alternate leaves. The hermaphrodite flowers are yellowish with 3 to 6 tepals and numerous stamens and pistils.

III.1.3 Species

Table 11 : different species of anonna **(Mabberley, 1990).**

Annona Species	Vernacular Name	Origin
A. cherimola Mill.	Eng. : cherimoya, custardapple* Spa. : chirimoya Fre. : chérimolier, chérimole Dut. : cherimoya Por. : cherimólia	**Andes of Peru and Ecuador**
A. diversifoliaSaff.	Eng.: ilama Spa.: ilama, anonablancaFre.: ilama	Central America
A. glabra L.	Eng.: pond apple, alligator apple Spa.: cayur, corcho Fre.: anone des marais, mamin Por. : araticú do brejo	Tropical America & West Africa
A. montanaMacfad.	Eng.: mountain soursop, wild soursop Spa.: guanábanacimarrona	Central America

	Fre.:corossolierbâtardDut. : boszuurzak Por. : araticumapé	
A. muricata L	. Eng.: soursop Spa.: guanábanaFre.: corossolierDut.: zuurzak Por. : graviola	Tropical America
A. purpurea Moc.et Sessé	Eng.: soncoya, negro head Spa.: soncoya, cabeza de negro Fre.: atier Por. : cabeça de Negro	Central America
A. reticulata L.	Eng.: bullock's heart, custard apple* Spa.: corazón, anón, mamónFre.: coeur de boeuf, cachimanDut.: custardappel, ossehart Por. : coração de boi	Tropical America
A. scleroderma Saff.	Eng.: poshté, cawesh Spa. : anona del monte	Central America
A. senegalensis Pers.	Eng.: wild custard apple	West Africa
A. squamosa L.	Eng.: sugar apple, sweetsop, custard apple* Spa.: anón, anonablanca, saramuyoFre.: pommecanelle, attierDut.: suikerappel, kaneelappelPor : ata, fruta do conde, pinha	Tropical America
A. squamosa × A. cherimola	Eng.: atemoya, custard	Artificial hybrid

	apple*	

III.2 Ecology

Cherimoya is mainly cultivated in the highlands from sea-level up to approximately 1400 m altitude. In Colombia and Ecuador, it grows naturally at elevations between 1400-2000 m where the temperature ranges between 17-200C **(Pepenoe, 1970).**

III.3 Nutritional composition

The nutritional composition of cherimoya fruit is that of a typical sweet fruit but with a high content of carbohydrates and low content of acids **(Table 12)**. Its vitamin A content is modest, but it is a good source of thiamine, riboflavin, niacin, iron, calcium and phosphorous **(Allen, 1967).**

Table 12 : nutritional composition of 100g pulp according to different authors.

Constituents	**Allen BM , 1967**	**FranciosiTijero, 1992**
Water(g)	77.1	77.1
Protein(g)	1.9	1.9
Fat(g)	0.1	0.1
Carbohydrates(g)	18.2	18.2
Fibre(g)	2.0	2.0
Ashes(g)	0.7	0.7
Calcium(mg)	32.0	32.0
Phosphorous(mg)	37.0	37.0
Iron(mg)	0.5	0.5
Vitamin A(IU)	0.01	0.0
Thiamin(vitaminB1)(mg)	0.1	0.1
Riboflavin(vitamin B2)(mg)	0.14	0.14
Niacin(mg)	0.9	0.9
Ascorbic acid(vitamin C)(mg)	5	5
Calories(kcal)	73	73

III.4 Principal caracteristics of anonaa cherimoya

III.4.1 Morphological characteristics

The cherimoya tree is erect but low branched and somewhat shrubby or spreading and ranging from 5 to 9 m **(Chetty et al., 2008).**

III.4.1.2 Leaves

Leaves are briefly deciduous to semi-deciduous due to the mitriform petiole concealing the bud. They are alternate, 2-ranked, with minutely hairy petioles; ovate to elliptic, short blunt pointed at the apex; slightly hairy on the upper surface, velvety on the lower surface **(Morton, 1987).**

III.4.1.3 Flowers

Fragrant flowers, solitary or in group of 2 or 3, on short hairy stalks, have 3 outer, greenish, fleshy, oblong petal-like tepals and 3 smaller inner tapels**(Chetty et al., 2008) .**

III. 4.1.4 Fruit

The syncarp fruit, formed by amalgation of pistils and receptacle, is conical or somewhat heart-shaped, 10 to 20 cm long and upto 10 cm in width, weighing on average 150-500 g, but extra-large specimens of 2.7 kg or more have been reported **(Farre, 1999).**The skin may be smooth with finger print-like markings or covered with conical or rounded protuberances. The fruit is easily broken or cut open, exposing the snow-white, juicy flesh of pleasing aroma and delicious, subacidflavour, and is containing numerous hard, brown or black bean like, glossy seeds, 1.25 to 2 cm long **(Morton, 1987).**

III.5 Phytochemical constituents

The plant is reported to contain alkaloids, flavonoids, glycosides, saponins, tannins, carbohydrates, proteins, phenolic compounds, phytosterols, and amino acids **(Adarsh et al., 2011)** The antistress activity of cherimoya is mainly attributed to these constituents with established antioxidant activity **(Adarsh et al., 2011)** The various chemical constituents isolated from stem and seeds of the plant including annocherine A, B, cherianoine, aromin-A, Ncis-caffeoyltyramine, dihydro-feruloyltyramine, N-transferu loylmethoxyty ramine and N-cisferu loyltylmethoxyty ramine **(Chen CY et al., 1997, 1998, 1999)** Specimens of cherimoya from Taiwan contained cherimoline**(Chen CY et al., 1997)** Seeds contain cyclo octapeptide, cherimola cyclopeptide A, and cherimola cyclopeptide B **(Wele et al., 2004).**

The volatile constituents of cherimoya bark were identified from the essential oil obtained by the steam distillation and studied by Gas chromatography and Mass spectrometry. The bark contains annonaine, an alkaloid which is found to posses many of the properties. The major compounds were identified as methyl butanoate (69.08%), butyl butanoate (56.56%), 3-methylbutyl butanoate (15.36%), 3-methyl butyl 3methyl butanoate (56.69%) and 5-hydroxymethyl-2furfural (71.82%) **(Liseth et al.,2009) .**

III.6 Antimicrobial activity

The chemical composition of the essential oils of leaves, flowers and fruits of A. cherimola were studied for its antimicrobial activity. Five bacteria (Staphylococcus aureus, Enterococcus faecalis, E. coli, Shigellasonei and Proteus mirabilis) and one fungus (Candida albicans) were selected for screening.

The screening results showed that highest zone of inhibition were observed in leaf extract against E.coli.**(Mariaet al,2003)** Volatile compound (cherimolacyclopeptide E) of this plant was also studied for its antimicrobial activity **(Rajiv, 2005)** The methanolic extracts of the leaves and a pure compound isolated from A. cherimola plant exhibit antiviral activity against herpes simplex type 2 (HSV-2) viruses **(Bentancur et al., 1999).**

III.7 Uses

Cherimoya is essentially a dessert fruit that is eaten fresh. It can also be used for making ice-cream, milkshakes or sorbets and is processed into yoghurt, flan fruit juice and wine **(NationalResearch Council, 1989)(Gardiazabal, 1993) .**Occasionally it is seeded and added to fruit salads or used for making sherbet or ice-cream. Due to its enzymatic characteristics, cherimoya fruits cannot be submitted to thermal processes and its processing should utilize refrigerating or freezing, with addition of antioxidants to avoid enzymatic oxidation and subsequent colouring**(Oleata, 1996)**Traditionally, cherimoya seeds are crushed and used as insecticide, mostly to kill lice and cure parasitic skin disorders **(Varea, 1992).**

Biochemically, cherimoya seeds are an important source for acetogenins**(Rajiv, 2007)** , a type of alkaloid, all of which show antiparasitic and cytotoxic activities that are used in pharmaceuticals (**Sanhpaz, 1996)**.The annonaceousacetogenins are a new group of

powerful bioactive agents and more than 300 of these compounds have been found. Properties attributed have been antimicrobial, antitumor, cardiotonic and insecticidal **(Sanewski, 1991).**

The fruit is very low in cholesterol and sodium. It is a good source of dietary fibre, vitamin B6 and potassium, and a very good source of vitamin C. The cherimoya fruit is known for its exceptional taste, its use in traditional medicine as an antimicrobial and insecticide, and as an effective treatment for digestive disorders such as stomach-ache and pancreatic ulcers [46]. In Jamaica, the dried flowers have been used as flavouring for snuff. Cherimoya is an immature fruits are used in vegetable curries. The entire immature fruit is used as a cooked vegetable. A decoction of the bark is used both as a tonic and a remedy for diarrhoea. The root is chewed to relieve toothache and a decoction from the root is used to treat fevers. A decoction of the leaves is used to treat worms. The leaves are used to tan leather **(Sanewski, 1991).**

IV. Database

A database is an organized collection of structured information, or data, typically stored electronically in a computer system. **(Database, 2019).**A database is built and maintained by using a database programming language. The most common database language is SQL, but there are multiple "flavors" of SQL, depending on the type of database being used. Each flavor of SQL has differences in the SQL syntax and are designed to be used with a specific type of database. For example, an Oracle database uses PL/SQL and Oracle SQL (Oracle's version of SQL). A Microsoft database uses T-SQL (Transact-SQL).

IV.1 Evolution of the Database

Databases have evolved dramatically since their inception in the early 1960s. Navigational databases such as the hierarchical database (which relied on a tree-like model and allowed only a one-to-many relationship), and the network database (a more flexible model that allowed multiple relationships), were the original systems used to store and manipulate data. Although simple, these early systems were inflexible. In the 1980s, relational databases became popular, followed by object-oriented databases in the 1990s. More recently, NoSQL databases came about as a response to the growth of the internet and the need for faster speed and processing of unstructured data. Today, cloud

databases and self-driving databases are breaking new ground when it comes to how data is collected, stored, managed, and utilized **(figure 6).**

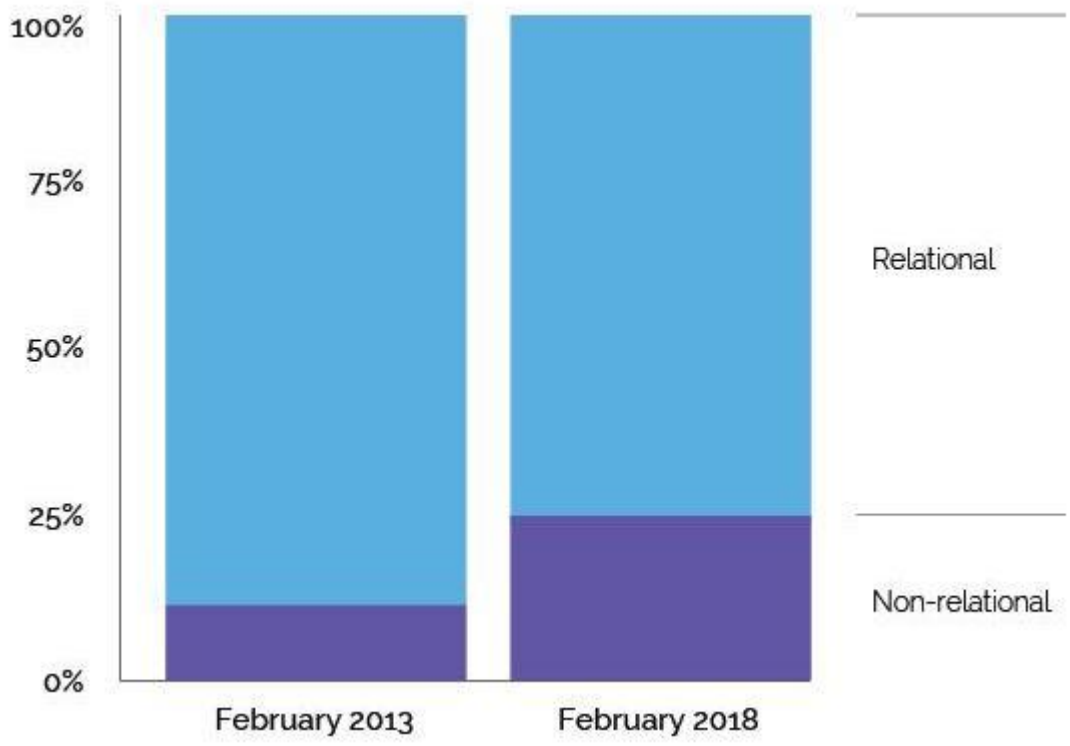


Figure 6 : Popularity (pourcentage) relationaldatabase vs non-relationaldatabase

IV.2 The Difference Between a Database and a Spreadsheet

Databases and spreadsheets (such as Microsoft Excel) are both convenient ways to store information. The primary differences between the two are:

- How the data is stored and manipulated
- Whocanaccess the data
- How much data can be stored

Spreadsheets were originally designed for one user, and their characteristics reflect that. They're great for a single user or small number of users who don't need to do a lot of incredibly complicated data manipulation. Databases, on the other hand, are designed to hold much larger collections of organized information—massive amounts, sometimes. Databases allow multiple users at the same time to quickly and securely access and query the data using highly complex logic and language.

IV.3 The Elements of a Database

A database is made up of multiple tables. Just like Excel tables, database tables consist of columns and rows. Each column corresponds to an attribute, and each row corresponds to a single record. Each table must have a unique name in a database.

For example, consider a database table that contains names and telephone numbers. You would probably set up columns named "FirstName," "LastName" and "TelephoneNumber." Then you would simply start adding rows underneath those columns that contain the data. In a table of contact information for a business with 50 employees, we'd wind up with a table that contains 50 rows.

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.

The data in a database is further protected by what are called *constraints*. Constraints enforce rules on the data to ensure its overall integrity. For example, a unique constraint ensures that a primary key cannot be duplicated. A check constraint controls the type of data you can enter — for example, a Name field can accept plain text, but a social security number field must include a specific set of numbers. Several other types of constraints exist, as well.

One of the most powerful features of a database is the ability to create relationships between tables using foreign keys. For example, you might have a Customers table and an Orders table. Each customer can be linked to an order in your Orders table. The Orders table, in turn, might be linked to a Products table. This kind of design comprises a relational database and simplifies your database design so that you can organize data by category, rather than trying to put all the data into one table, or just a few tables.

IV.4 Types of Databases

There are many different types of databases. The best database for a specific organization depends on how the organization intends to use the data.

1. **Relational databases.** Relational databases became dominant in the 1980s. Items in a relational database are organized as a set of tables with columns and rows. Relational database technology provides the most efficient and flexible way to access structured information.
2. **Object-oriented databases.** Information in an object-oriented database is represented in the form of objects, as in object-oriented programming.

3. **Distributed databases.** A distributed database consists of two or more files located in different sites. The database may be stored on multiple computers, located in the same physical location, or scattered over different networks.
4. **Data warehouses.** A central repository for data, a data warehouse is a type of database specifically designed for fast query and analysis.
5. **NoSQL databases.** A NoSQL, or nonrelational database, allows unstructured and semistructured data to be stored and manipulated (in contrast to a relational database, which defines how all data inserted into the database must be composed). NoSQL databases grew popular as web applications became more common and more complex.
6. **Graph databases.** A graph database stores data in terms of entities and the relationships between entities.
7. **OLTP databases.** An OLTP database is a speedy, analytic database designed for large numbers of transactions performed by multiple users.

These are only a few of the several dozen types of databases in use today. Other, less common databases are tailored to very specific scientific, financial, or other functions. In addition to the different database types, changes in technology development approaches and dramatic advances such as the cloud and automation are propelling databases in entirely new directions. Some of the latest databases include :

- **Open source databases.** An open source database system is one whose source code is open source; such databases could be SQL or NoSQL databases.
- **Cloud databases.** A cloud database is a collection of data, either structured or unstructured, that resides on a private, public, or hybrid cloud computing platform. There are two types of cloud database models: traditional and database as a service (DBaaS). With DBaaS, administrative tasks and maintenance are performed by a service provider.
- **Multimodel database.** Multimodel databases combine different types of database models into a single, integrated back end. This meanstheycanaccommodatevarious data types.

- **Document/JSON database.** Designed for storing, retrieving, and managing document-oriented information, document databases are a modern way to store data in JSON format rather than rows and columns.
- **Self-driving databases.** The newest and most groundbreaking type of database, self-driving databases (also known as autonomous databases) are cloud-based and use machine learning to automate database tuning, security, backups, updates, and other routine management tasks traditionally performed by database administrators.

IV.5 Biology/bioinformatics databases

Central databases are to the molecular biology research and development, consider a sampling of the public bioinformatics databases listed in Table 2-1. Perhaps the bestknown of the hundreds of DNA sequence databases accessible through the Internet are the international nucleotide sequence database collaborators GENBANK, supported by the National Center for Biological Information (NCBI), the DNA DataBank of Japan (DDBJ), and the European Molecular Biology Laboratory (EMBL). Another major database, PubMed, which is maintained by the U.S. National Library of Medicine, is a key resource for biomedical literature.

Table 13 : Public BioinformaticsDatabases Accessible via the Internet.

Database Type	Example	Note
Nucleotide Sequence	GenBank	One of the largest public sequence databases
	DDBJ	DNA DataBank of Japan
	EMBL	European Molecular Biology Laboratory
	MGDB	Mouse Genome Database
	GSX	Mouse Gene Expression Database
	NDB	Nucleic Acid Database

Protein Sequence	SWISS-PROT TrEMBL TrEMBLnew PIR	Swiss Institute for Bioinformatics and European Bioinformatics Institute Annotated supplement to SWISSPROT Weekly, pre-processed update to TrEMBL
3D Structures	PDB MMDB Cambridge Structural Database	Protein DataBank Molecular Modeling Database For small molecules
Enzymes and Compounds	LIGAND Chemical	compounds and reactions
Sequence Motifs (Alignment)	PROSITE BLOCKS PRINTS Pfam ProDOM	Sequence motifs Derived from PROSITE A superset of BLOCKS Protein families database of alignments and hidden Markov models Protein Domains
Pathways and Complexes	Pathway Metabolic and	regulatory pathway maps
Molecular Disease OMIM	Online Mendelian	Inheritance in Man
Biomedical Literature	PubMed Medline	Contains Medline Medical Literature
Vectors	UniVec	Used to identify vector contamination
Protein Mutations	PMD	Protein Mutant Database
Gene Expressions	GEO	Gene Expression Omnibus
Amino Acid Indices	Aaindex	Amino Acid Index Database
Protein/Peptide Literature	LITDB	Literature database for proteins and peptides
Gene Catalog	GENES	KEGG Genes Database

The nucleotide sequence databases and PubMed represent the extremes of the spectrum from sequences of base pairs to their relevance in disease and the practice of medicine. Other online databases, such as the protein sequence database SWISS-PROT, and the Online Mendelian Inheritance in Man (OMIM) database—a molecular disease database that links human genes and genetic disease—provide data that is somewhere between the two ends of the spectrum. For example, SWISS-PROT contains sequence motifs (where a motif is a small structural element that is recognizable in several proteins, such as the alpha helix) that are often associated with particular functions, linking structure and function. Popular representatives of so-called alignment databases are PROSITE and BLOCKS, for sequence motif and motif alignment data, respectively.

Public structural databases are represented by the Cambridge Structural Database for small molecules and the Protein Data Bank (PDB) for macromolecules. The PDB, which is maintained by the Research Collaboratory for Structural Bioinformatics (RCSB), includes publicly available 3D structures of proteins, nucleic acids, and carbohydrates, as determined by X-ray crystallography and NMR spectroscopy. The PDB serves as the source data for other databases, such as the Molecular Modeling Database (MMDB), which is used to construct 3D images of the molecules involved.

IV.6 Database Challenges

Today's large enterprise databases often support very complex queries and are expected to deliver nearly instant responses to those queries. As a result, database administrators are constantly called upon to employ a wide variety of methods to help improve performance. Some common challenges that they face include:

- **Absorbing significant increases in data volume.** The explosion of data coming in from sensors, connected machines, and dozens of other sources keeps database administrators scrambling to manage and organize their companies' data efficiently.
- **Ensuring data security.** Data breaches are happening everywhere these days, and hackers are getting more inventive. It's more important than ever to ensure that data is secure but also easily accessible to users.
- **Keeping up with demand.** In today's fast-moving business environment, companies need real-time access to their data to support timely decision-making and to take advantage of new opportunities.

- **Managing and maintaining the database and infrastructure.** Database administrators must continually watch the database for problems and perform preventative maintenance, as well as apply software upgrades and patches. As databases become more complex and data volumes grow, companies are faced with the expense of hiring additional talent to monitor and tune their databases.
- **Removing limits on scalability.** A business needs to grow if it's going to survive, and its data management must grow along with it. But it's very difficult for database administrators to predict how much capacity the company will need, particularly with on-premises databases.
- Addressing all of these challenges can be time-consuming and can prevent database administrators from performing more strategic functions.

CHAPTER II : Materials and Methods

I Material and Methods

I.1 Biologic material

I.1.1 Bacterial Material

Five 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**)** 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.

Two bacterial strains; *Clostridium difficile* and *Mycobacterium tuberculosis* were isolated locally in standard hygienic conditions at the microbiology laboratory of Saida University. However, the bacterium *Mycobacterium tuberculosis* was not used in this project.

I.1.2 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.

I.2 Plant Material:

The ripen fruits of *Annona cherimolla* (*mill*), commonly known as Cherimoya, were imported from spain and obtained from local market in Saida city, Algeria.

I.2.1 Collection of plant material:

Cherimoya seeds were harvested from the fresh ripen fruit.

a. Drying:

The seeds were then separated from the pulp **(Figure 1)**, then dried up under a shade area at room temperature to guarantee total drying and to prevent the breakdown of pharmacological elements **(Figure 2).**

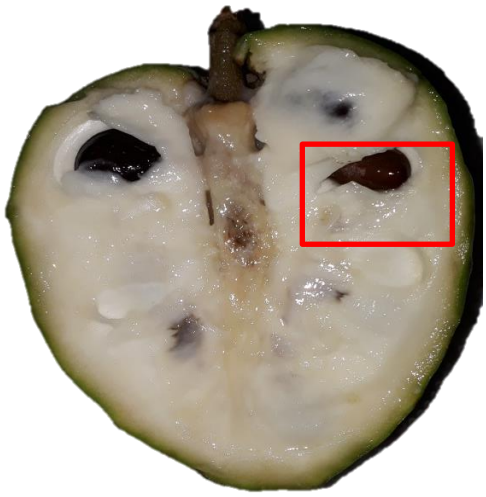


Figure 1 : Seeds still in the pulp flesh of the Cherimoya fruit before harvesting and drying.

Figure 2 : Cherimoya seeds after drying

b. Grinding:

The dried out seeds were then milled into a fine powder using an electric grinder **(Figure 3)** The powdered material was put in sealed glass bottle and stored in cold room.

