Screening of antimicrobial and antibiofilm compounds against *Acinetobacter baumannii* and illustration of quorum sensing molecules

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Department of Biotechnology and Bioinformatics

Project report submitted in fulfillment of the requirements for the degree of

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In

Biotechnology

By

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Under the Guidance of

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WAKNAGHAT, Solan (H.P)

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CERTIFICATE

This is to certify that the work which is being presented in the title of **"Screening of antimicrobial and antibiofilm compounds against *Acinetobacter baumannii* and illustration of quorum sensing molecules"** for the end semester of B.Tech in Biotechnology and submitted in the department of biotechnology and bioinformatics, **Jaypee University of Information Technology, Waknaghat** is an authentic record of work carried out by Shivangi Kapoor (141843) during the period of July 2017 to May 2018 under the supervision of **Dr. Jitendraa vashistt**, Assistant Professor, department of Biotechnology and Bioinformatics, Jaypee University Of Information Technology, waknaghat.

The above statement made is correct to the best of my knowledge.

Date: May 2018

_____

Dr. Jitendraa vashistt

Assistant Professor

Department of Biotechnology

ACKNOLEDGEMENT

I would like to express my sincere gratitude first and foremost to the institution, Jaypee University Of Information Technology for giving me a prospect for achieving my goals and fulfilling my dreams.

I am highly beholden to my supervisor Dr. Jitendraa Vashistt for taking me under his guidance and I am also thankful to my P.H.D scholars Miss. Nutan Thakur and Miss. Monika Choudhary and my senior Miss Natasha Thakur for helping me throughout the year.

I also give my gratitude to the department of Biotechnology and Bioinformatics where I received all the facilities for pursuing my project and to the fellow staff members for supporting me for the same.

I am thankful to Dr. Sudhir Kumar, HOD, Department of Biotechnology and Bioinformatics, Jaypee University Of Information Technology for providing me the desirable guide Dr. Jitendraa Vashistt.

At last but not the least, I would like to recall my obligation from the core of my heart to my respected and beloved parents for providing me moral support, incessant encouragement in all spheres of life.

DECLARATION

I hereby declare that the present work on **Screening of antimicrobial and antibiofilm compounds against *Acinetobacter baumannii*** is a record of original work done by me under the guidance of **Dr. Jitendraa Vashistt** at **Jaypee University of Information Technology, Waknaghat,** solan (H.P), from July 2017 to May 2018 at Microbiology lab in the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, solan.

I also declare that no part of this report has previously been submitted to any University for acquiring any degree.

DATE: SHIVANGI KAPOOR

PLACE: B.TECH Biotechnology

LIST OF FIGURES

SUMMARY

Total 4 strains of *Acinetobacter baumannii* were taken for the semiquantitative analysis of *CsuAB* gene which is responsible for Quorum sensing.

Acinetobacter baumannii has ability to form Biofilms on biotic as well as abiotic substances and was susceptible to common antibiotics but 1970 onwards it has developed into a MDR bacteria. According to clinical reports, most of the isolates has become XDR and PDR and hence it is difficult to treat associated infections.

The antimicrobial activity was checked against *Acinetobacter baumannii* using different chemical compound and a traditional compound was used i.e. *Juglan regia* which is used as datum in the local area of kangra in Himachal Pradesh.

Then the antibiofilm activity was also checked for the same compounds against *Acinetobacter baumannii*.

CHAPTER 1: INTRODUCTION

Acinetobacter baumannii is a Gram-negative bacillus which is aerobic, pleomorphic and is a not motile organism. It is an opportunistic pathogen, which belong to the family Neisseriaceae. When it is observed under the microscope it appears intermediate involving a rod and a sphere. *A. baumannii* is a comparatively novel pathogen and is considered its initial appearance in martial management amenities for the period of the "Iraq War". It is included as a MDR. This incident of multidrug-resistant (MDR) pathogens has progressively more happen to a root for staid fear with regard to both "nosocomial" and community-acquired infections. Undeniably, the World Health Organization (WHO) has freshly recognized antimicrobial confrontation as one of the three mainly imperative troubles facing human health. The majority familiar and staid MDR pathogens have been covered within the ellipsis "ESKAPE", which stands for *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* sp.