Figure 3 : seeds after grinding

I.3. Data Preparation & Programing Tools:

A number of software tools were implemented in the realization of the bioinformatics part of this project included the following:

- Microsoft Excel 2010 has been used in creating all of the histograms and graphs related to the statistical analysis of the web-lab results. It has also been used for data formatting and preliminary storing prior to the database creation.
- Notpad++ for programming codes writing and editting.
- XAMPP server vesion maria for MySql in-house database creation.
- PhpMyAdmin for PHP scripts interpretation and MySql in-house database handling, storing data and results retrieving

I.3.1. Software and Data preparation:

- **Microsoft Excel 2010:**

Microsoft Excel 2010 comes within Microsoft Windows software installed on own Personal Computer as part of the Microsoft Office software tools. This Excel version has been used store and format the various data generated by this project. All of the Excel files were formatted in the Comma-Separated Values of CSV format for easy handling by the PHP programming scripts **(Figure 4).**

Figure 4: Microsoft Excel 2010 used as Microsoft Office software tools.

◆ **XAMPP installation:**

XAMPP is an easy to install Apache distribution containing MySql Database (called MariaDB in this version), PHP code interpretor, and Apache server platform. A free software version was download and installed. The XAMPP server is run in the background of the computer processes to be able to provide the framework of the tools mentioned above, see **(Figure 5)**.

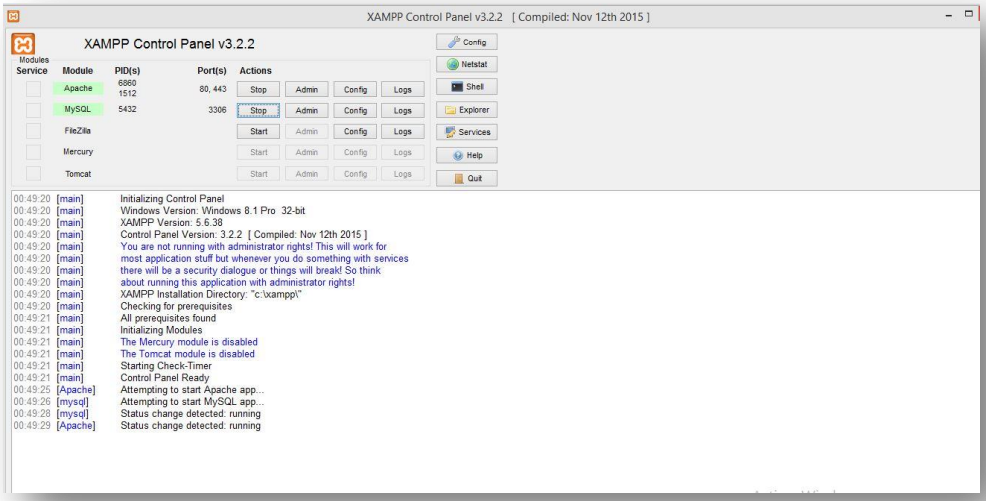


Figure 5: XAMPP started. The figure show that two essential processes are activated, the Apache server and MySql database necessary for the in-house database creation and PHP coding tasks.

◆ **Notepad++ Installation:**

A free version of NotePad++ tool was downloaded from internet and installed. This tools has been used as the primary editor for creating the various PHP codes used in this project, see **Figure 6**.

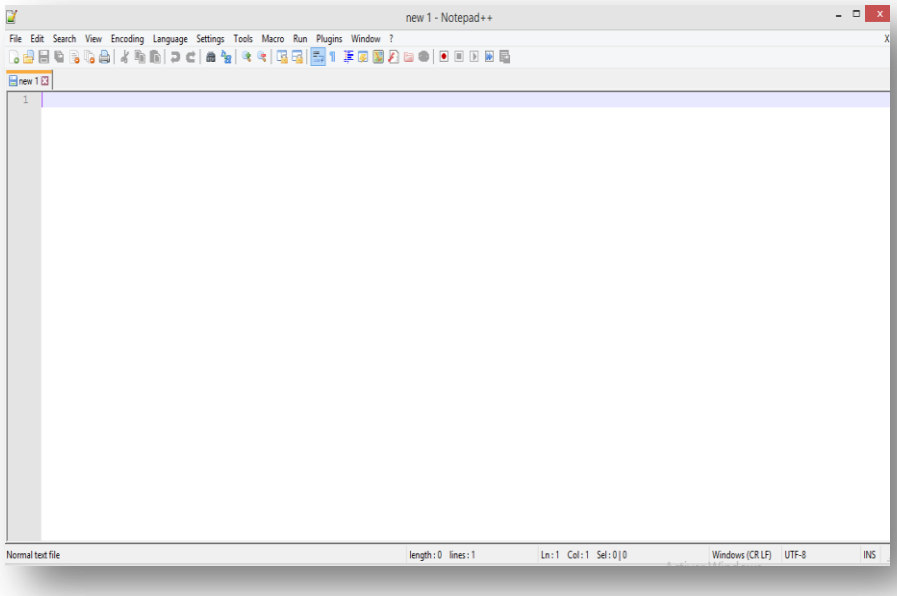


Figure 6: Note pad++ installed and run.

II. Methods

II.1 Purification and isolation of bacterial strains

II.1.2 Confirmation of bacterial

Strains purity

Fresh bacterial cultures were prepared by sub-culturing stock bacterial cultures into freshly prepared nutrient agar and incubating at 37°C for 24 hours **(figure)**.

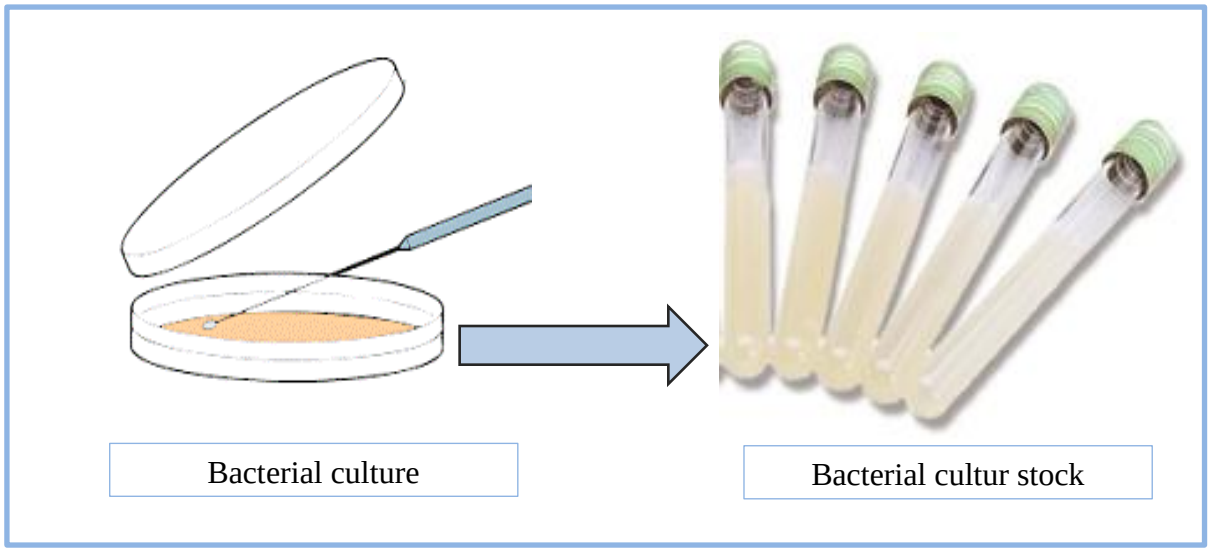
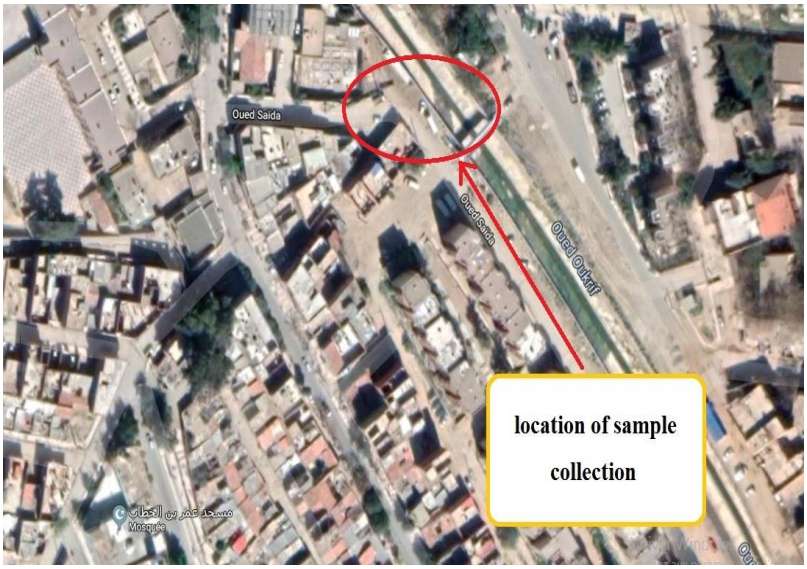


Figure 7: stock bacterial culture

II.1.2 Isolation of clostridium from municipal sewage water sample

II.1.2.1 Collection of Sample

The water sample was collected aseptically from Municipal sewage water of “graba el oued” (the colloquial name of a residential region in Saida, figure ?). Sample was collected in a sterile bottle and transported to the Saida University laboratory for identification.



This sample was stored in clean place. 1 mL of water sample was used for isolating the bacteria through serial dilution and pour plate culture technique.

II.1.2.2 Serial dilution

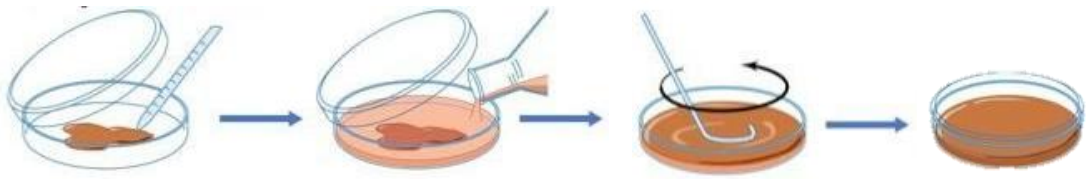
This method is based on the principle that when water sample along with bacterial colonies taken, the result is obtained in the form of reduced number of bacterial colonies which leads to obtaining pure colonies.

The collected sample has been serially diluted to 10^6 with sterile distilled water; 1mL of the initial suspension was added to 9 mL of distilled water and mixed carefully maually for 10 seconds.

Figure 8 : Serial dilution

II.1.2.3 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.
- Incubated in anaerobic jar for 24hours at 37 °C in an inverted position.
- After incubation, presence of sulfate reducing germs should show up in black colonies.
- Black colonies were identified.



Sample is pipetted into sterile plate	Sterile medium is added	Mixed well with inoculum	Medium added second time

II.1.2.4 Characterization of bacterial culture

II.1.2.4.1 Morphological studies

a. Gram staining

An important taxonomic characteristic of bacteria is their response to Gram stain.

The Gram-staining procedure begins with the application of a basic dye, crystal violet. A solution of iodine is then applied; all bacteria will be stained blue at this point in the procedure. The cells are then treated with alcohol. Gram-positive cells retain the crystal violet– iodine complex, remaining blue; gram-negative cells are completely decolorized by alcohol.

As a last step, a counterstain (basical fushin) is applied so that the decolorized gram-negative cells will take on a contrasting color; the gram-positive cells now appear purple. (**Geo et al, 2013)**

b. Shape, Size, Colour

- Shape the bacteria studied by microscopic observation; shape will be Round, Road, Coccid. Shape of the colony is identifying by observing the margin and elevation.
- Colour of the bacteria will be identifying by observing the colony.

II.1.2.4.1 Biochemical tests

a. Catalase test

Catalase test is used to detect the presence of enzyme catalase in a bacterium. The enzyme catalase catalyzes the breakdown of hydrogen peroxide with the release of free oxygen. It is found in most aerobic and facultative anaerobic bacteria.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2\uparrow$$

In this test, a small amount of culture to be tested is picked up from a nutrient agar plate with a sterile platinum loop or glass rod and this is inserted into hydrogen peroxide solution held on a slide or in a tube. Immediate production of air bubbles in the solution denotes a positive test and no bubbles indicate a negative test.

b. Oxidase test

This test determines the presence of enzyme oxidase in many bacteria. The enzyme oxidase catalyzes the oxidation of reduced cytochrome by molecular oxygen. Kovac's oxidase reagent that contains tetramethyl-p-phenylenediamine dihydrochloride is the main reagent used in the oxidase test.

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. In a positive test, the smeared area on the filter paper turns deep purple within 10 seconds. No color change indicates negative test.

c. Urease test

Urease test is used to determine the ability of an organism to split urea to ammonia by the enzyme urease. Production of ammonia makes the medium alkaline; thus the indicator phenol red changes to red or pink in color. The test is performed in Christensen's urease medium. The medium is inoculated with the bacterial colony and incubated at 37°C. Urease-positive bacteria produce a pink color.

$$\text{Urée} + 2\,H_2O \xrightarrow{\text{UREASE}} 2\,NH_4^+ + CO_3^{2-}$$

d. Indole test

Indole test is used to detect the ability of bacteria to decompose amino acid tryptophan to indole, which accumulates in the medium. Tryptophan or peptone broth is the medium used for indole test .The test is performed by inoculating the medium with bacteria, incubating at 37°C for 24hours. Then, 5 drops of Kovac's reagent 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.

e. Nitrate reduction

Nitrate reduction test is used to determine the presence of enzyme nitrate reductase in the bacteria. The enzyme reduces nitrate to nitrites or free nitrogen gas. The test is carried out by inoculating the broth containing 1% potassium nitrate (KNO3) and incubating at 37°C up to 5 days. Then 1–2 drops of a reagent that consists of a mixture of 1 mL of naphthylamine and 1 mL of sulfanilic acid is added. Red color developing within a few minutes signifies positive reaction, while absence of color indicates negative reaction. **(Parija, 2012)**

f. Mannitol motility

Mannitol Motility Test Medium is designed to differentiate bacteria on the basis of their motility and ability to ferment mannitol **(MacFaddin, 2000),** 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.

- With a sterile straight loop, touched a young colony(24h) growed on agar medium Single stab down the center of the tube to about half the depth of the medium. Incubated at 37°C **(Patricia et al, 2011).**

II.1.3 Mycobacterium tuberculosis detection :

Using the **'Programme National de Lutte Conter la Tuberculose'** protocol **(PNLCT, 2011),** the clinical diagnosis of tuberculosis is supported by laboratory diagnosis and other tests including radiographic evidence of pulmonary disease. Definitive diagnosis of tuberculosis is made by detection of M. tuberculosis from clinical specimens by microscopy or culture. **(Parija, 2009),** our case the bacteria detection was not completed / done because of indisponibility of the material.

II.1.3.1 Sputum collection

Sputum is the specimen of choice for pulmonary tuberculosis. Sputum, not saliva, is collected in the morning into a clean wide-mouthed container, such as sputum cup. Collection of morning sputum specimen is ideal.

II.1.3.2 Smear preparation

- smear is prepared directly from a fresh sample (without prior centrifugation) using an disposable loop, select and pick up the yellowish purulent particles of sputum.
- Prepare the smear in an oval shape in the centre of the slide. The smear size should be 2 cm in length and 1 wide, which will allow 100 fields to be counted in one length.
- fixed by passing the slide 3-4 times through the flame of a Bunsen burner

II.1.3.3 Ziehl-neelsen stain

Primary stain

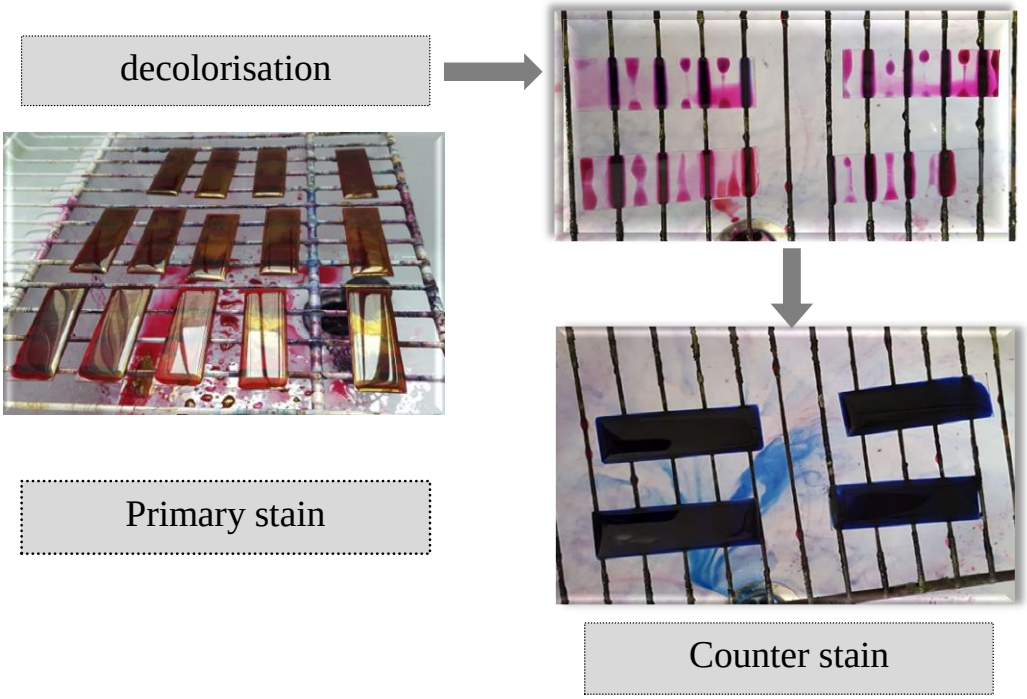
- Place the slide on staining rack and pour carbol fuschin over smear and heat gently underside of the slide by passing a flame under the rack until fumes appear (without boiling!). Do not overheat and allow it to stand for 5 minutes.
- Rinse smears with water until no color appears in the effluent.
- Allow the stain to remain on the slide to complete ten minutes. Adequate time is required for to penetrate and stain the cell well (twice for each time 3 min).

Decolorization

- Cover each slide with acid alcohol; wait eight minutes:
- Pour acid, wait for three minutes then rinse .
- Pour alcohol and wait for another five minutes.
- Rinse slides again carefully with water and tilt each slide to remove excess water.

Counter stain

- Flood the slide with the methylene blue counterstain for one minute.
- Wash off the stain with clean water and place them in a draining rack for the smear to air-dry (not blot dry).
- Examine the smear microscopically, using the 100x oil immersion objective



When acid-fast bacilli seen in a smear, they were counted . According to the number of acid-fast bacilli seen, the smears are classified as 3+, 2+, or 1+ **(Table 1)**. The greater, the number and the more infectious patient.

Table 1 : Smear Classification.

Smear interpretation	Smear Result (Number of AFB observed at 1000X magnification)	Semi-quantitative notation
negaiveNegative BAAR	0	0 BAAR
Weakly positive Precise no of BAAR/100 fields	±	1-9 BAAR/100 fields
Moderately positive (Positive BAAR)	+ or (1+)	10-99 BAAR/I100 field
Moderately positive (Positive BAAR)	++ or (2+)	1-10 BAAR / field
Strongly positive (Positive BAAR)	+++ or (3+)	10 BAAR/ field

II.1.3.4 Culture:

a. decontamination of sputum samples

- Marked the wall tube of specimen, the same number and the inoculation date writed on two tubes of media.

- 2 ml of sputum transferred into a centrifuge tube. Added a double volume of NALC-NaOH solution(4 ml). Mix for no more than 20 seconds, Centrifuge at 3000g for 15 minutes.
- Keep in centrifugion at 20–25°C for 15 minutes to decontaminate .poured the supernatant
- Fill the mix tube to within 4ml with distilled water.
- Centrifuge at 3000g for 15 minutes.
- Carefully poured off the supernatant into a discard bottle containing the appropriate disinfectant .
- Re-suspend the deposit and inoculate onto two slopes of LJ medium Using a pipette,
- Inoculate each slope with 2 drops.

b. Culture incubation

M. tuberculosis is a slow-growing bacillus with an average generation time of 14–15 hours. Prolonged incubation is therefore necessary for demonstrating growth of the bacteria. The colonies usually appear in almost 2 weeks, but sometimes require incubation up to 8 weeks to appear **(Parija, 2009)**

Inoculated solid cultures should be incubated with caps loosened in a slanted position for at least one week to ensure an even distribution of the inoculum. Caps should then be tightened to prevent desiccation of the media and, if space is needed in the incubator, the tubes can be placed upright. Tops should be tightened to minimise evaporation which can result in the media drying out.

c. Culture examination

All cultures should be examined 48 hours after inoculation in order to:

- Check absorption of liquid inoculated;
- Tighten caps to prevent drying out of media; and detect early contaminants. Cultures should then be examined on a weekly basis or, if this is not feasible, at least three times during the eightweek incubation period.
- Seven-day check: To detect rapidly growing mycobacteria.

- Three-to-four-week checks to detect positive cultures of M. tuberculosis as well as other slow-growing mycobacteria.
- End of culture check (after eight weeks) to detect very slow-growing mycobacteria, including M. tuberculosis, before discarding and reporting the culture as negative **(WHO; 2004).**

d. Confirmation test

Ziehl neelsen staining was repeated second time to confirm the presence and the purity of *Mycobacterium tuberculosis* **(Figure 9).**

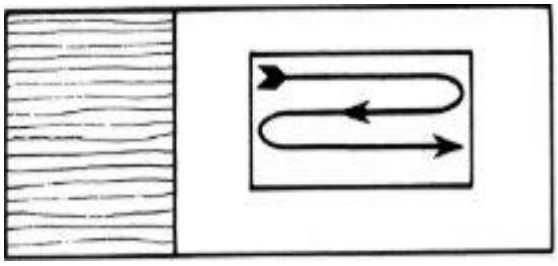


Figure 9 :Examining Slide.

II.2 Antibiotic sensitivity test method (The Kirby-Bauer disc diffusion):

This method was used to test antibacterial resistance. A total of 14 antibacterial agents belonging to 7 antibacterial classes **(Table 2).**

Table 2: 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
	Gentamicine	Cn (10UI)/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

All bacteria for which the evaluation of antibacterial activity are shown in the below table (**Table 3)**. As mentioned eralier mycobacterium tuberculosis is not shown as is not part of the experiments carried out in this project.

Table 3 : Bacterial strains used to evaluate antibacterial activity.

Tested			**References**
Bacteria	Gram (+)	*Staphylococcus aureus*	ATCC 25923
		Clostridium difficile /spp	*Isolated*
	Gram (-)	*Pseudomonas aeruginosa*	ATCC 27853
		Klebsiella pneumoniae	ATCC 70603
		Escherichia coli	ATCC 25922

Mueller-Hinton agar was prepared according to the manufacturer‟s protocol (liofilchem srl Zona Ind.Le- Roseto d. Abruzi (TE) – ITALY).

The bacteria were purified on nutrient agar. Using a sterile, round-wire inoculating loop, a loop-full of the bacterial colonies was collected and standerized using the spectrophotometer to reach the final concentration of 10^6 CFU/ml.

- This was then streaked onto the surface of the Muller Hinton agar plate until its surface was thoroughly covered, under Bunsen flame to ensure a sterile environment. Using a pair of sterile forceps, the antibiotic discs were removed from

the dispensers, and thengently placed on the agar, making sure each disc was fixed on agar surface.

- The discs were placed equidistant from each other with only four antibiotic discs placed per plate to ensure clarity of results. The plates were then placed upside down and incubated for 24hrs at 37°C **(NCCLS, 2000**).
- The sensitivity of each isolate was then read by measuring the clear, circular diameter around each disc. These results were recorded in millimetres and later classified as susceptible, intermediate and resistant.

II.3. *Annona cherimoya* seeds activity:

II.3.1 Preparation of seeds extracts

Extraction is the crucial first step in the analysis of medicinal plants, because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization. **(Fabricant and Farnsworth, 2001)**

II.3.1.1 Maceration

Maceration is a method that commonly used for extraction of bioactive components from natural products.This extraction method was chosen for the first preliminary study because of its simplicity and manageability **(Figure 10)**.

a. Methanolic extract

10g of seeds powder were macerated with 100 ml methanol at room temperature for 2 4 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.

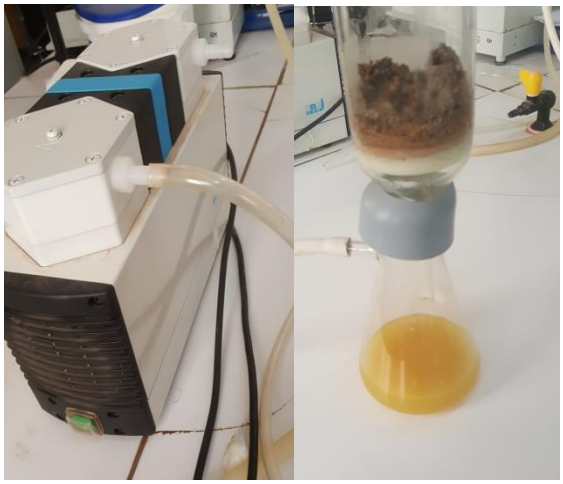
b. Hydromethanolic Extract

10 g of the dry fruit seeds was weighed and extracted with 80% methanol and distilled water (80ml methanol / 20ml 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 10g of the powdered material was leached in 100 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.

Maceration



Filtration

Evaporation

Figure 10: Extraction by maceration method.

II.3.1.2 Decoction

The dried form of powder was mixed with sterile distilled water (10 g powder in 100 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 **(Figure 11)**

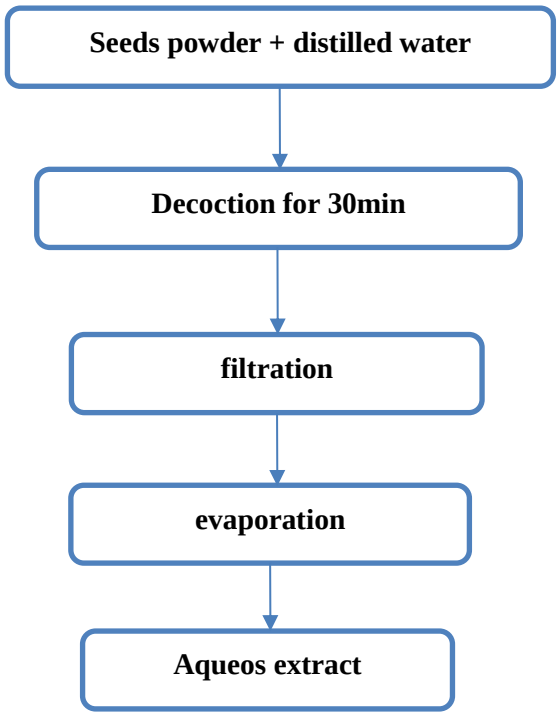


Figure 11: Decoction method main steps.

II.3.2 The extraction yield

The final dry weight was used to calculate extraction yield.

The extraction yield (%) was expressed as shown in equation bellow suggested by **(Walsh et al.2003).**

Extract yield % = W1 / W2 × 100

W1 = Net weight of powder in grams after extraction

W2 = Total weight of wood powder in grams taken for extraction.

II.3.3.1 Phytochemical Screening

Phytochemical Analysis of *Annona cherimoya* seeds powder obtained was subjected to qualitative analysis for the presence of Phenolic groups, Glycosides, Alkaloids, Flavonoids, Tannins, Terpenoids, Saponins, Oils and gums as described by the method of **(kokate , 2005), (Oloyede, 2005)** as specified in the book of Practical Pharmacognosy.

II.3.3.2 Detection of Alkaloids:

- 1g of powder 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.

II.3.3.3 Detection of Tannins: A small quantity of powder 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.

II.3.3.4 Detection of Anthocyanins : The presence of anthocyanins has been demonstrated by adding 2 mL of the plant 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.

II.3.3.5 Detection of Flavonoids

1 g of the plant powder 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.

II.3.3.6 Detection of Quinones

2g of powder 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.

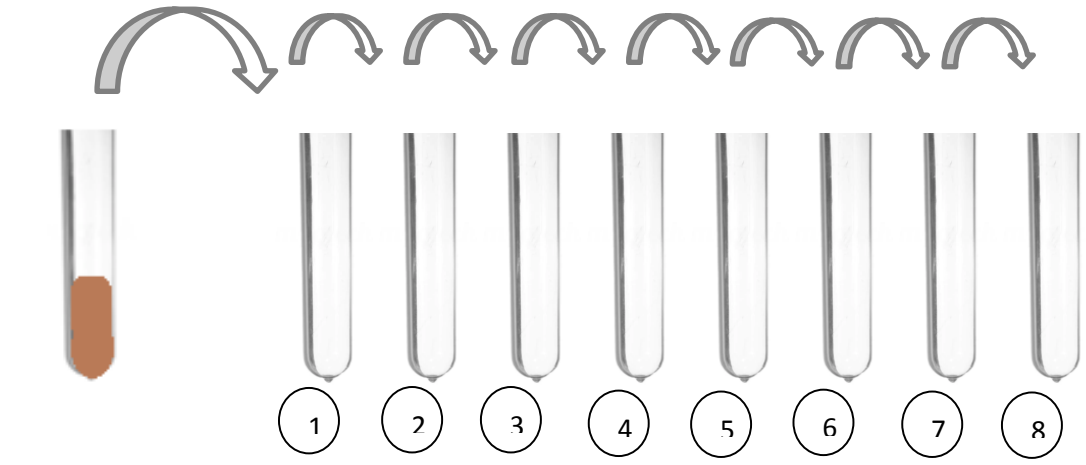
II.4 Antibacterial activity

In this study, we determined antimicrobial activity using various concentrations of different extracts of cherimoya seeds against Gram-positive bacteria (staphylococcus aureus) and gram-negative bacteria (Pseudomonas aeroginosa, Escherichia coli, klebseilla pneumoniae). For these studies, one bacterial sample were collected from sewage water (clostridium dificille). The bacterial sample were identified and screened on the basis of Gram staining method . All these antimicrobial studies were conducted under CLSI guidelines.

II.4.1 Disc diffusion method

II.4.1.1 Seeds Extracts dilution

For dilution preparation 1g of each extract was dissolved in 2ml dimethylsulpoxide (DMSO) and agitated then filtered by using a sterile 0.45 millipore filter to obtain 8 serial concentrations .Negative control used was DMSO.



The first concentration

1 ml transfered from each tubes to the other serially

A total of four exracts were produced and used in the antibacterial activity testing of cherimoya seeds and are listed with labels given to each in the following table (**Table 4)**.

Table 4. The final four extracts used in the antibacterial activity testing of cherimoya seeds.

The Four Extract Types	**Lables**
Hydromethanolic extract	HME
Methanolic extract	ME
Aqueous extract (maceration) - Cold	HEC
Aqueous extract (decoction) - Infusion	HEI

II.4.1.2 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).

II.4.1.3 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).

II.4.1.4 Screening of Antimicrobial activity

- 10ml of sterilized Mueller-Hinton agar medium were poured into the each sterile petridish.
- After solidification, the sterile cotton swab was dipped into the culture or broth of *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Staphylococcus aureus* and *Clostridium dificile*.
- 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.

- 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.

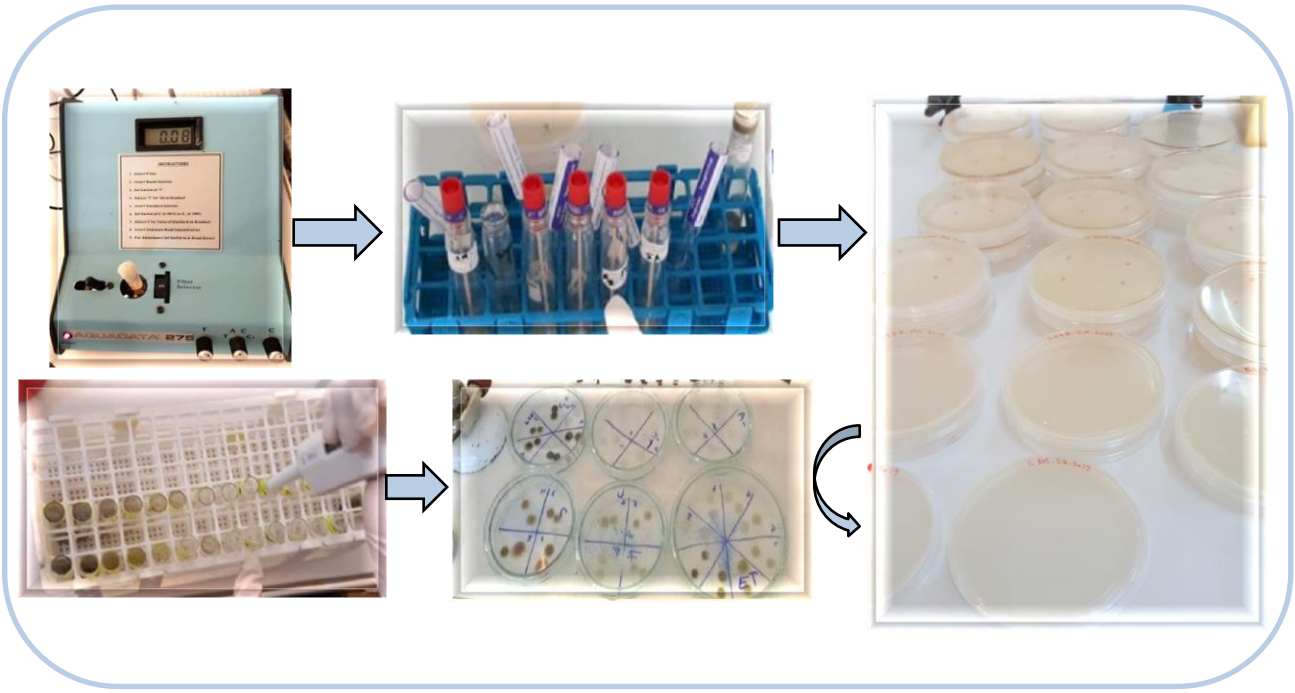


Figure 12: discs diffusion method.

II.4.2 Minimum Inhibitory Concentration assay (MIC)

MIC is the lowest concentration of a compound that inhibits microbial growth. MIC can be determined by agar dilution or broth dilution method usually following the guidelines of a reference body such as the Clinical and Laboratory Standard Institute (CLSI), British Society for Antimicrobial Chemotherapy (BSAC) or European Committee of Antimicrobial Susceptibility Testing (EUCAST).

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

⇒ Column 11 contained 50 µl of standardised inoculum and Column 12 contained 100 µl of the medium broth (as a control to monitor sterility),

⇒ 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.

⇒ The tested concentrations of the different extracts achieved through serial dilutions from columns 10–1.

⇒ 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.

⇒ After incubation for 24 h at 37 °C, microplates read with spectrophotometer of ELISA type.

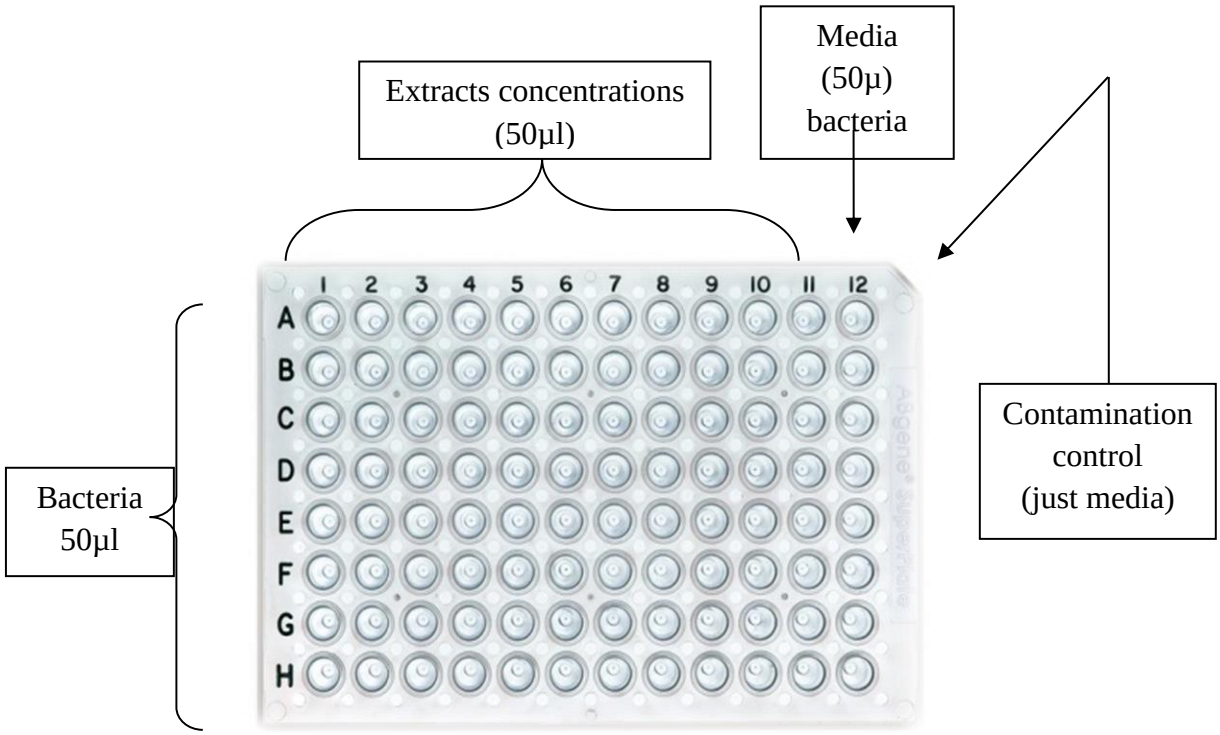


Figure 13: Minimum Inhibitory Concentration (micro dilution method).

II.5 Bioinformatics Tools and Methods:

The database contained a number of tables with Primary Keys to insure data integrity and inter-related to each other, when necessary, via the implementation of Foreign Keys to facilitate the find out of relationships between the different types of data.

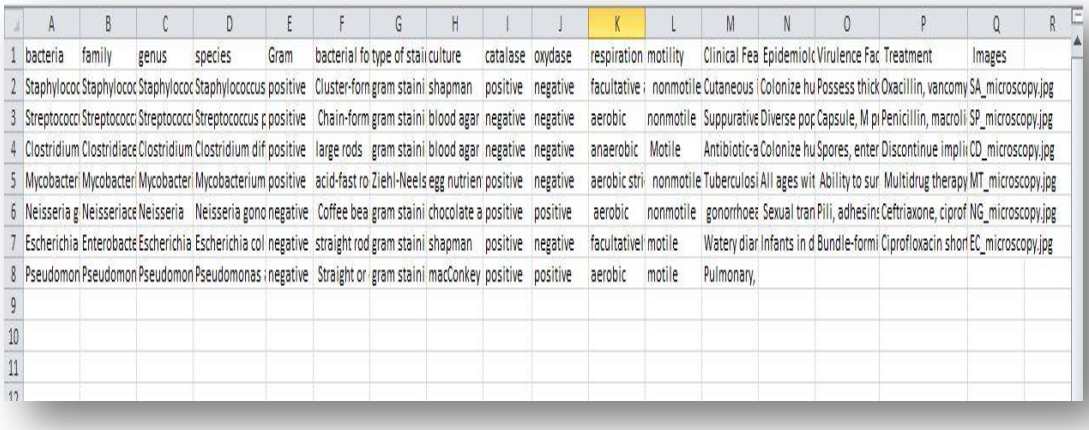
The database work space which has been generically named as "BARID" (Bacterial Antibiotics Resistance Investigation Database) and related tables were all created using PHP and SQL script codes specifically written during the project development.

The project generated a fair amount of data both quantity, types and quality which needed annotation, story and relationship identification between the different types of data; bacterial tests, antibiotic tests and antibacterial effects of cherimoya extracts. To achieve such goals data needed preparation, database storing and data querying. Data preparation & In-house Database Schema creation:

An In-house Database, which is a localized production relational database, was created to develop the framework for the project management and development.

II.5.1 Data Preparation & Flat-files creation

Data files were created using the Excel 2010. The CSV files were also further processed for editing and corrections purposes using the Notepad++ software. The figures below show examples of the Excel and Notepad++ files.



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
1	bacteria	family	genus	species	Gram	bacterial fo	type of stai	culture	catalase	oxydase	respiration	motility	Clinical Fea	Epidemiolc	Virulence Fac	Treatment	Images	
2	Staphylococ	Staphylococ	Staphylococ	Staphylococcus	positive	Cluster-forn	gram staini	shapman	positive	negative	facultative	nonmotile	Cutaneous i	Colonize hu	Possess thick	Oxacillin, vancomy	SA_microscopy.jpg	
3	Streptococci	Streptococci	Streptococci	Streptococcus p	positive	Chain-form	gram staini	blood agar	negative	negative	aerobic	nonmotile	Suppurative	Diverse pop	Capsule, M pi	Penicillin, macroli	SP_microscopy.jpg	
4	Clostridium	Clostridiace	Clostridium	Clostridium dif	positive	large rods	gram staini	blood agar	negative	negative	anaerobic	Motile	Antibiotic-a	Colonize hu	Spores, enter	Discontinue impli	CD_microscopy.jpg	
5	Mycobacteri	Mycobacteri	Mycobacteri	Mycobacterium	positive	acid-fast ro	Ziehl-Neels	egg nutrien	positive	negative	aerobic stri	nonmotile	Tuberculosi	All ages wit	Ability to sur	Multidrug therapy	MT_microscopy.jpg	
6	Neisseria g	Neisseriace	Neisseria	Neisseria gono	negative	Coffee bea	gram staini	chocolate a	positive	positive	aerobic	nonmotile	gonorrhoea	Sexual tran	Pili, adhesins	Ceftriaxone, ciprof	NG_microscopy.jpg	
7	Escherichia	Enterobacte	Escherichia	Escherichia col	negative	straight rod	gram staini	shapman	positive	negative	facultativel	motile	Watery diar	Infants in d	Bundle-formi	Ciprofloxacin short	EC_microscopy.jpg	
8	Pseudomon	Pseudomon	Pseudomon	Pseudomonas	negative	Straight or	gram staini	macConkey	positive	positive	aerobic	motile	Pulmonary,					
9																		
10																		
11																		
12																		

Figure 14: Bacteria card data in Excel.

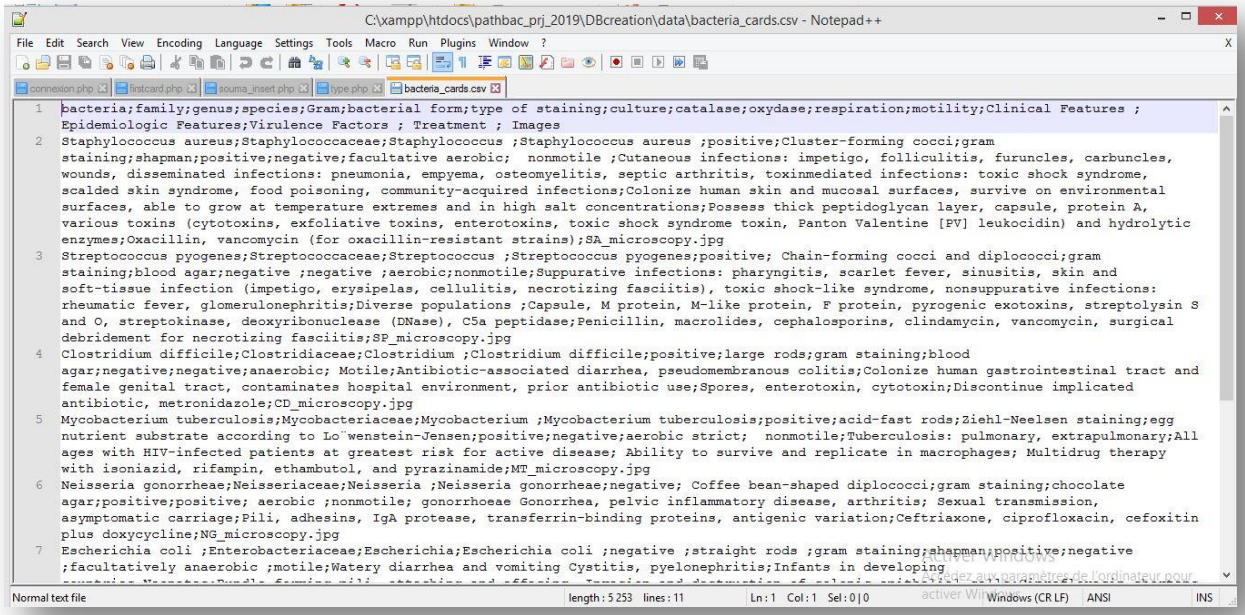


Figure 15: Bacteria card data edited with Notepad++.