A famous micro-biologist named Beijernick initially isolated the organism in 1911 from soil by minimal media enriched by calcium acetate. In the beginning it was depicted as *Micro-coccus calco-acetiicus*, the genre Acinetobacter was anticipated almost 43 years afterward by Brasov and Privet to distinguish it from the motile organisms contained by the genre Achromobacter

The genre Acinetobacter, is now divergent, clinch ,Gram-negative, austerely ae-robic, not-fermenting, can grow in specific media, not-motile, catalane-positive, oxidazse-negative bacteria which has a DNA G + C contented 39% to 47%. Ensuing DNA-DNA cross lessons executed by Bouvet and Grimnot in 1986, the Acinetobacter genre at the present consist of 26 species and nine genomic variety. Four variety of *Acinetobacters* (*A. calco-aceticus, A. baumannii*, Acinetobacter genomic species 3 plus Acinetobacter genomic species 13TU) have phenotypic connection that they are complicated to make a distinction, and as such are habitually referred to as the *A. calcoaceticus*-complex. This categorization can be ambiguous as the ecological species *A. calcoaceticus* has not been caught up in medical ailment, whilst the additional three species in the *A. calcoaceticus*- multifaceted are conceivably the most

clinically noteworthy species, being drawn in mutual community-acquired and nosocomial infections.

It is now become as XDR-PDR which is called as extensive drug resistant and pandrug resistant respectively. Available options for treatment colistin, tigecyclines.

Figure1.1: Representing the colonies of *Acinetobacter baumannii* in Macaconkey agar plate

CHAPTER 2: REVIEW OF LITERATURE

2.1 Natural Habitat:

Organisms which fit in to the genre Acinetobacter are habitually painstaking to be universal in surroundings known that they can be recovered from nearly all soil and facade water sample.[13] This perceptive has donated to the frequent mistaken belief that *A. baumannii* is also ubiquitous.[26] *A. baumannii* exclusively aims humid tissues like mucous membranes or some parts of the skin that are because of any calamity or injury. Regardless of its connection with skin contagion, *A. baumannii* has been found infrequently as part of the usual skin.

2.2 Epidemiology:

2.2.1 Europe

In this place *A. baumannii* contagion is a munificent remedial distress. By molecular epidemiological typing methods hospice outburst of the infections in many countries including England, Germany, Netherlands, Spain, and Italy are scrutinized. In the majority of cases, one or two epidemic strains were detected in a given epidemiological setting. Transmission of such strains has been observed between hospitals, most doubtless via transfer of colonized patients. [7]Spread of multidrug-resistant *A. baumannii* is not confined to hospitals within a city but also occurs on a national scale. Paradigm are the increase of South-east clone and the Oxa-23 clones 1 and 2 in South-east Eng-land (9,5), the dissemination of a multidrug-resistant *A. baumannii* clone in Portugal (11), the interhospital spread of a VEB-1 ESBL-producing *A. baumannii* clone from a total of 55 medical centers in northern and southeastern France (3), and the spread of an amikacin resistant *A. baumannii* clone observed in nine hospitals in various regions in Spain (83). International transfer of colonized patients has led to the introduction and subsequent epidemic broaden of multidrug-resistant *A. baumannii* strains from Southern into Northern European countries, such as Belgium and Germany (42, 4). Intercontinental spread of multidrug-resistant *A. baumannii* has also been described between Europe and other countries as a corollary of airline travel (38, 42). These proceedings highlight the importance of appropriate screening and possible isolation of patients reassign from countries with soaring rates of drug-resistant organisms.

Carbapenem conflict in *A. baumannii* is currently a concern in scores of European countries. Information on the pervasiveness of carbapenem fighting in assorted European countries is thorny to acquire, excluding it materialize commenc0ing the rash journalism that carbapenem opposition rates are premier in Turkey, Greece, Italy, Spa-in, and England and are immobile fairly stumpy in other states. Carbapenem resistance in Eastern Europe emerges to be mounting (12, 6). Tariff emerge to be buck in Scandinavia, though intermittent isolates have been testimony from patients convey from away, counting fatalities of the Indi-an Ocean tsunami (28). In an industry supported surveillance report (MYSTIC) from 48 European hospitals for the period 2002-2004, just 73.1% of isolates were susceptible to meropenem and 69.8% were susceptible to imipensem (5). Propensity to other antibiotics was also dreadfully stumpy, with 35.4%, 29.4.0%, and 45.6% being predisposed to cezidime, ciprofloxacin, and gensamicin, respectively (5). *A. baumannii* isolates resistant to the polymyxins have been sensed in Europe, although at present these remain rare . For a comprehensive examination of phenotypic resistance in *Acinetobacter* spp. throughout Europe, readers are referred to an tremendous analysis by Van Looveren and Goossens (5).