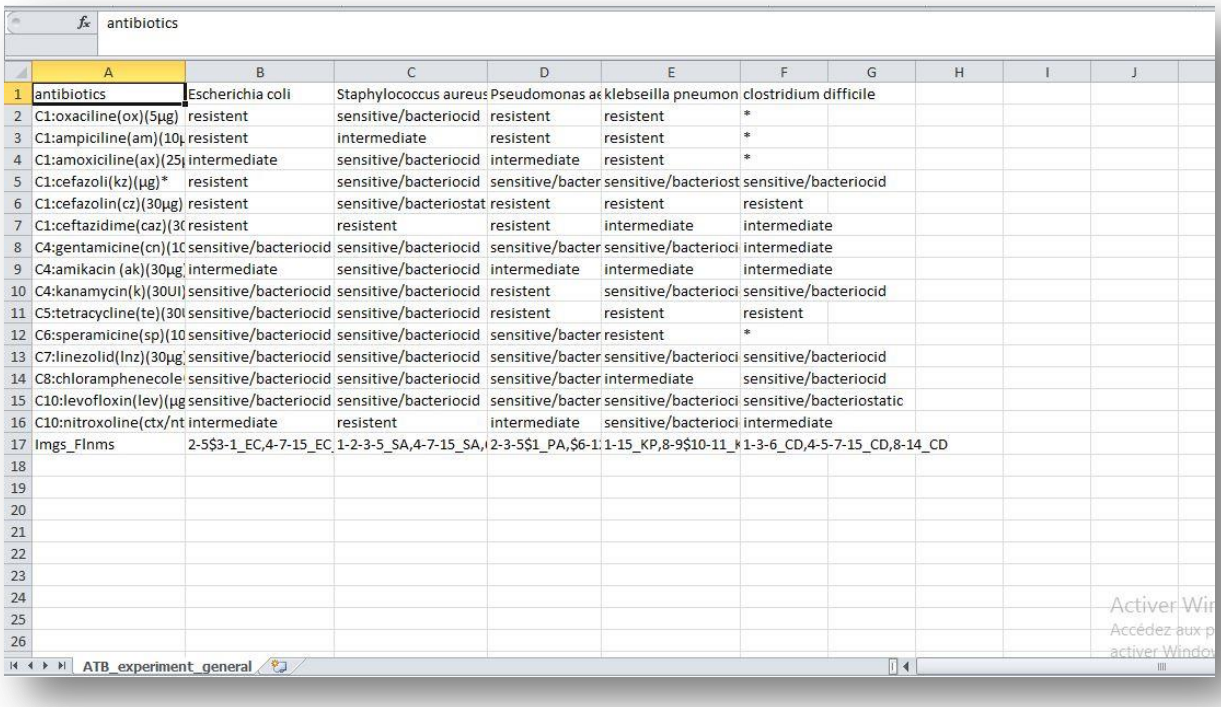


Figure 16: Antibiotics data table in Excel.

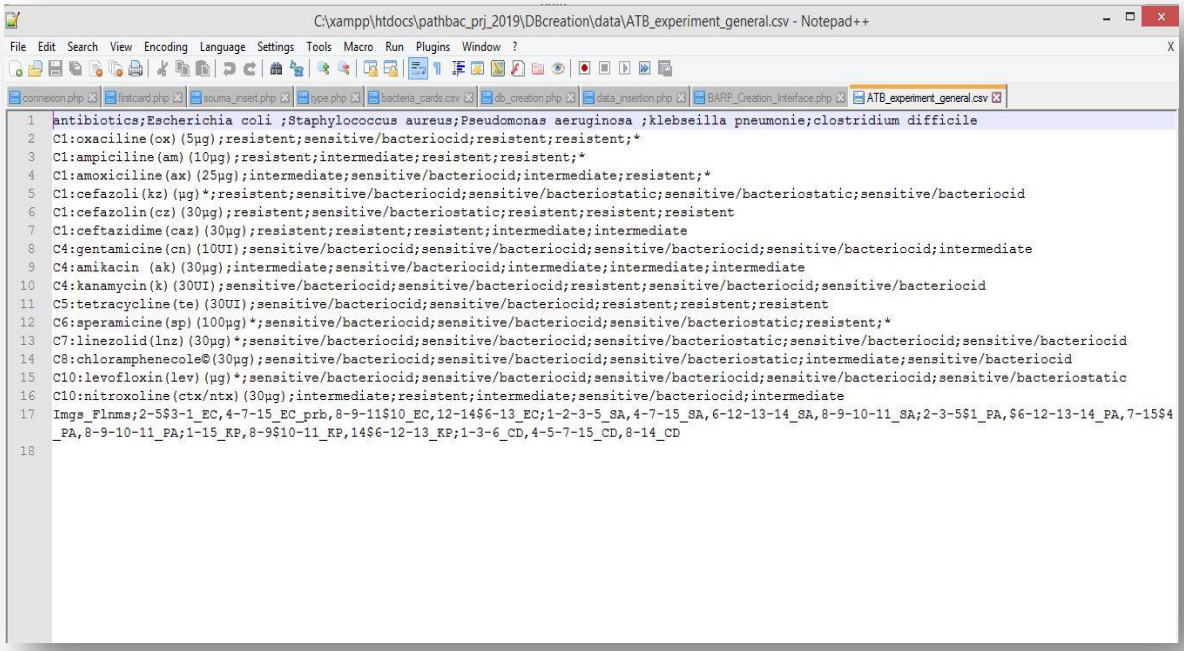


Figure 17: Antibiotic data table in Notepad++.

Data types included also the types of Cherimoya seeds extract shown in **Table 4** (above section of this chapter)

II.5.2 In-House Database and related Tables creation

The phpMyAdmin system allows for the creation of databases though the use of specific menu tools, see **(Figure 18)**. However, this manual method (click-through procedures) is not convenient when creating complex and dynamically updated

databases.

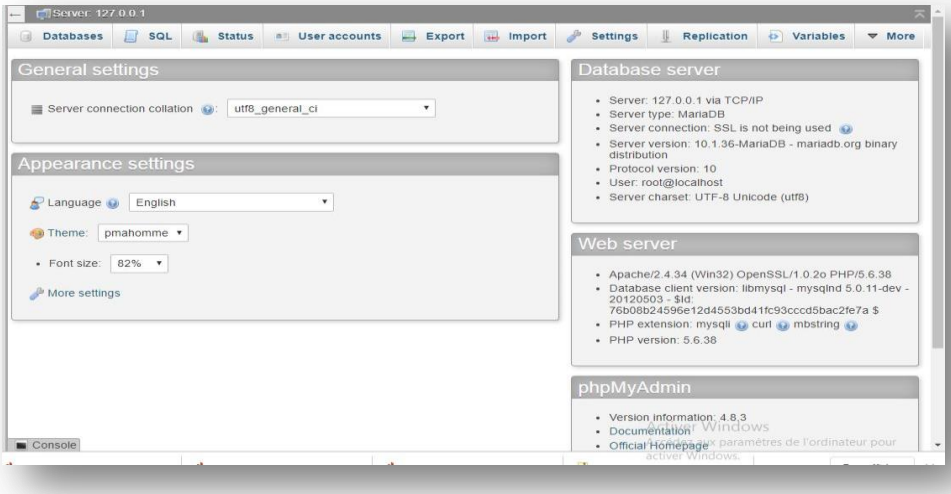


Figure 18: The interface of PhpMyAdmin tool which allows click-through creation of databases**.**

Throughout the project, the database and tables together with data insertion has been done via PHP and SQL programming though code modules each written for relevant tasks. Refer to the following figures for show example about some of the PHP codes.

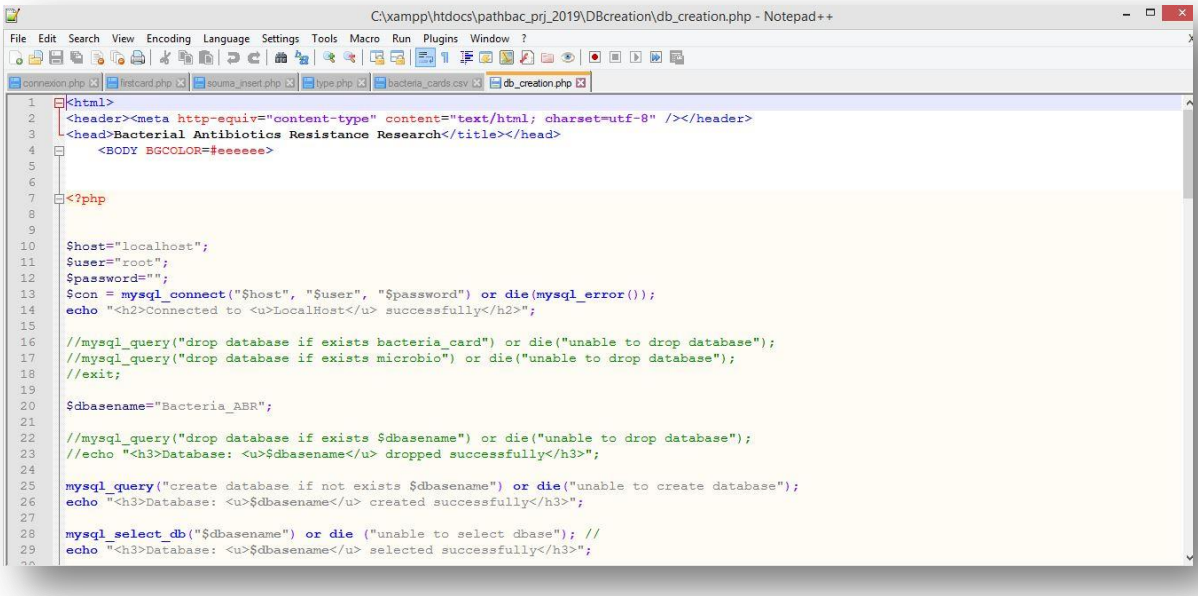


Figure 19: Depiction of partial PHP/SQL script codes used to create the database and tables Schema.

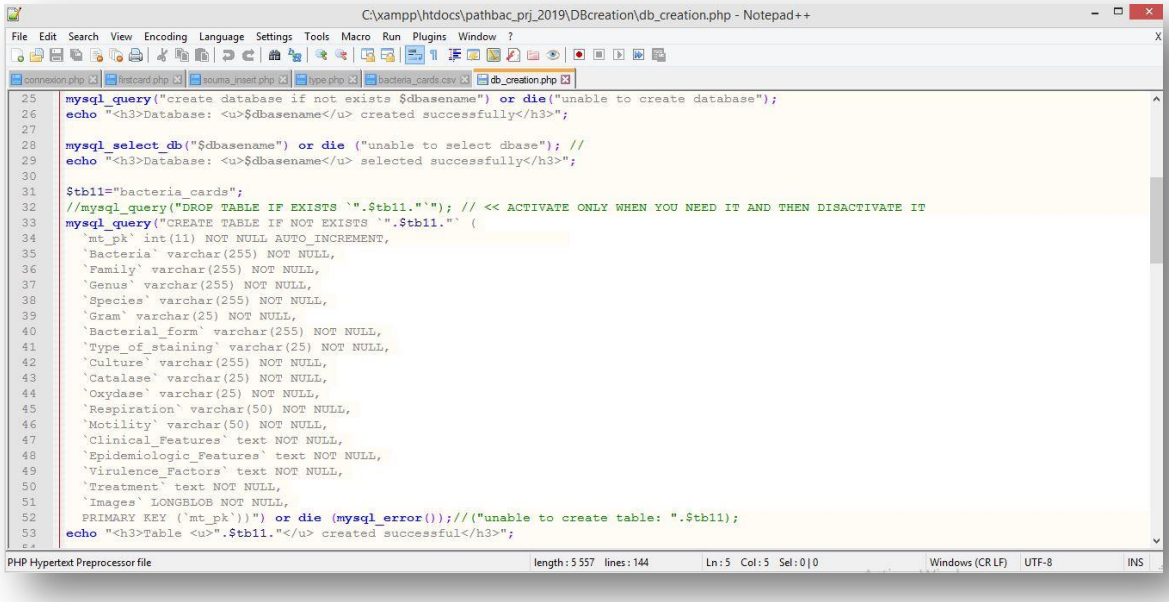


Figure 20 : Add data into data base with database validation.

II.5.3 Data insert into the database

The prepared data were inserted into each the created tables using SQL scripts via PHP coding; the SQL statement **INSERT INTO**, for example, is used to insert new rows of data in a database tables by calling the function "**mysql_query()**" through a PHP script, **(see figures below)**.

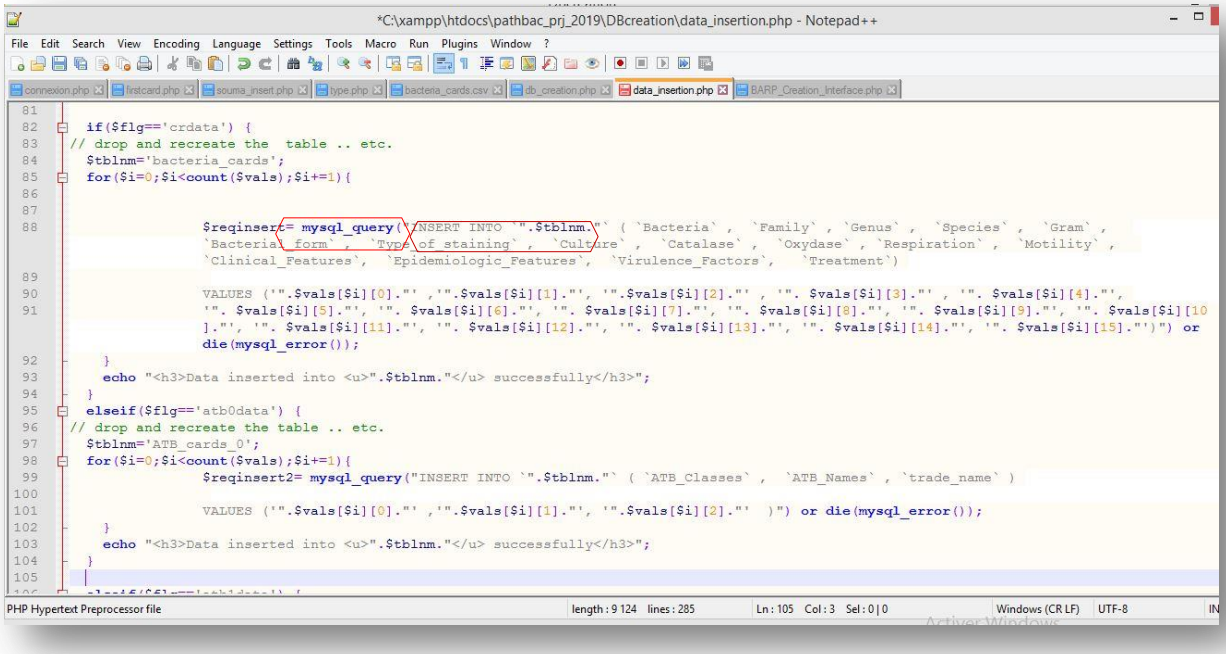


Figure 21: Depiction of PHP script with functions that implements SQL statements (highlighted in red) for data insertion.

After execution of PHP scripts, such as the example depicted in **Figure 21** , data will be inserted in the relevant tables and can be examined manually using the phpMyadmin interface, **Figure 22**

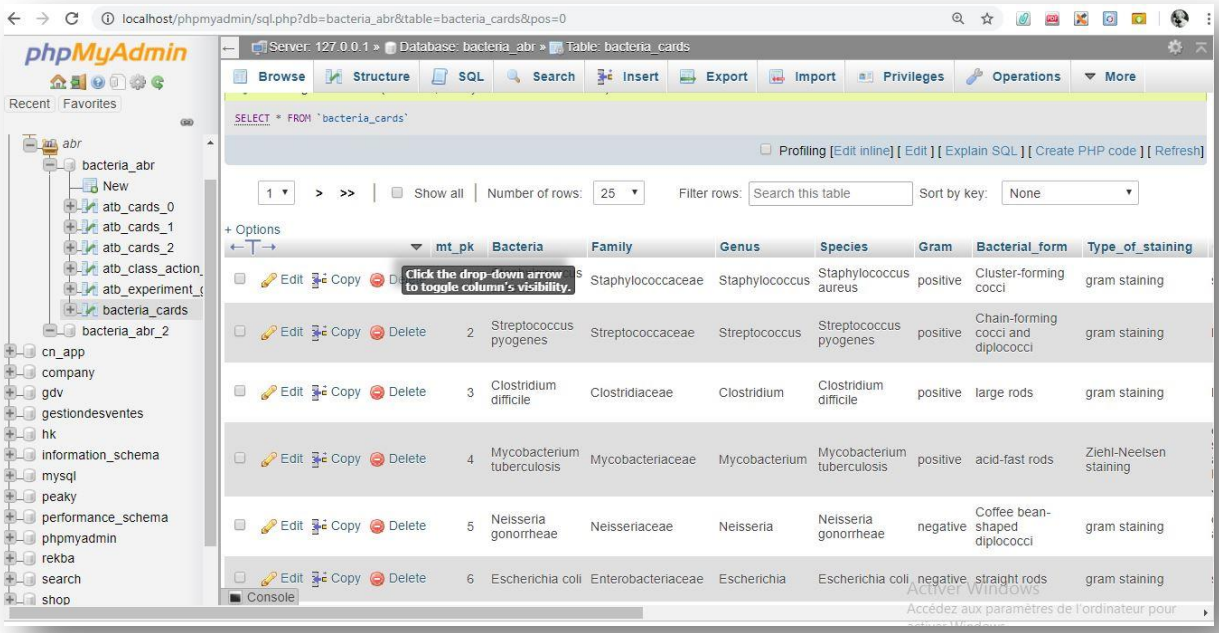


Figure 22: A depiction of a database table showing its content in **phpMyAdmin** after data insertion step.

II.5.2 Operational (Online) Database Creation:

After the production database (the In-house database) was successfully created and relevant data stored. The next step was to create an operational database which can be used to **query** and **retrieve** results in meaningful presentation with proper linkage of query results for the maximum benefit from the database and the valuable data stored there in.

This step was beyond the-time frame for the student to learn and implement and hence was done by the supervisor of this project Dr. Abdelkrim Rachedi.

The database was installed on an online server maintained by the supervisor and was given the web-address as follows:

http://bioinformaticstools.org/prjs/barid (see **Figure 23** below)

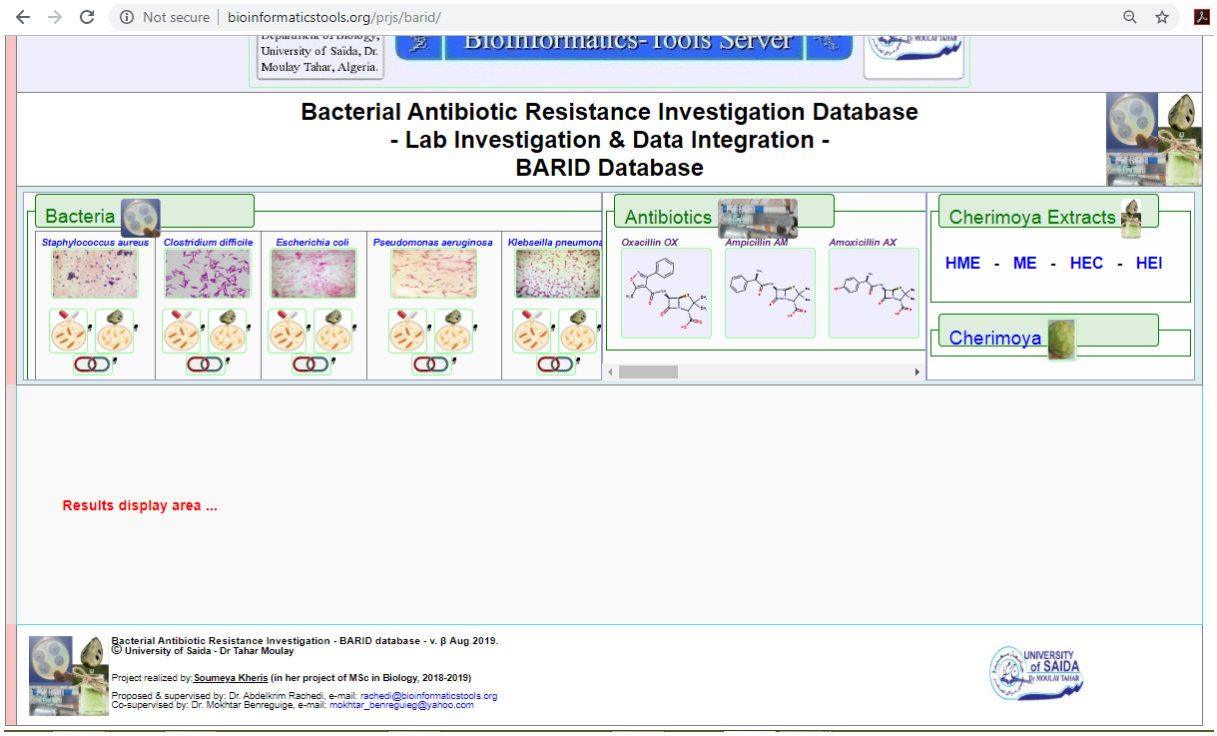


Figure 23. The BARID database main interface page.

Refer to next chapter, **Results & Discussion**, for more details on the BARID database system.

CHAPTER III : Results and Discussion

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

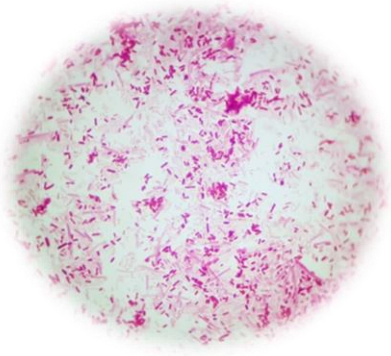
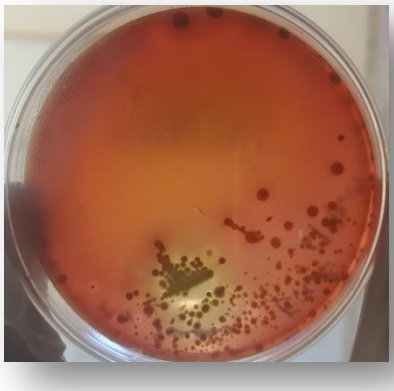
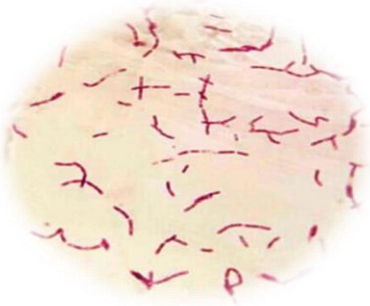
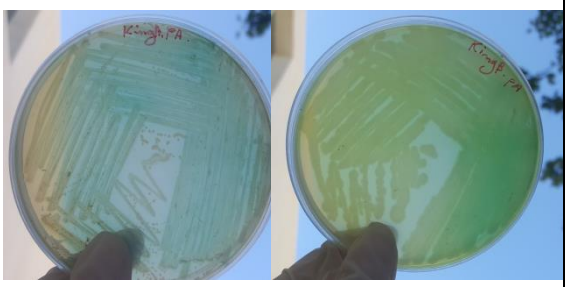
The first part is related to the wet-laboratory research work which includes Bacteriology, Antibiotics and Anti-bacterial activity of the Cherimoya seeds' extracts experiments. The second part exposes the bioinformatics related results as obtained from the annotation of the data generated by the first part.

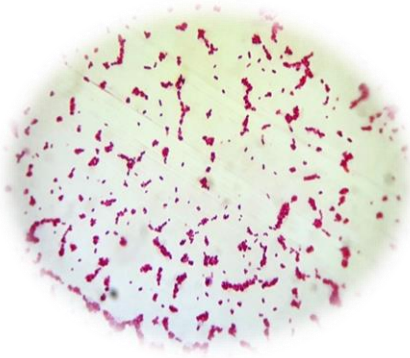
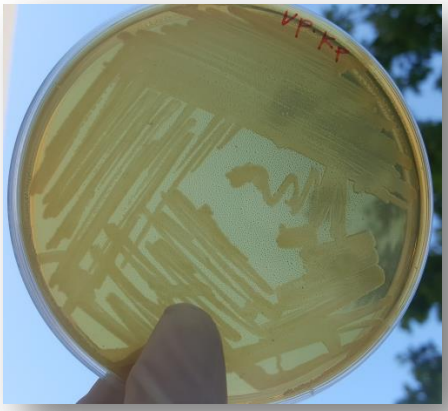
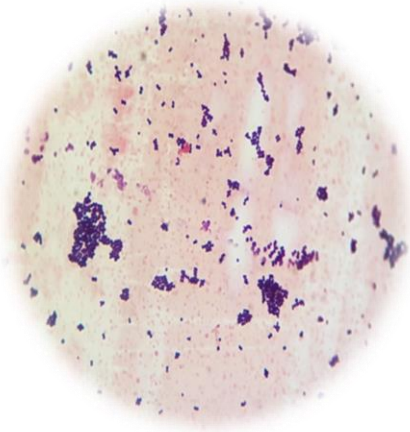
I. Wet-laboratory Experiments Results

I.1 Purification and isolation of bacterial strains

I.1.1 Confirmation of bacterial strains purity

Table 1: Purification and confirmation of bacterial strains used

bacteria	Gram	Culture
E. coli		
P. aeroginosa		

K. pneumoniae		
S. aureus		

I.1.2 Clostridium isolation

Table 2 : Characterization of bacterial culture **.**

Morphological studies	**Result**	**Observation**
Gram staining	Positive	

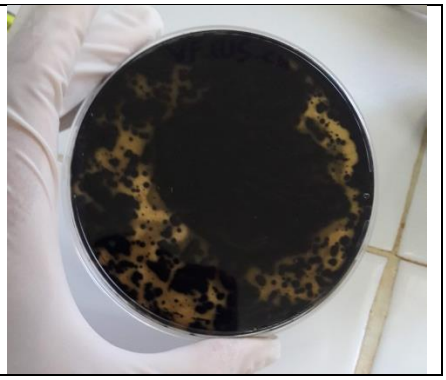
Culture	Presence of black colonies in meat liver agar indicate sulfite reducing germs presence	

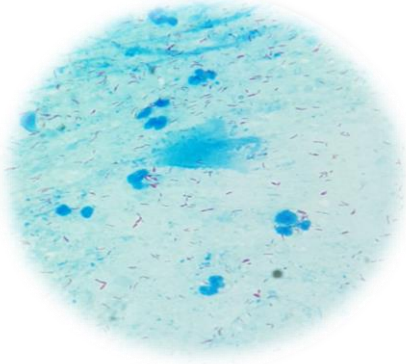
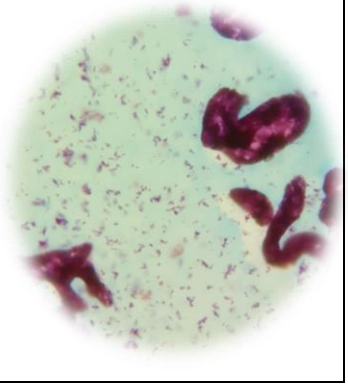
Table 3: biochemical tests results of *Clostridium difficile*.

Biochemical tests	**Observation**	**Results**
Catalase test	no bubbles observed.	Negative
Oxidase test	No color change change observed	Negative
Mannitol motility	Observation of bacteria diffused growth throughout the medium wich indicates it's motility. changing of the media color from the red to yellow indicats fermentation of mannitol	Positive
Nitrate reduction	absence of color	Negative
Indole test	No color change	Negative
Urease test	Absence of pink color observed	Negative

I.1.3 *Mycobacterium tuberculosis* detection:

Ziehl-neelsen results of stain Smear taken from sputum sample and the culture showed in **Table 4.**

Table 4 : morphologic study of *Mycobacterium tuberculosis* .

Ziehl-neelsen stain	**Culture**	**Confirmation test**
		

I.2 Antibiotic sensitivity test method (The Kirby-Bauer disc diffusion):

KB tests are performed under standardized conditions and standard-sized zones of inhibition have been established for each antibiotic. KB test results are usually reported as sensitive, intermediate, or resistant, based on the size of the zone of inhibition. If the observed zone of inhibition is greater than or equal to the size of the standard zone, the bacteria is considered to be sensitive to the antibiotic. Conversely, if the observed zone of inhibition is smaller than the standard size, the microorganism is considered to be resistant.

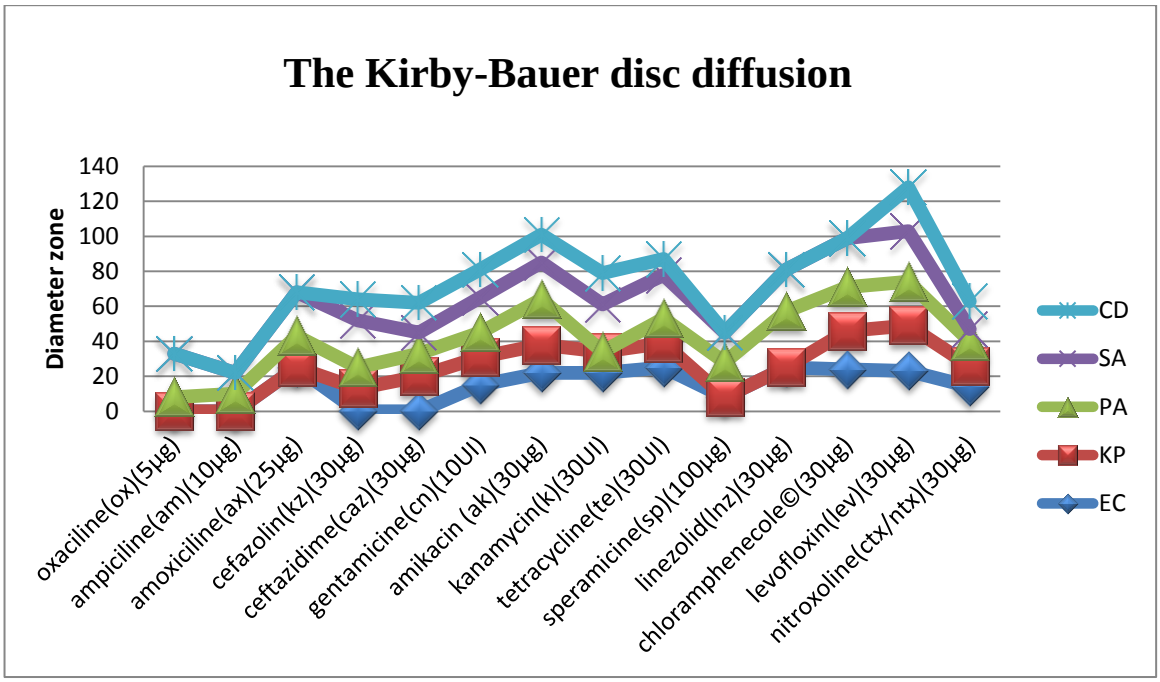


Figure 1 : Results of antibiotic sensitivity test method on the tested bacteria.

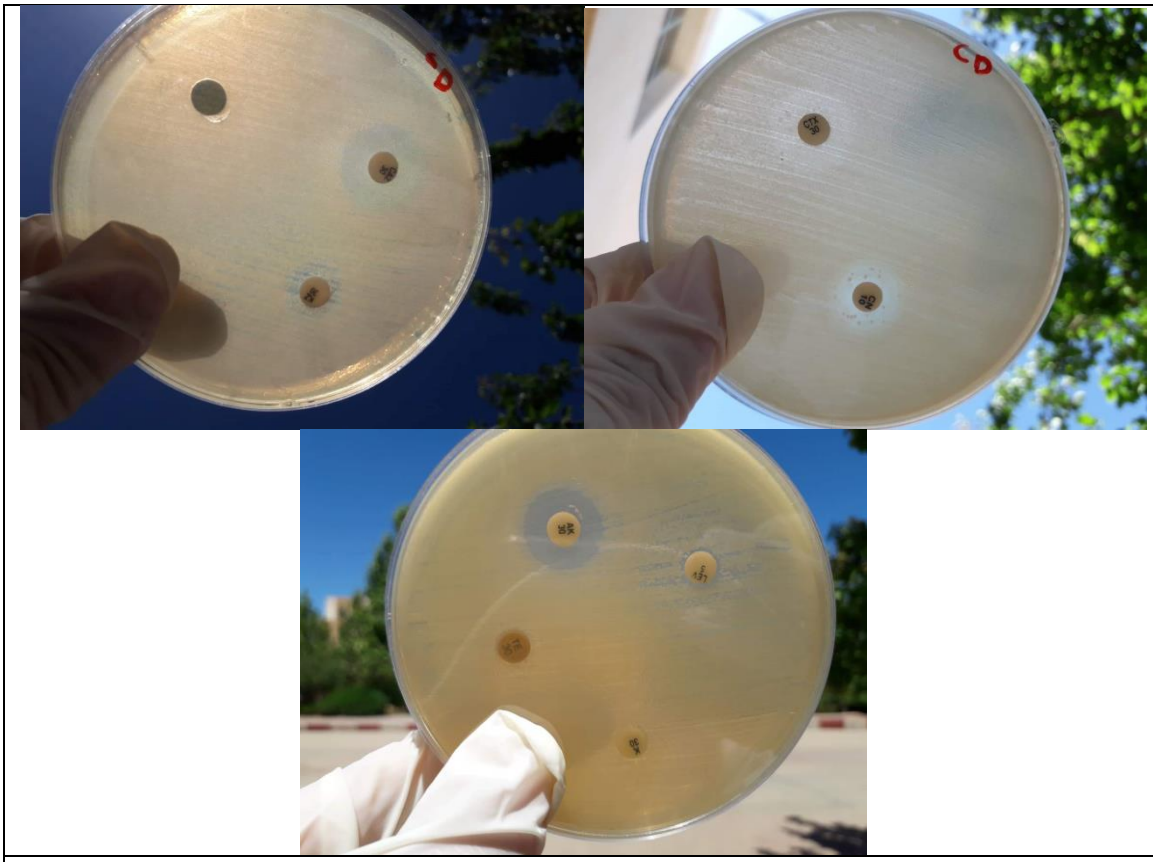


Figure 2: Results of antibiotic sensitivity test on *C.difficile.*

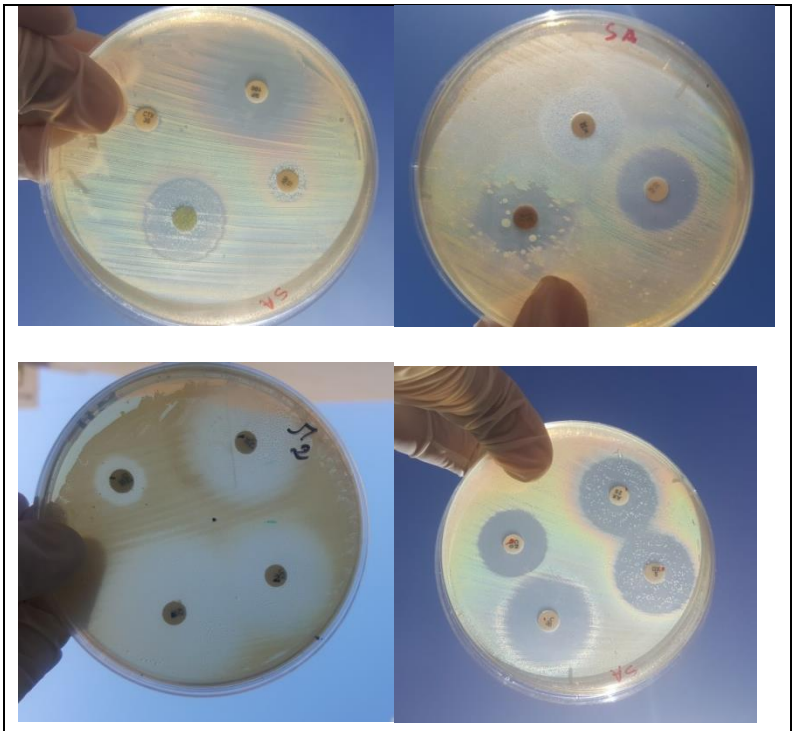


Figure 3: Results of antibiotic sensitivity test on *S.aureus*.

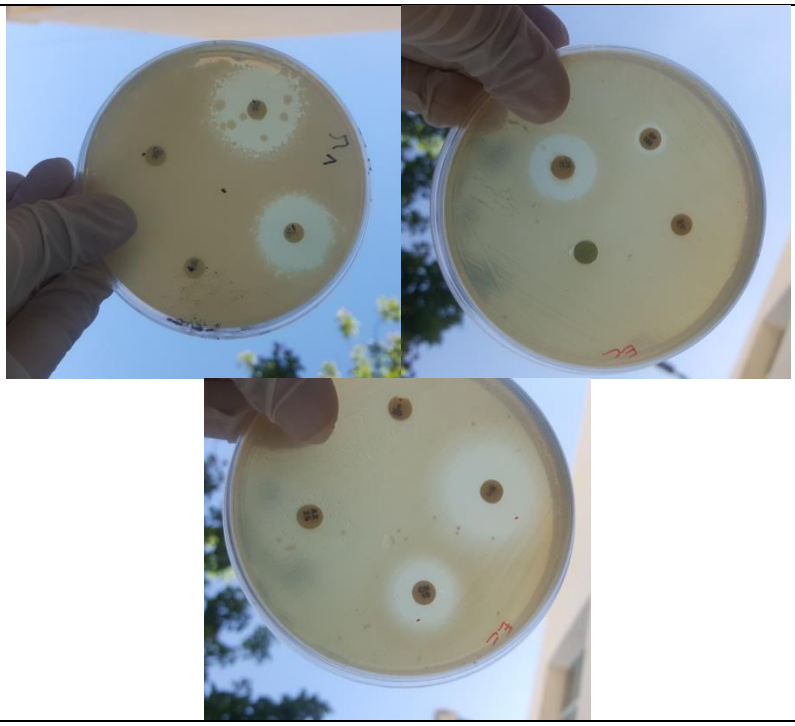


Figure 4: Results of antibiotic sensitivity test on *E.coli*

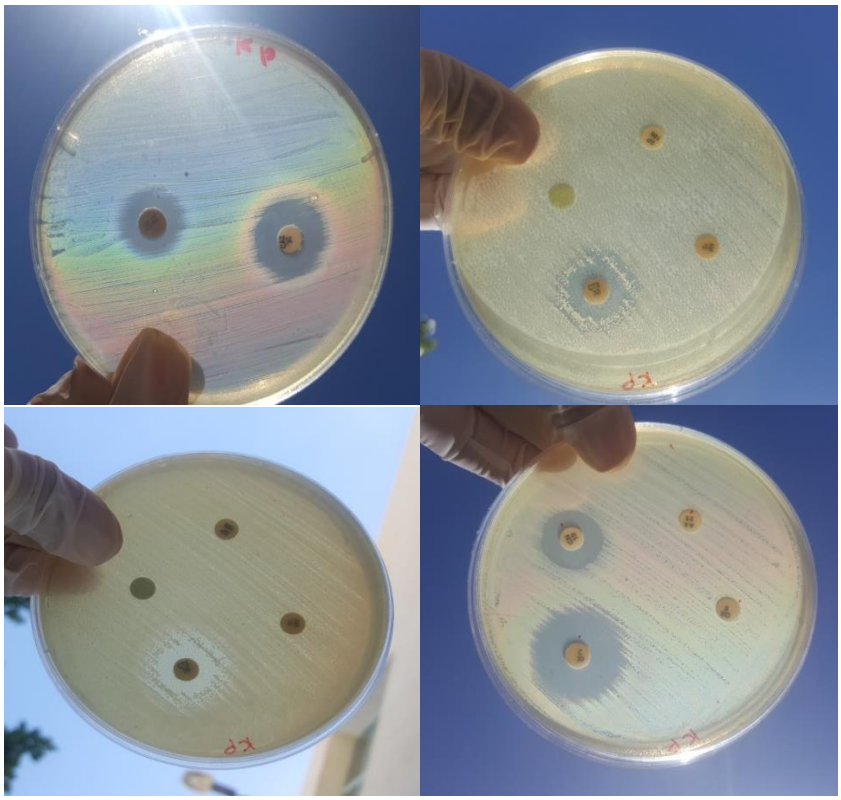


Figure 5 : Results of antibiotic sensitivity test on *K.pneumoniae*

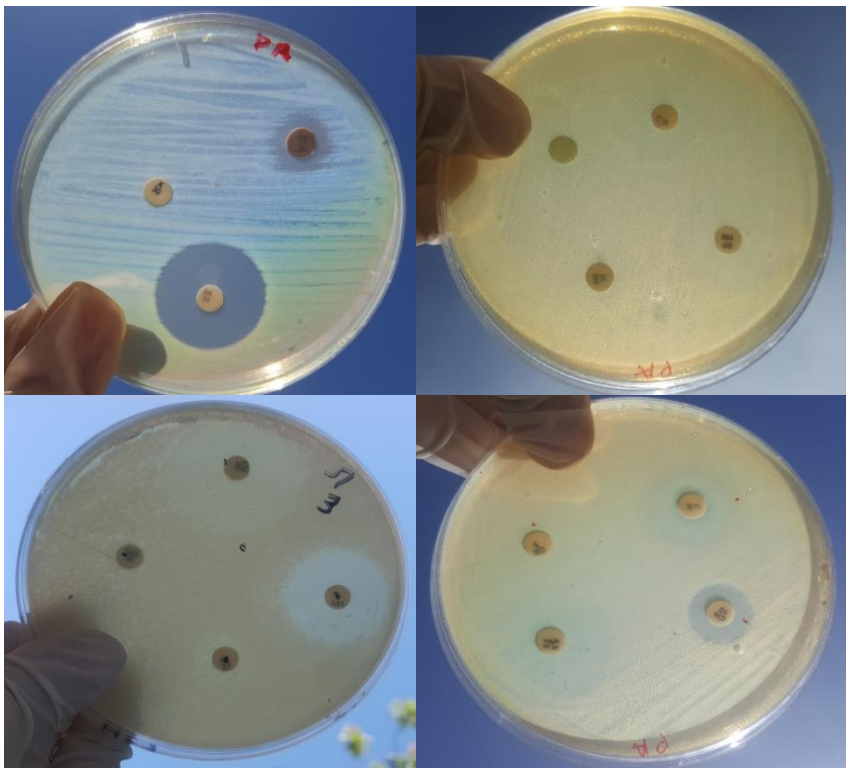


Figure 6 : Results of antibiotic sensitivity test on *P.aeroginosa*

I.3 Cherimoya seeds activity

I.3.1 Extraction yield

The yields obtained for different extracts are shown in **Table 5** and **Figure 7**.

Table 5 : different yields extraction results.

Extract	Solvent	Yield extract
Methanolic extract	Methanol	5.12%
Aqueous extract (decoction)	Distilled water	3.53%
Hydrometahnolic extract	Methanol/ water	3.43%
Aqueous extract (maceration)	Distilled water	3%

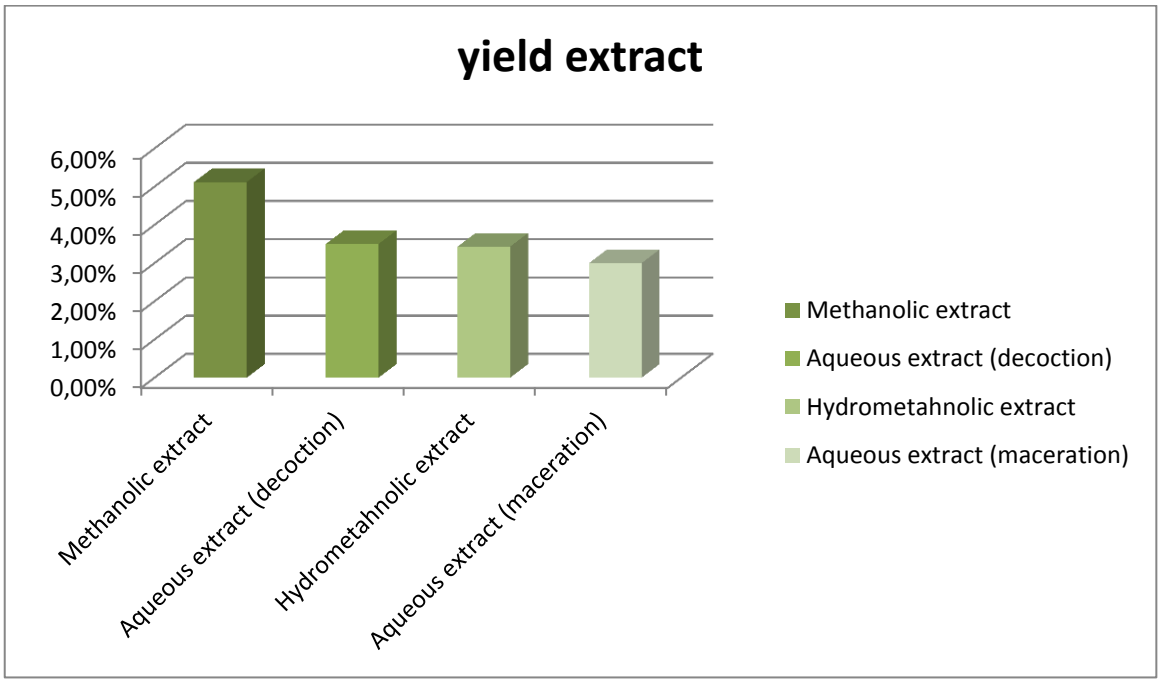


Figure 7: The yield amounts variation resulted from the different processes of extraction.

The four types of extracts studied show substantially similar yields, the highest yield was obtained from methanolic extract of 5.12% while the least yield was of 3%.

I.3.2 Phytochemicals screening

The phytochemical screening of *Anonna cherimola mil* seeds powder revealed the presence of secondary metabolite such as alkaloids, shown in (**Table 6)**.

Table 6 : showed results of phytochemical screening.