2.2.2 Latin America

Duty of non-susceptibility to meropenem, imudipenem, cefadzidime, pipraeracillin, ciprofloxacin, and gentametamicin in LA emerge to be surrounded by the utmost in the planet. For instance, just 70% of isolates were prone to merfeopenem or imipenem in an judgment from a scrutiny curriculum in the episode 2001-2005 . In a scrutiny cram concerning Argentina, Brazil, Chile, and Colombia commencing 1994 to 2001, conflict tariff were premier in Argentina, but no parts were secure multi-drug-resistant isolates As illustrate beforehand, a multiplicity of carbpenem have been notorious in *A. baumannii* isolates in LA, including IMfP-1 and IMfP-6 in Brssazil OXA-23 in Brazil and Colombia (11,), and OXA-58 in Argentina . Captivatingly, the SsPM- and VIdM-type MBLs, which are insidious in Brazil (SPM) and other part of LA (VIM) in *P. aeruginosa* strains, have not yet been reported for *A. baumannii* strains in this expanse, to our acquaintance.

2.2.3 Africa

Information on the coverage of antibiotic fighting in *A. baumannii* in Africa are chiefly inadequate to South Africa at the present time, although in attendance are spread hearsay from other countries[25, 39, 47]. Brink and contemporaries have exposed that about 32% of *A. baumannii* bloodstream isolates in South Africa are carbapenem defiant, 40% are opposing to cefepime and piperacillin-tazobactam, and 30% are unwilling to ciprofloxacin and levofloxacin [5]. "Such anti strains are widespread in some units (for example, burns and ICUs) and have been broadening from association to association.

2.2.4 Asia and the Middle East

Numerous spate of pan drug-resistant *A. baumannii* have been accredited in Asian and Middle Eastern infirmary and a assortment of carbapenemases have been portrayed to instigate there[2, 3, 28,]. Rates of non-susceptibility in sentinel isolates (2001-2004) surpass 25% for imipenem and meropenem, 40% for cefepime and ceftazidime, 40% for ampicillin-sulbactam, 35% for amikacin, and 45% for ciprofloxacin. Regrettably, confrontation to tigecycline [38] and polymyxin B [17, 29] previously subsist in this expanse.

2.2.5 Australia and Pacific Islands

Initial reports of *A. baumannii* from Australia same from the Northern Territory, where community-acquired infections are well described (8, 9). Such infections have a vastly different epidemiology from that seen in hospital-acquired infections, with male gender, age of >45 years, Aboriginal ethnic background, cigarette smoking, alcoholism, diabetes mellitus, and chronic obstructive airway disease being important risk factors(9). Also, these community-acquired strains are significantly more disposed to anti microbials (9). Gorge stage coach and micro aspiration may be implicated in the pathogenesis of these infections (8).

2.3 CLINICAL EXPRESSION OF *ACINETOBACTER BAUMANNII* INFECTIONS

2.3.1 Hospital acquired Pneumonia

It has been reported that *A. baumannii* isolates are from the respiratory tracts of individuals who are suffering from, in the medical institutions. It is seen that, it is in

many circumstances, it is extremely intricate to discriminate superior airway colonization from factual pneumonia. Though there is not any disbelief, that factual ventilator-associated pneumonia because of *A. baumannii* take place. From different studies in many countries, it has been reported that 6%-11% of cases of ICU- acquired pneumonia were because of this very organism (18). Though, it has been seen that in many medical houses the ratio for this particular disease that is ICU-acquired due to this very bacteria is exceptionally high. The sufferers from *A.baumannii* illness have had to stay for long time in ICU (18), even though in outburst condition, prior attainment of illness may arise.

2.3.2 Community-acquired Pneumonia

In the parts of Australia and Asia, Community-acquired pneumonia is common because *A. baumannii*. (8, 9, 39,). When there is a rainy period the disease takes place to those who have a past of alcohol abuse and they refer to ICU at times (8). This infection is thus followed by the completion of medical path, 2^{o}bloodstream infection and transience rate of 45-65 (3). This infection is arrived from throat that take place to the alcohol consumptives i.e. approx 12%. (8).

2.3.3 Bloodstream Infection

From many studies in America from 1995-2003) , it is cleared that the bloodstream infections that are derived from hospitals, *A. baumannii* was very widespread causer which comes under top 10 and is , being conscientious for 1.4% of all single microbial hospital derived bloodstream infections (5). A ratio of 1.6 to .9 respectively of ICU-acquired infection of bloodstream from *A.baumannii* to Non ICU-acquired is obtained from a journal. Death rate from this infection is known to be 30-45% in ICU and around 17% external the ICU. *A. baumannii* bloodstream infection is in the top 3 utmost death rate in the ICU. *A. baumannii* infections were the latest of all bloodstream infections to occur during hospitalization, occurring a mean of 26 days from the time of hospital admission (5). It is therefore not certain if the high crude mortality rate represents its occurrence in patients with ongoing underlying critical illness or whether the organism does have significant attributable mortality. Sources of bloodstream infection were not described in the study mentioned above but are typically line related or attributed to underlying pneumonia, UTI, or wound infection (5). It is notable that 102 patients had bloodstream infections at sites treating U.S.

military members injured in Iraq or Afghanistan from 1 January 2002 and 31 August 2004 (5). The sites of origin of these infections were not described in this report.

2.3.4 Traumatic Battlefield and Other Wounds

A. baumannii may sporadically grounds skin/pliable tissue contagion slight of the martial inhabitants. The life form grounds 2.11% of ICU-acquired pelt/pliable tissue infections in one judgment. It is a well branded pathogen in smolder element and possibly will be complicated to exterminate from such patients. Yet, its involvement to pitiable upshot in smolder patients is deliberated. *A. baumannii* is universally secluded from lesion of warfare fatalities from Iraq. It was the frequently inaccessible organism (33.5% of belongings) in one consideration of struggle fatalities with unlock tibial fissure. However, it appears to be of low pathogenicity at this site after initial treatment, the organism was never isolated from follow-up cultures in any of the patients with open tibial fractures and did not appear to contribute directly to persistent nonunion or need for amputation.

2.3.5 UTI

A. baumannii is a sporadic cause of UTI, being dependable for just 1.6% of ICU-acquired UTIs in one revision (6). Normally, the individual is allied with catheter-associated infection or immigration. It is not usual for this individual to cause straightforward UTI in hale and hearty out-patients.

2.3.6 Meningitis

Nosocomial, post-neurosurgical *A. baumannii* meningitis is progressively more imperative entity. The microbial epidemiology of nosocomial meningitis is embryonic to incorporate additional gram-negative pathogens (5,8), so it is not astounding that multidrug-resistant *A. baumannii* is amid the pathogens occupied Archetypal patients have undergone neurosurgery and have an peripheral ventricular deplete . Transience may be as elevated as 73%, while the cause of transience is often thorny to discriminate .

Other Manifestations

A petite integer of case hearsay of *Acinetobacter* endocarditic subsist [10,15]. Mostly but not all cases have concerned prosthetic regulator. *Acinetobacter* spp. may root

endophthalmitis or keratitis, at times allied to contact lens exercise or subsequent eye surgery (A solitary case tale exists of a toxic generating *A. haemolyticus* strain, which was coupled with gory diarrhea in a 3-month-old preschooler. Memorandum that fixed type ID relics a concern in these information.

2.4 Clinical impact

Fascinatingly when upshot from *A. baumannii* bacteremia was evaluated unswervingly to folks who had bacteremia with supplementary gram-negative organisms, counting *Klebsiella pneumoniae*, a noteworthy enlarge in humanity was eminent for *A. baumannii* (26, 46). An auxiliary reading confirm a considerable boost in transience with multi-drug-resistant *A. baumannii* migration or infectivity balanced to that with multi-drug-resistant *Pseudomonas aeruginosa* migration or contagion, using a Kaplan-Meier scrutiny (19). However, not even a single study worn a ceremonial, unvarying scheme to fiddle with cruelty of infirmity or co morbidities, such as an APACHE, McCabe, or Charlson score Whether the disproportion between these can be enlightened wholly by procedural discrepancy is anonymous. Conversely, it is chief that all *A. baumannii* results engross a single geographic area, and thus the impending for a pathogen-specific capricious, such as virulence, to foundation the miscellany in results is doable. This impression is more sustained by the extensively shoddier outcome pragmatic in folks tainted with *A. baumannii* from the society than those for folks tainted in the sanatorium setting, counting a lofty commonness of bacteremia, keen respiratory pain syndrome, circulated intra-vascular coagulation, and fatality. “Community-acquired *A. baumannii* infections materialize to be a matchless proven entity stirring chiefly in stifling weather (9, 32).