Tests		interpretation	results	Obsevation
Alkaloids	Mayer's test	Formation of a white/ cream precipitate indicates the presence of alkaloids.	+++	
	Wagner's test	brown/ reddish brown precipitate indicates the presence of alkaloids.	+++	
	Dragendorff 's test	Formation of orange precipitate indicates the presence of alkaloids.	+++	

Phynoliic componds	Flavonoid	No transformation to red /pink color indicates the absence of flavonoid	-	
Phynoliic componds	Tannins	Absence of dark green color formation indicates the absence of tannins.	-	
	Anthocine		-	
Quinons		Absence of red/ purple color indicates the absence of Quinons	-	

The phytochemical compounds detected are known to have medicinal importance. For example, alkaloids have been reported as powerful poison, and many alkaloids derived from medicinal plants show biological activities such as anti-inflammatory **(Souto, 2011)**,

antimalarial **(Dua VK et al., 2013)**, antimicrobial, cytotoxicity, antispasmodic, and pharmacological effects **(Ameyaw et al., 2009**, **Thite et al., 2013).**

I.3.3 Antibacterial activity

I.3.3.1 Disc diffusion method:

Evaluation of the antibacterial activity of the four different extracts from *anonna cherimola mil* seeds was determined initially by the disc diffusion method against the three gram negative bacteria (*E.coli, P.aeroginosa, K.pneumoniae*) and two gram positive (*S.aureus, C.difficile).*

These organisms were frequently encountered in infectious diseases. This study showed that almost all of the extracts exhibited a varying degree of antibacterial activity against all test bacteria **(Table 7)** showing inhibition zones.

Table 7: Inhibition zones diameters resulting from the antibacterial activity of *annona cherimola mil* on five pathogen bacteria. The value '0' the table indicates no inhibition zone formation.

Extract	**Concentrations (mg/ml)**	**Bacteria (mm)**				
Methanolic		EC	PA	KP	SA	CD
	First concentration	7	0	8	7	6
	100	10	13	0	10	0
	50	7	7	0	13	0
	25	9	14	0	12	0
	12.5	10	14	0	15	0
	6.25	8	10.5	0	0.8	0
	3.12	7	8.5	7	14	0
	1.56	7	12.5	7	7	7
	0.78	8	13	7	8	7
Aqueous (decoction)	First concentration	6.5	42.5	7	7	0
	100	6.5	7	0	0	0
	50	6.5	8	0	0	0
	25	6.5	14.5	0	7	0
	12.5	0	11.5	0	7	0
	6.25	0	7	0	12	0
	3.12	0	7	0	8	7
	1.56	0	7	7	13.5	7
	0.78	0	7	8	0	8
Hydromethanolic	First concentration	10	28	0	0	7
	100	7	7	0	0	0

	50	7	7	0	0	0
	25	8	7	0	0	0
	12.5	0	6.5	0	7	8
	6.25	8	9.5	7	8	7.5
	3.12	7	11	7	10	0
	1.56	8	8.5	8	7	0
	0.78	9	12	0	7	0
Aqueous (maceraion)	First concentration	7	50	0	0	0
	100	0	35	0	0	0
	50	0	26.5	0	0	0
	25	0	25.5	0	0	0
	12.5	0	7	0	0	0
	6.25	0	13	0	0	0
	3.12	0	13	0	0	0
	1.56	0	7.5	0	0	0
	0.78	0	14	0	0	0

The following figures shows the results of discs diffusion test of the four extracts (methanolic, hydromethanolic, aqueous decoction and maceration on the tested bacteria **(Figure 8**, **Figure 9**, **Figure 10**, **Figure 11).**

Figure 8. Antibacterial activity of methanolic extract P.aeroginosa

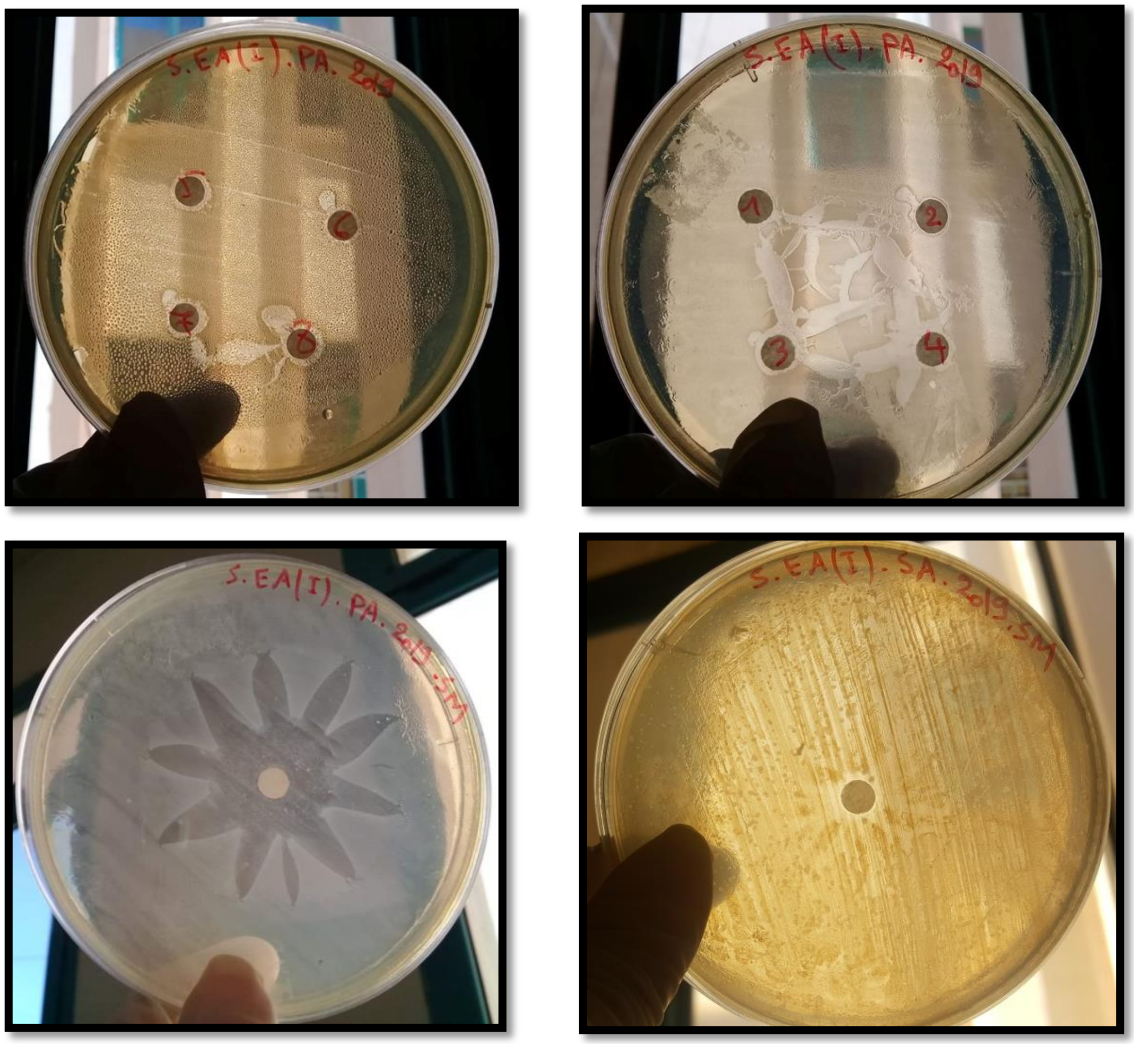
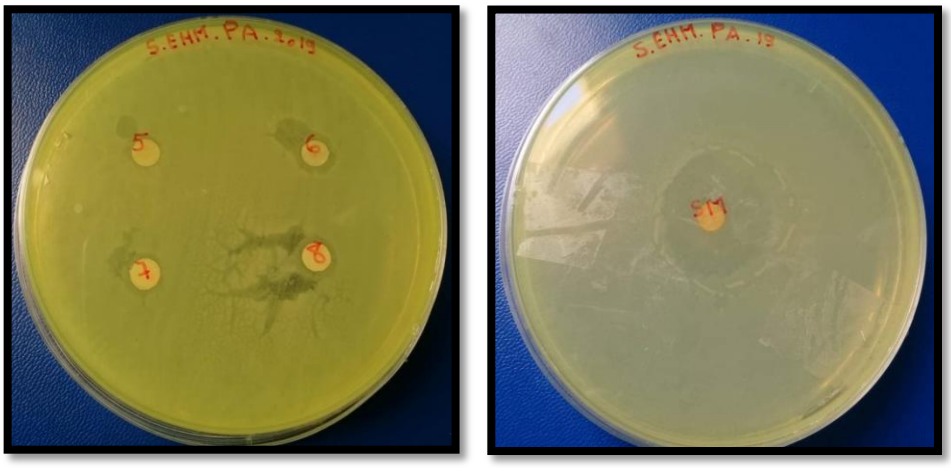


Figure 9. Antibacterial activity of Aqueous extract (difusion) on pseudomonas aeroginosa and staphylococcus aureus.



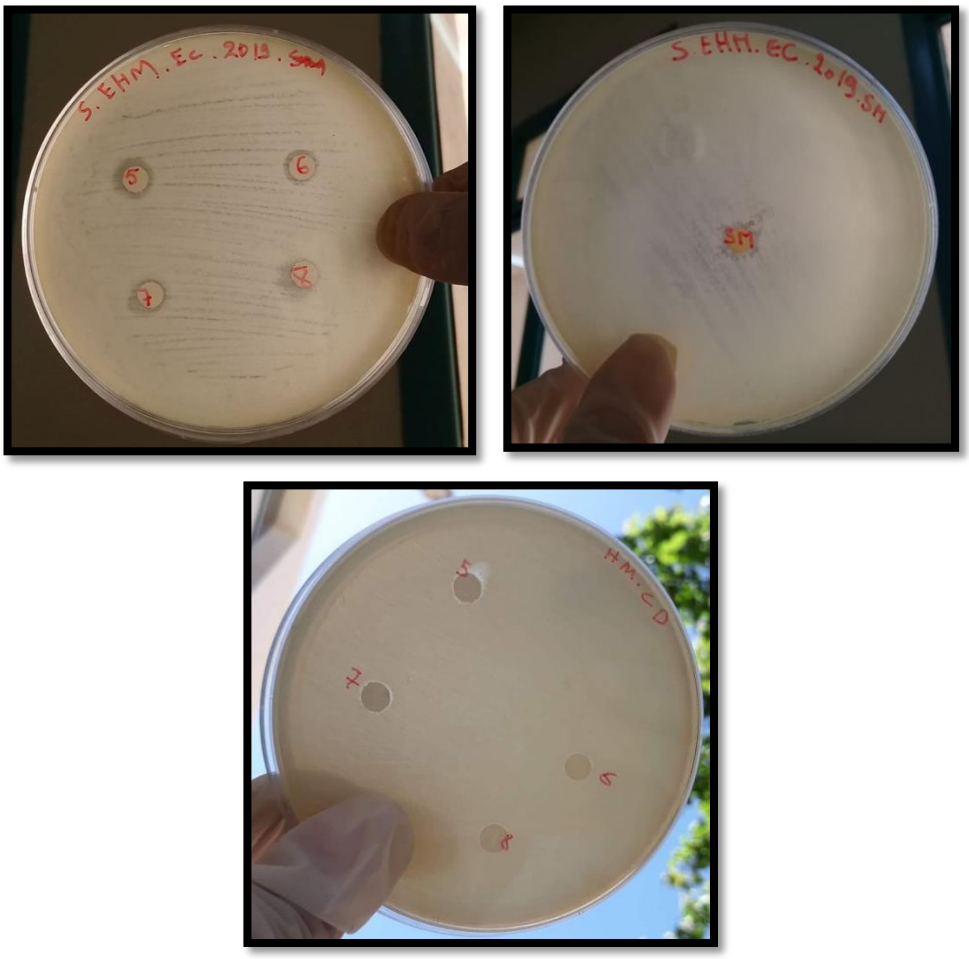


Figure 10: Antibacterial activity of Hydromethanolic extract on P.aeroginosa, E.coli and C.difficile.

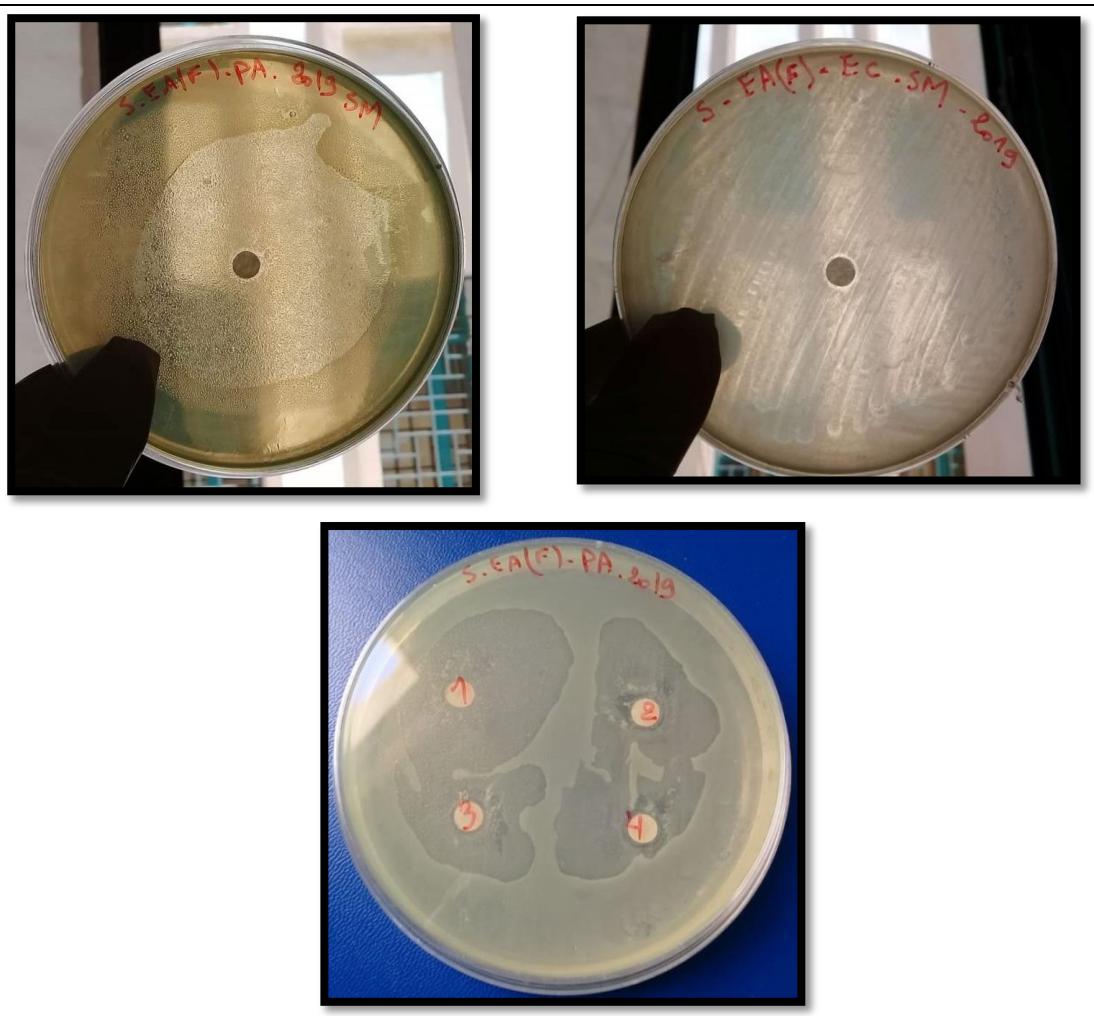


Figure 11 : Antibacterial effect of Aqueous extract (maceration) on *P. aeroginosa* and *E coli*.

The following figures (**Figure 12**, **Figure 13**, **Figure 14**, **Figure 15**) show the effects of the different *Annona cherimoya mil* seeds extracts on all of the five tested bacteria.

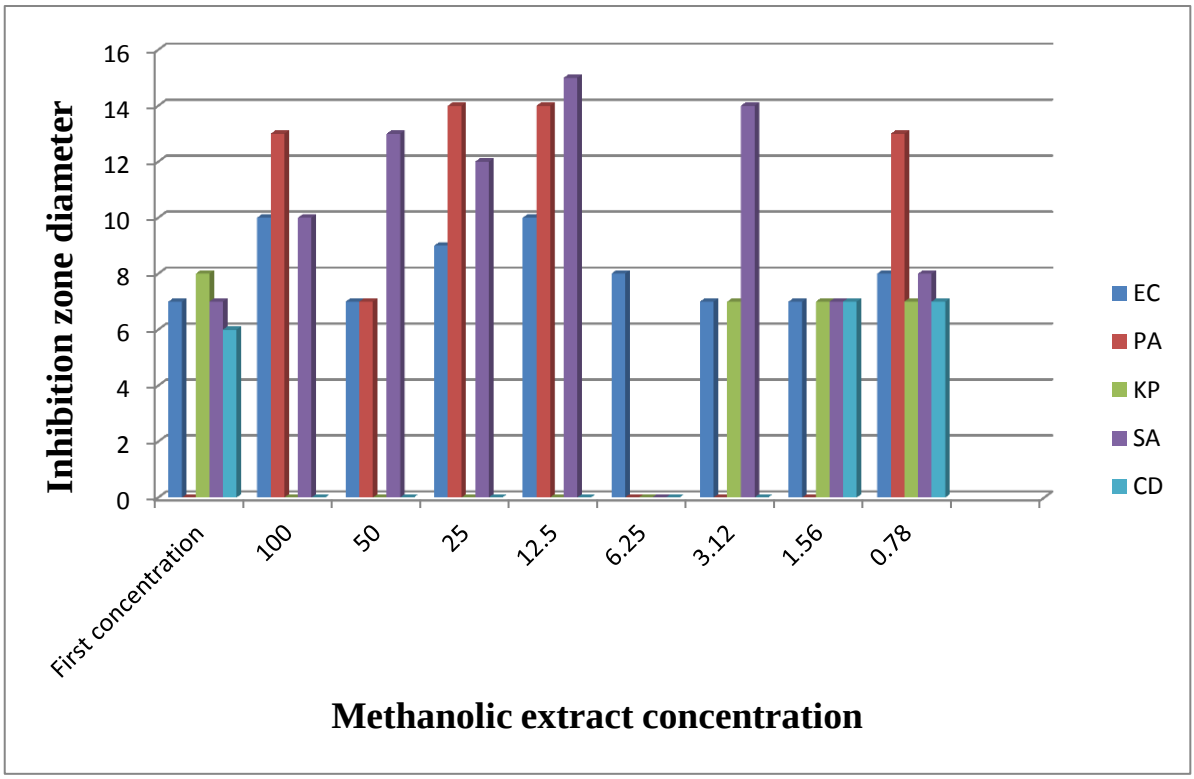


Figure 12: Methanolic exctract of *Annona cherimoya mil* seeds effect on the bacteria tested.

Diameter of inhibition zones obtained were marked from 7 to 10mm on *E.coli* and 8 to 14 mm on *P. aeroginosa* and 15mm on *S. aureus* (the largest one) in 12.5 µl concentration while it was small to absent in *K. pneumoniae* and *C. difficile*.

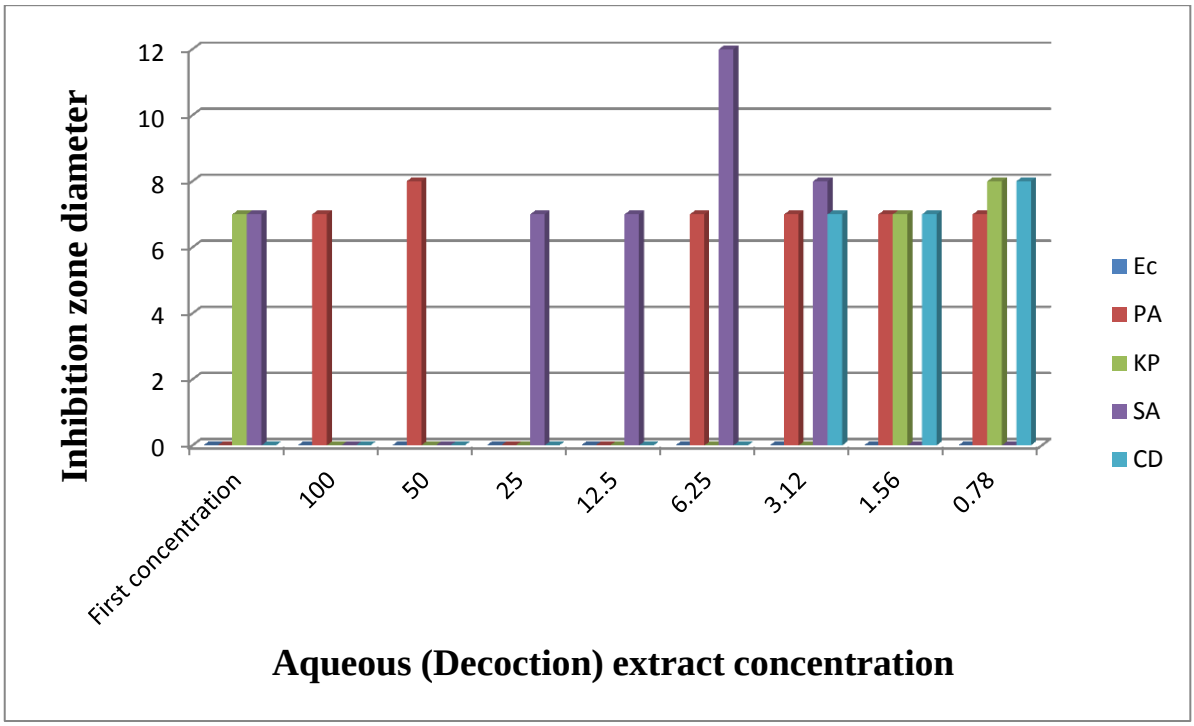


Figure 13: Aqueous (Decoction) exctract of *Annona cherimoya mill* seeds effect on the bacteria tested

In aqueous extraction (decoction) parsley had antibacterial effects on *P. aeroginosa* by exhibition the zone of inhibition between 8 to 42.5 mm in the fours highest concentrations (first concentration, 100µl, 50µl, 25µl, 21.5µl).

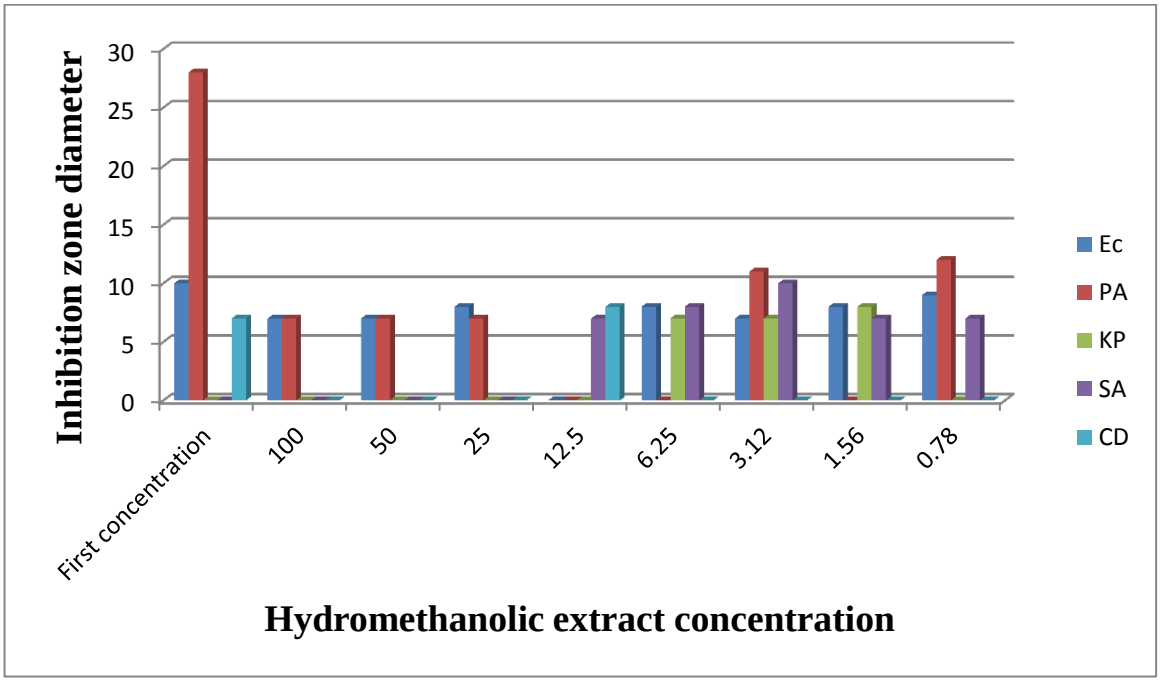


Figure 14 : Hydromethanolic exctract of *Annona cherimola mill* seeds effect on the bacteria tested.

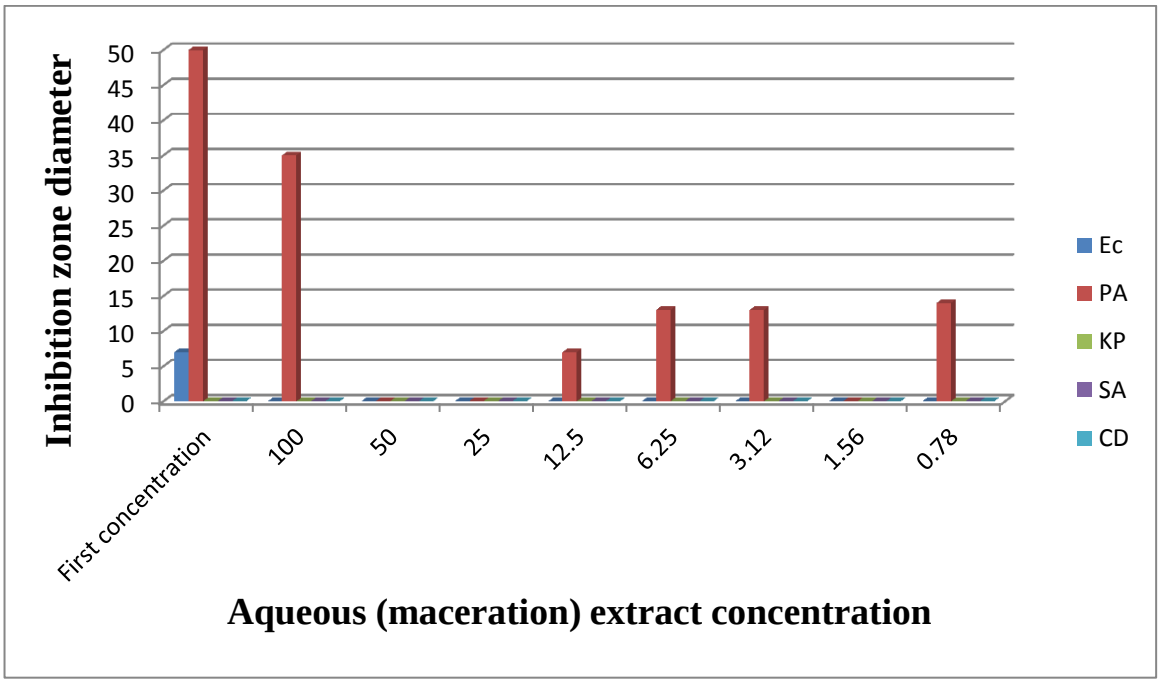


Figure 15. Aqueous extract of *Annona cherimola mill* seeds effect on the tested bacteria.

The results showed that both extracts (hydromethanolic and aqueous (maceration)) exhibited remarkable activity against P.aeroginosa.

The hydromethanolic extract was found to be more active than the aqueous extract on the tested bacteria . while contrary on P.aeroginosa was high activity in all the concentrations . the average inhibitory zone diameter was 14mm in the 0.78µl concentration to 50mm in the first concentration .

I.3.3.2 Minimal Inhibitory Concentration (MIC)

The effectiveness of the extracts in tested bacterial strains was determined by measuring the minimum inhibitory concentration. MIC was performed for only those organisms which showed a zone of inhibition and were sensitive to the plant extracts in the previous antibacterial assays by agar discs diffusion method.

Among all extracts tested to show strong antimicrobial activity (**Figure 16**)

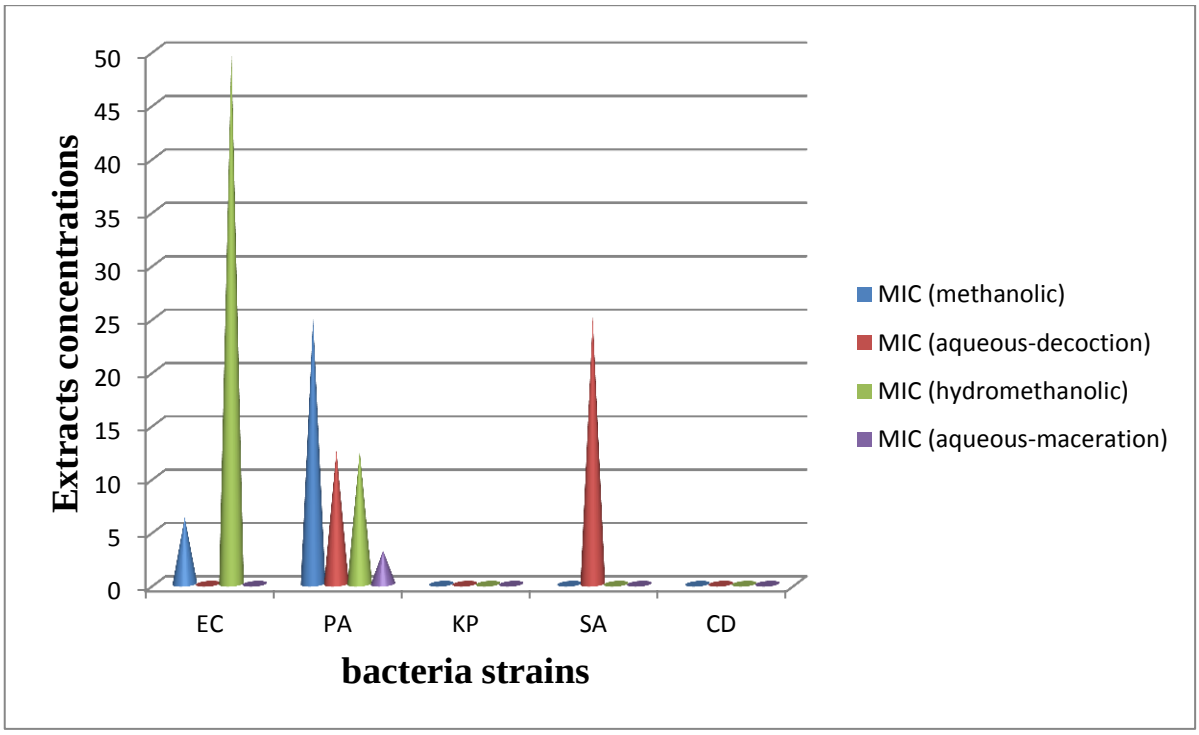


Figure 16. MIC of *Annona cherimola mill* seeds extracts on the tested bacteria.

The OD of bacterial growth contained the seeds extracts compared with growth of bacterial culture, which contained no extracts. The results showed that all extracts could inhibit the growth of *P. aeroginosa* but with different sensitivity.

Extracts showed strong activity against *P. aeroginosa* at (12.5 mg/ml) concentration for hydromethanolic extract and aqueous extract (decoction 12 mg/ml) (maceration 3.12 mg/ml) methanolic extract (25mg/ml).

While the values of methanolic extract with the best MIC at 6.25 mg/ml on *E. coli* and no inhibition for the other bacteria strains.

II. Bioinformatics Results

As reported in the previous chapter, the data generated by the experimental part of this project, outlaid above, have been processed and stored in an in-house database (development database). The in-house database has been further developed into an operational one and made available online. The following text presents the online database, its different way of usage and discussion.

II.1 The Online Database

The production database, named in the project as BARID for Bacterial Antibiotic Resistance Investigation Database has been mounted on a web-server to provide the data and results to local and international community of researcher including students (**Kheris *et al.*, 2019**).

II.1.1 Web access and Querying

The web address for the BARID database is: http://bioinformaticstools.org/prjs/barid which available for free. **Figure 1**7, shown, below show the main interface page of the database and highlighted are the main three different ways of querying; Bacteria-tab, Antibiotics-tab and Cherimoya Extracts-tab.

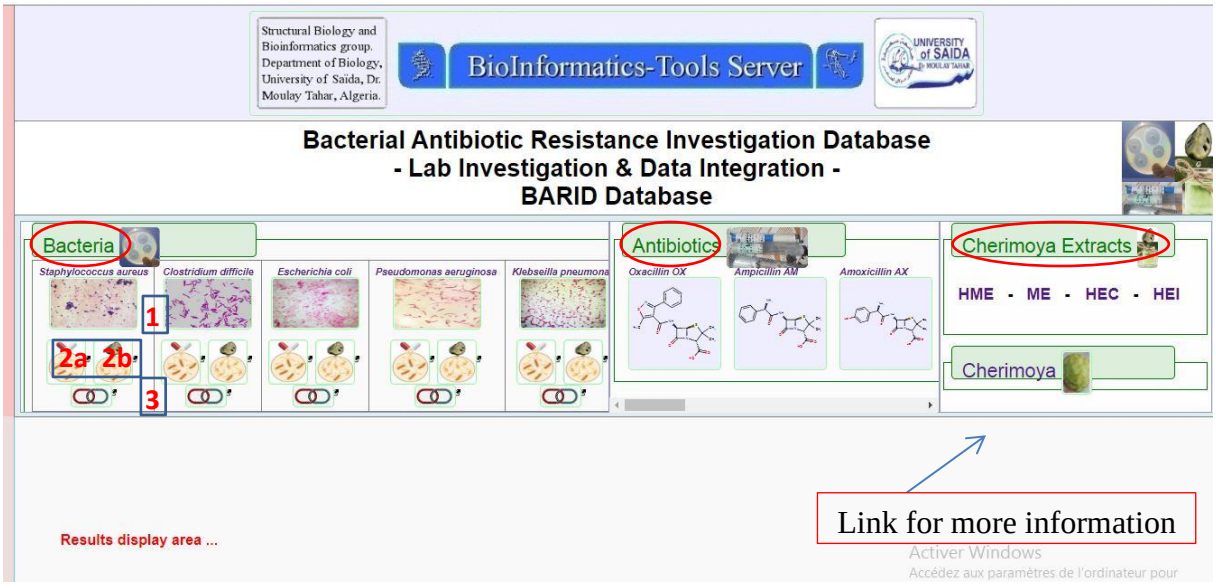


Figure 17: The main interface of the BARID database system. Three ways of access/querying the database are highlighted in red; **Bacteria**, **Antibiotics**, **Cherimoya** (seeds) **Extracts** tabs. Plus an additional link "Cherimoya" to explore the *Annona cherimola mill* plant.

II.1.1.1 Querying BARID via the Bacteria-tab

There are 4 links; denoted as 1, 2a, 2b and 3 in (**Figure 17)** associated with this tab, that allow for 4 different types of results content.

1. Data results are displayed for every bacteria under study in this project. This is generated once the name or the icon image of the bacteria is clicked.

   The bacteria generated data incudes its classification, principal characteristics, gram stain images. In addition, the results may contain useful data associated with proteins, genes, 3d-structures (when available), drugs information and related publications **(Figure 18).** This additional category of results is generated through the Data Integration Module (The system is based on an improved version of the Data Integration algorithms published in the GABagent application paper; **(Rachedi et al., 2000)** of the BARID system.

2. a Clicking this link will generate the discovered results pertaining to the effect of all tested antibiotics on the bacteria mentioned in the Tab. This type of results helps exposing whether the bacteria, in question, show any Antibiotic Resistance against any of the antibiotics listed there in **(Figure 19).**

   b This link generates the reported results related to the Anti-bacterial activity of all the four extracts on the bacteria in relation to this tab **(Figure 20)**. Such result type exposes whether the Cherimoya seeds' extracts have any anti-bacterial effects.

3. Through the use of this link, **Joint Inspection** (denoted by the icon image ), a direct side by side inspection of antibiotics reaction and extracts effects is made possible **(Figure 21).** This allows direct comparison and conclusion drawing potential, refer below to the Discussion section.

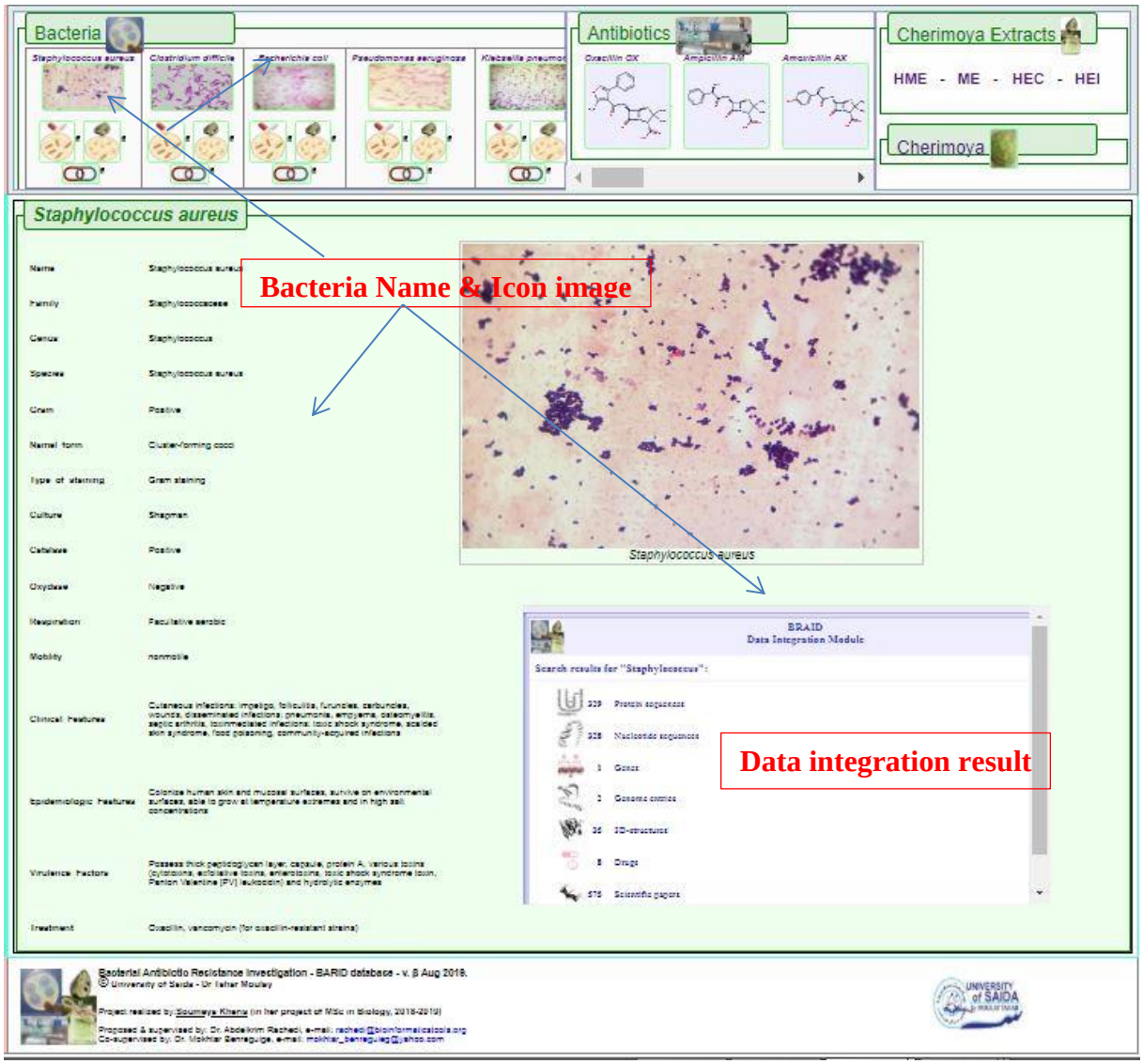


Figure 18. Display of bacteria results data type after clicking the Bacteria-tab on the region marked 1 (shown in **Figure 17**).

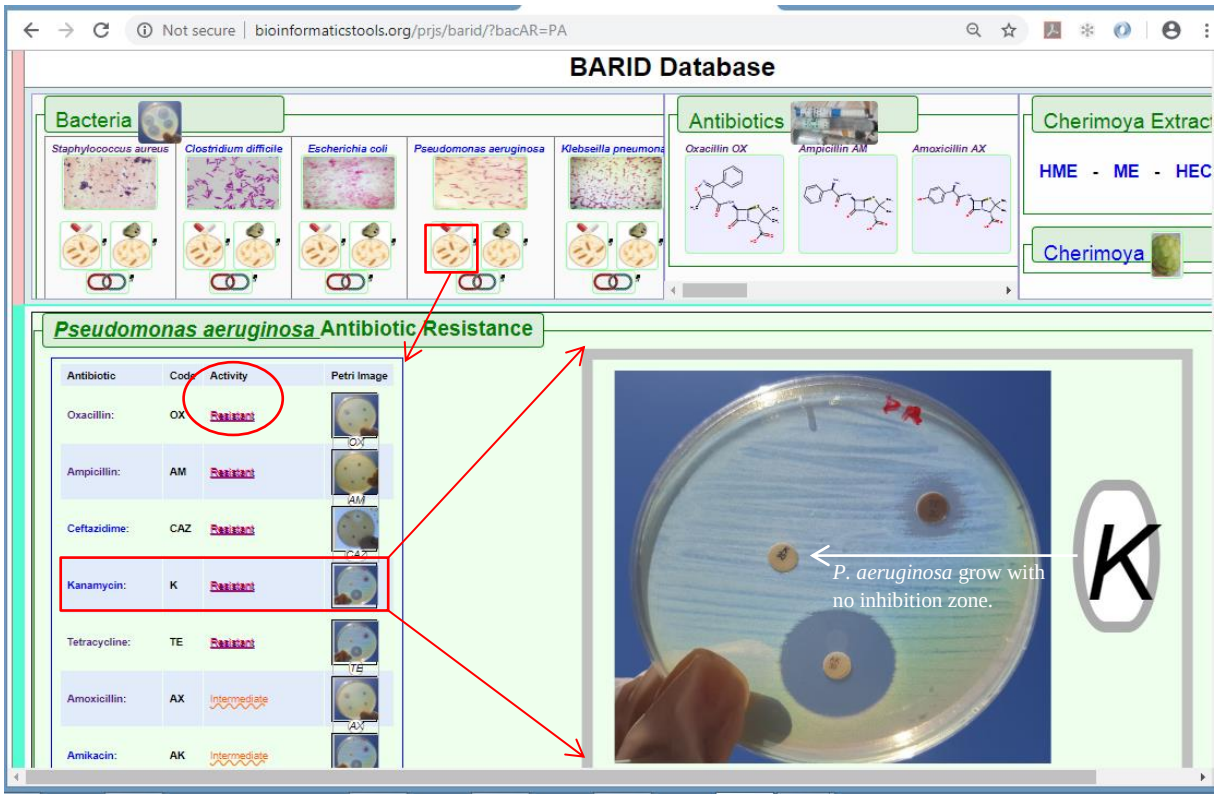


Figure 19. Display of Antibiotic Resistance results data type where a summary of the bacteria reactions to all list of antibiotics used in the study is displayed. This is shown after clicking the Bacteria-tab on the region marked 2 a (shown in **Figure 17**). **Hovering** with the **mouse pointer** over any of the peeridish icon images will zoom in the petridish image for better visualization.

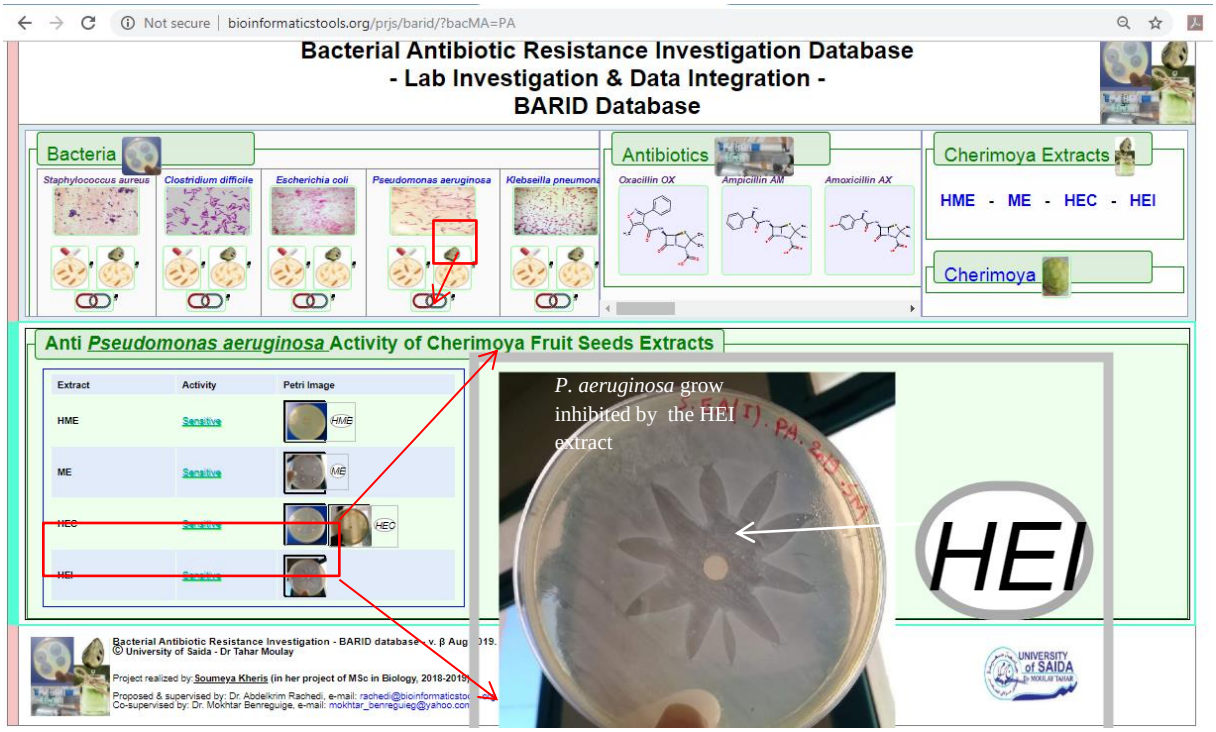


Figure 20. Display of Anti-bacterial Activity results data type where a summary of the bacteria reactions to the four types of Cherimoya seeds' extracts is displayed. This is shown after clicking the Bacteria-tab on the region marked **2 b** (shown in **Figure 17**). **Hovering** with the **mouse pointer** over any of the peeridish icon images will zoom in the petridish image for better visualization.