More recently, the clinical impact of empirical therapy on patient outcomes with *A. baumannii* bacteremia has been analyzed. Several studies report that receipt of inactive empirical therapy is an independent predictor of increased mortality (3), whereas others have not been able to confirm these findings (5,). “Such differences may relate to the small patient numbers included in these studies and the resulting lack of statistical power. Finally, in spite of *A. baumannii* is the most frequent type foremost to quantifiable contagion; very a small number of information subsists on the assessment of conclusions amid *A. baumannii* and other *Acinetobacter* species. In a topical study from Korea, 30 patients with this caused by *Acinetobacter* species

slight the *A. baumannii* cluster, principally *A.lwoffii, A.haemolyticus*, and *A. calcoaceticus*, were balanced to 120 patients in the midst of *A. baumannii* bacteremia (8). Past amending for relentlessness of infirmity", "percentage of patients with poly-microbial bacteremia, and satisfactoriness of antibiotic therapy, no momentous divergence was pragmatic in transience". "However, the length of hospital study was significantly longer for those with *A. baumannii* infection". "Unfortunately, species identification in this study was not based on reliable methods, and therefore it is difficult to make definite conclusions".

It now appears that the damage of *A. baumannii* as a low-virulence pathogen vis under extreme scrutiny. The organism is clearly evolving, as determined by genomic comparative studies (17h2), and with the acquisition of drug resistance determinants, which impairs our ability to use active empirical therapy, acquisition of virulence determinants may also be occurring.

2.5 Antibiotic Resistance

2.5.1 AbaR resistance islands

AbaR-type resistance islands are typical of drug-resistant *A. baumannii*, and different variations may be present in a given strain. Each consists of a transposon backbone of about 16.3 Kb that facilitates horizontal gene transfer. Transposons allow portions of genetic material to be excised from one spot in the genome and integrate into another. This makes horizontal gene transfer of this and similar pathogenicity islands more likely because, when genetic material is taken up by a new bacterium, the transposons allow the pathogenicity island to integrate into the new microorganism's genome.

2.5.2 Beta-lactamase

A. baumannii has been shown to produce at least one beta-lactamase, which is an enzyme responsible for cleaving the four-atom lactam ring typical of beta-lactam antibiotics. Beta-lactam antibiotics are structurally related to penicillin, which inhibits synthesis of the bacterial cell wall. The cleaving of the lactam ring renders these antibiotics harmless to the bacteria. The beta-lactamase OXA-23 was found to be flanked by insertion sequences, suggesting it was acquired by horizontal gene transfer.[47]

2.5.3 Efflux pumps

Efflux pumps are protein machines that use energy to pump antibiotics and other small molecules that get into the bacterial cytoplasm and the periplasmic space out of the cell. By constantly pumping antibiotics out of the cell, bacteria can increase the concentration of a given antibiotic required to kill them or inhibit their growth when the target of the antibiotic is inside the bacterium. *A. baumannii* is known to have two major efflux pumps which decrease its susceptibility to antimicrobials. The first, AdeB, has been shown to be responsible for aminoglycoside resistance.[48]

2.6 Biofilm formation

A. baumannii has been renowned for the perceptible potential to endure on synthetic facade for an extensive phase therefore letting it to persist in the hospice surroundings. This is considerd to be because to its capability to structure Biofilms. For various biofilm forming bacteria, the progression is interceded by flagella. Nevertheless, for *A. baumannii*, this progression appears to be interceded by pili. Auxiliary, distraction of the alleged pili chaperone and shepherd genes *csuC* and *csuE* were exposed to reduce biofilm formation. The creation of biofilms has shown to amend the metabolism of microbes inside the biofilm, accordingly tumbling their sensitivity to antibiotics. This is because less nutrients are obtainable deeper inside the biofilm. A leisurely metabolism can thwart the bacteria from captivating up an antibiotic and performing a crucial gathering express adequate for exacting antibiotics to have an achieve. They too afford a corporeal obstacle alongside better molecules and might avert aridness of the microbes.