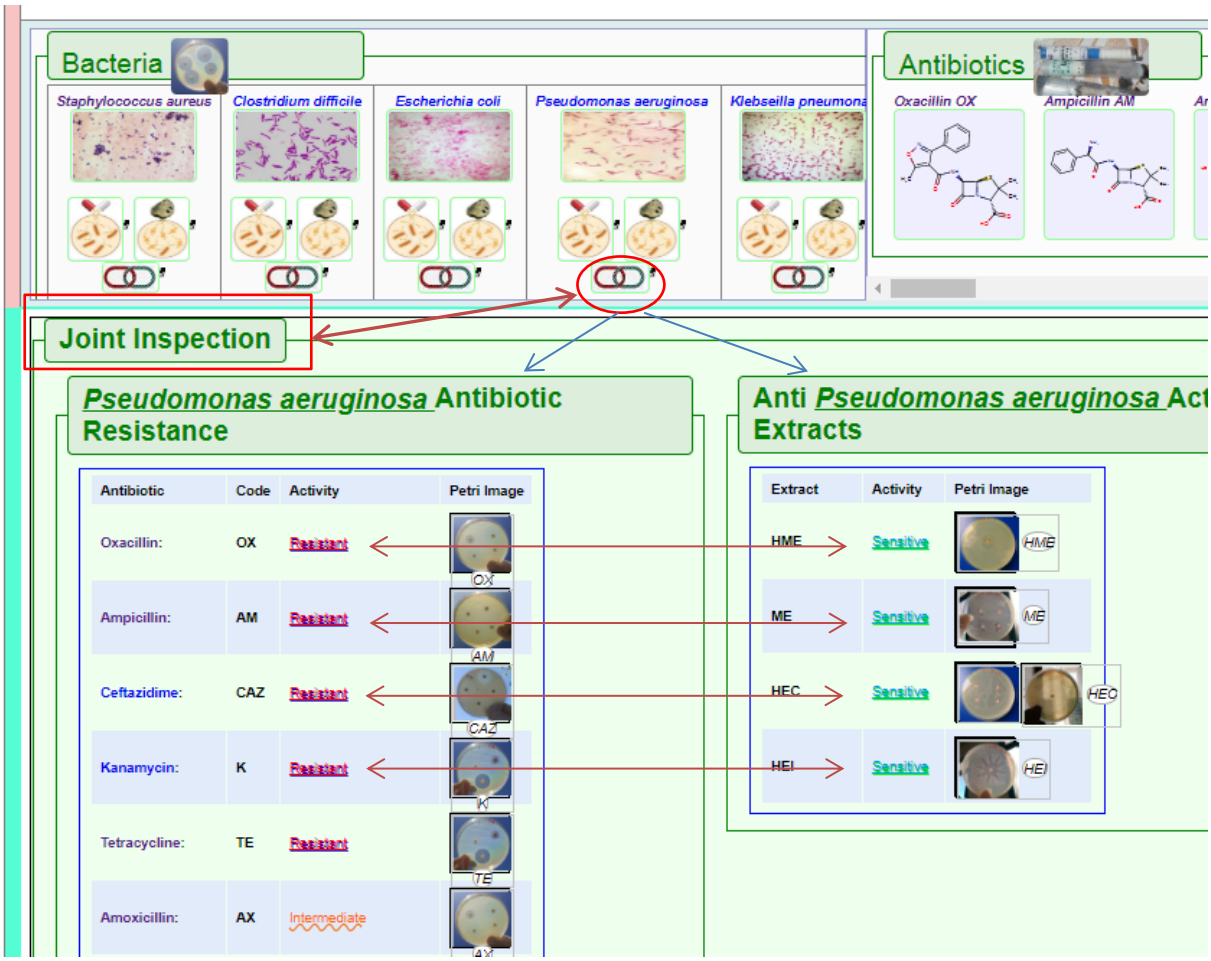


Figure 21: The Joint Inspection feature allows for the display of side by side Antibacterial Resistance and Antibacterial effects. As seen in above figures, hovering with mouse pointer will zoom in the petridish icon images for better visualization.

II.1.1.2 Querying BARID via the Antibiotics-tab

Researching the database can also be done by click on the Antibiotics list provided undr the Antibiotics-tab, see next figure (**Figure 22**).

Clicking on antibiotics by names generates summary information relevant to the antibiotic, class, type and its chemical/structure composition. Also displayed are the reactions of all of the five bacteria under study to the antibiotic in question.

Data Integration results are also generated including related proteins, genes, 3d-structures (when available), further drugs information and related publications.

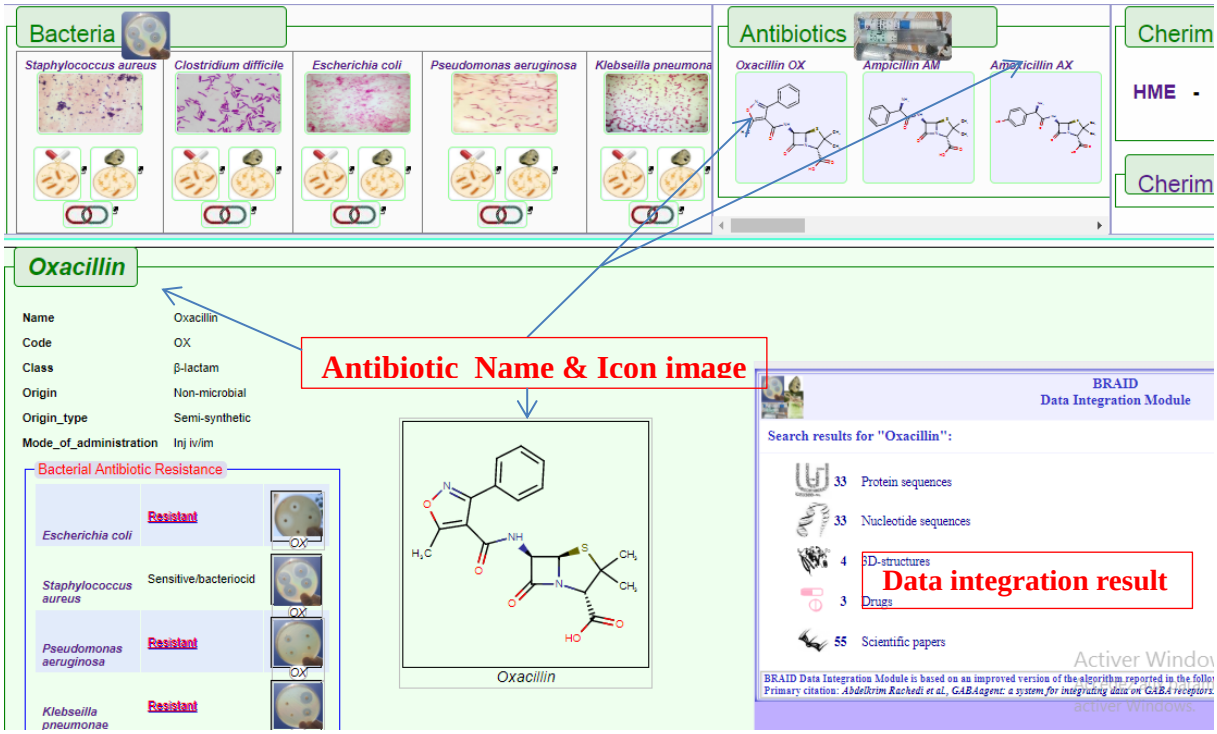


Figure 22. Results shown when the Antibiotics list is used to query the database. As seen in above figures, hovering with mouse pointer will zoom in the petridish icon images for better visualization.

II.1.1.3 Querying BARID via the Cherimoya Extracts-tab

This features display the ani-bacterial activity of the Cherimoya extracts against all of the five bacteria under the study as shown below in **Figure 23**.

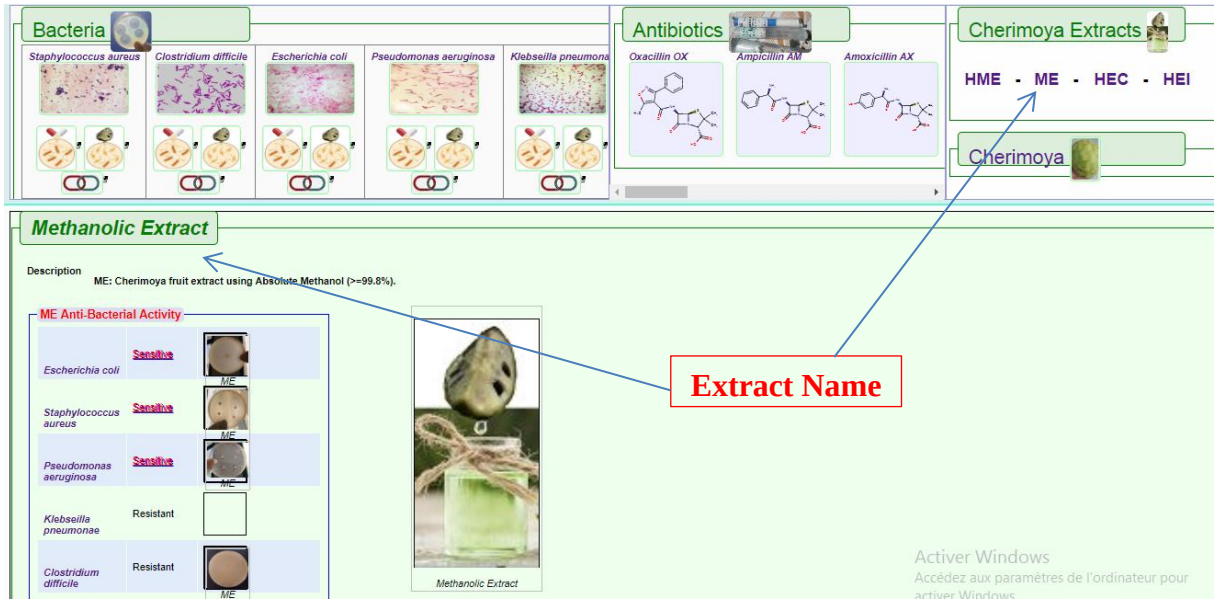


Figure 23. Results shown when the Cherimoya Extracts list is used to query the database. As seen in above figures, hovering with mouse pointer will zoom in the petridish icon images for better visualization.

II.1.1.4 Querying BARID via the Cherimoya Fruit-tab

This feature allows for displaying details about the *Annona Cherimola mill* plant as shown below in **Figure 24**.

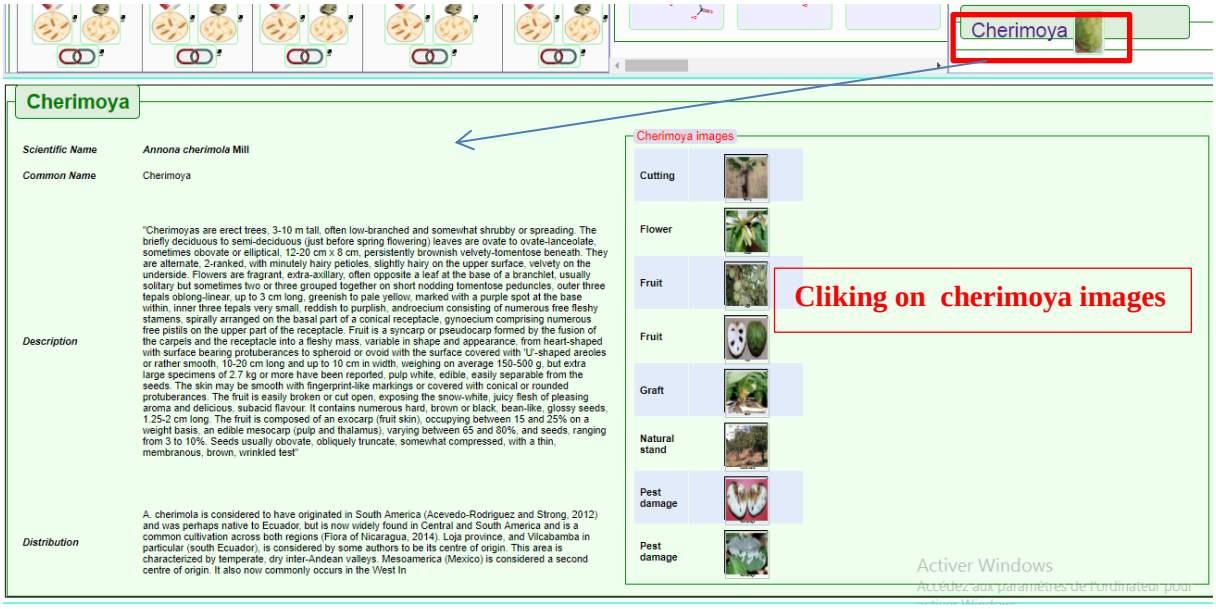


Figure 24. Results shown when the Cherimoya Fruit-tab is used to query the database. Here also, hovering with mouse pointer will zoom in the petridish icon images for better visualization.

II.1.1.5 Data Integration Results

Almost all of results mentioned above, like the shown in figures **18** and **22**, would be endorsed with related useful data associated with proteins and nucleic primary sequence, genes, 3d-structures (when available), drugs information and related peer reviewed publications**.** This additional category of results is generated through the BARID's **Data Integration Module** which is based on an improved version of the Data Integration algorithms published in the GABagent application paper; **(Rachedi et al., 2000)**.

II.1.2 Discussion

Due to time-frame limitation given to master projects, the discussion side where comparison of this project results with other international research groups who might have published their discoveries is not fully undertaken except the mention of few references. However, this is left to future plans that might lead to journal publications.

The discussion here concerns with the potential benefits of both venturing into such important research axes and the power of bioinformatics and its tools in exposing the scientific value found in the results data.

In addressing this subject within the context provided by the results data of obtained by the wet-lab research undertaken in this project, as represented in the BARID database, it is here preferred to take one of the virulent bacteria which is *Pseudomonas aeruginosa* treated as a study case and explore what the results are pointing to.

II.1.2.1 Antibiotic Resistance

In the above case represented by **Figure 19**, region 2 a of the Bacteria-tab (as noted first in **Figure 17**) associated with *Pseudomonas aeruginosa* is marked by the larger red square which when clicked showed the Antibiotics Resistance of this bacteria against the list of antibiotics (only partial list is shows in the image above) pointed to by the red arrow. Hovering with the mouse pointer over any petridish images will zoom in the petridish image; in this case the zoomed in image show that *P. aeruginosa* is grown normal, with no zone of inhibition in the presence of the antibiotic Kanamycin (K).

This is a clear illustartion that *P. aeruginosa* shows antibiotic resistance and is a conformation of previous published work (**Mathai D *et al.*, 2001).** Same can be discovered easily in BARID about the other antibiotics and their effects on the other four bacteria.

II.1.2.2 Anti-bacterial Activity

The case presented in **Figure 20**, region **2 b** of the Bacteria-tab (also noted first in **Figure 17**) associated with *Pseudomonas aeruginosa*, marked by the larger red square, which when clicked it provides users with the Anti-bacterial Activity of all the Cherimoya seeds' extracts on the *P. aeruginosa* pointed to by the red arrow.

The zoomed in image shows a clearly visible flower-shaped zone of inhibition which is mean that *P. aeruginosa* is **sensitive** to the **HEI** Cherimoya seed extract type (Refer to Table 4, previous chapter).

While this study shows clear inhibition zone meaning obvious anti-bacterial activity of the extracts, results by recent study about the effect of Cherimoya extracts on (**Norma *et al.*, 2017)** on *P. aeruginosa* show only moderate antibacterial activity.

II.1.2.3 Antibiotic Resistance and Anti-bacterial Activity

The BARID database system provide a very simple but efficient way to compare between the results discussed above via the **Joint Inspection** feature (see **Figure 2**) which brings the two sets of results together and sort them out systematically to enabling direct

identification of where antibiotic ‘Resistance’ versus anti-bacterial or ‘Sensitivity’ to the Cherimoya seed extracts.

This originally novel exploration process through the BARID system is available to all of the five bacteria, antibiotics and Cherimoya extracts enabling easy and efficient investigation of the subject and help in conclusions drawing.

Bacterial infectious diseases represent an important cause of morbidity and mortality worldwide. An antibiotic resistant bacterium is a threat which is becoming increasingly common. The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs/antibiotics in the future is still uncertain. The search for antibacterial from natural sources has received much attention and efforts have been put in to identifying compounds that can act as suitable antibacterial /antimicrobials agent to replace synthetic ones

Screening antibacterial activity as well as for the discovery of new antibacterial compounds calls for a return to natural substances is an absolute need of our time

In this project, confirmation of 'Antibiotics Resistance' reported in scientific literature have been verified. In the hand high antibacterial activity of all the extracts of *Annona cherimola mill* on the virulent bacteria *Pseudomonas aeroginosa* is quite remarkable while also effective against the other bacteria.

Phytochemicals derived from *Anonna cherimoya mill* seeds serve as a prototype to develop more effective medicines in controlling the growth of bacteria .

Phytochemical screening in qualitative analysis, tests for phytochemicals were carried out and the results confirmed the presence of alkaloid. Such compounds have significant therapeutic applications against human pathogens including bacteria.

In a general conclusion, the project results clearly points to the fact that Cherimoya seeds contain a potent antimicrobial agents that need further future investigation, identification, hopefully purification and possibly chemical synthesis. Such natural products can constitute possible alternatives to antibiotics to help fight the ever growing global problem of antibiotics resistance.

This study also showed that bioinformatics and the implementation of programming and database developments are potentaily essential in dealling with data generated in biology reasearch for in managing, analysis, value adding and giving meaning to the data for maximum benefit of science and it applications in many areas of life inclding medicine, disease fight and biotechnology.

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Culure media composition

1- GN medium :

Ingredients per liter of deionized water :

Pancreatic Digest of Casein……..…….10.0gm

Peptic Digest of Animal Tissue……..10.0gm

Sodium Chloride…….……..5.0gm

Sodium Citrate……..……5.0gm

Dipotassium Phosphate……4.0gm

Mannitol…….……..2.0gm

Monopotassium Phosphate……...……1.5gm

Dextrose……..…..1.0gm

Sodium Deoxycholate……..…….0.5gm

Final pH 7.0 +/- 0.3 at 25ºC.

2- Chapman medium :

Ingredients per liter of deionized water:

`Lab-Lemco' powder……1.0gm

Peptone……10.0gm

Mannitol……..10.0gm

Sodium chloride……...75.0gm

Phenol red…….……0.025gm

Agar……15.0gm

pH 7.5 ± 0.2 at 25°C.

3- **Muller Hinton agar medium :**

Ingredients per liter of deionized water:

beef extracts powder…….……2gm

acid digest of casein ……17.5gm

strach ……...……..1.5gm

agar ……1.7gm

4- Muller Hinton broth :

Ingredients per liter of deionized water:

Beef, dehydrated infusion from……..…….300.0gm

Casein hydrolysate…….17.5gm

Starch……1.5gm

5- King B medium :

dipotassium hydrogen phosphate……1.5 g/L

magnesium sulfate…….1.5 g/L

mixed peptone…… 20 g/L

pH 7.2 ± 0.2 at 25°C.

6- KING A medium :

Agar…….15 g/L

gelatine peptone (pancreatic)…….20 g/L

magnesium chloride……...1.4 g/L

potassium sulfate……10 g/L

pH 7.2 ± 0.2 at 25°C.

7- Hecktoen medium :

Ingredients per liter of deionized water

Protease peptone…….12.00gm

Yeast extract……..3.00gm

Lactose……...12.00gm

Sucrose…….2.00gm

Salicin…….…..9.00 gm

Bile Salts mixture…….9.00gm

Sodium chloride……5.00gm

Sodium thiosulfate…….5.00gm

Ferric ammonium citrate……1.50gm

Acid fuchsin……..…….0.10gm

Bromothymol blue……..0.065gm

Agar…….……..14.00gm

Final pH (at 25°C): 7.5±0.2

8- Mannitol mobility :

Agar……..… …… 3.0 g/L

Peptone ……..…. 20.0 g/L

Mannitol ……..... 2.0 g/L

Potassium nitrate…….…. 1.0 g/L

1% Phenol red solution…… 4 ml/L

pH: 7.6

9- Meat Liver Agar :

Ingredients per liter of deionized water

Agar…… 11.0 g/L

ammonium ferric citrate……. 0.5 g/L

D(+)-glucose……. 0.75 g/L

meat liver base……20.0 g/L

sodium sulfite……1.2 g/L

starch…….0.75 g/L

Final pH (at 25°C): 7.5±0.2

10- Indol :

Ingredients per liter of deionized water

Casein enzymic hydrolysate……10.000gm

Dextrose……..1.000gm

Sodium chloride …… 5.000gm

Phenol red……..…… 0.010gm

Agar……. …2.000gm

Final pH (at 25°C) 6.8±0.2

11- Urease :

Ingredients per liter of deionized water

Urea ……..…….20.0 gm

Sodium Chloride……..…5.0 gm

Monopotassium Phosphate…….. 2.0 gm

Peptone……...….. 1.0 gm

Dextrose……..…….. 1.0 gm

Phenol Red……...……..0.012 gm

Agar……15.0 gm

Final pH 6.7 +/- 0.2 at 25 degrees C.

12- Crystal violet :

Ingredients per liter of deionized water :

Sodium polypectate…… ……..… 18.000 gm

Sodium hydroxide …… . …… 0.360 gm

Sodium nitrate …… …… 2.000 gm

Calcium chloride.H2O …… …… 0.600 gm

Crystal violet …… ...…… 0.0015 gm

Sodium lauryl sulphate……..…. 0.100 gm

Agar …… …… 4.000 gm

Final pH (at 25°C) 7.2±0.2.

Zieil neelsen stain reactifs