2.7 Quorum sensing in Bacteria

- to converse microbes use signaling molecules
- bacterial talk and bacterial cross talk
- AHL (N-acyl homoserine lactones)

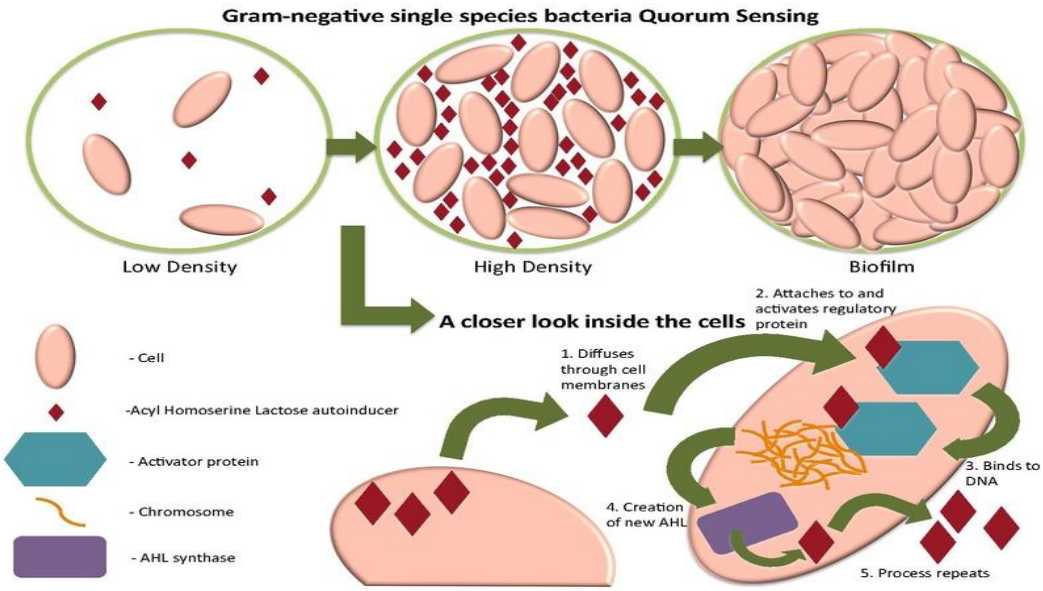


Figure 2.1: this figure depicts the quorum sensing in gram negative bacteria

Source: Miller, M.B. and Bassler, B.L., 2001. Quorum sensing in bacteria. Annual Reviews in Microbiology, 55(1), pp.165-199.

2.8 Bacterial Talk and Cross Talk

QS facilitate microbes to synchronize their deeds. As ecological circumstances repeatedly revolutionize hurriedly, microbes necessitate to counter swiftly in regulate to stay alive. These rejoinders restrain edition to accessibility of nutrients, security against micro-organisms which could contend for the equivalent nutrients and the dodging of venomous amalgam potentially treacherous for the bacteria. It is incredibly imperative for pathogenic bacteria throughout contagion of a horde to harmonize their virulence in categorize to smash out the impervious rejoinder of the horde in tidy to be proficient to launch a triumphant contagion.

Unusual bacterial variety exploits singular molecules to correspond. Here are numerous diverse module of signaling fragment. With-in apiece grouping there are moreover trivial deviation such as extent of elevation manacles. In several gear a lone bacterial sort can encompass supplementary than one QS scheme and consequently

utilize additional signal molecule. The bacterium may counter to apiece glimmer in a diverse loom. In this sagacity the signal molecules can be reflection of language surrounded by an idiom.

Substantiation that inter-species contact through QS canister happens. This term is well known as quorum sensing cross talk. Cross talk has allusion in countless vicinity of microbiology as in temperament bacteria nearly for eternity exists in diverse kind populations such as Biofilms.

QS Investigate has several impending relevance, the majority of these rivet scheming bacteria by inquisitive with their signalling systems. For paradigm numerous germs rely on QS to manage the phrase of the genes which grounds malady. If we canister wedge the QS systems we may be capable to thwart these germs from being precarious.

2.9 Gallic Acid

Gallic acid is white in color and do not have any odor. It is a phenolic coumpond. This is being used in photography, pharmaceutical

Studies have shown that there is antimicrobial activity of gallic acid against *A.baumannii* and additional bacterial species too. Gallic acid is dissolved in many solvents such as ethanol, methanol, water. Although, it was fully dissolved in 30% ethanol.[17]

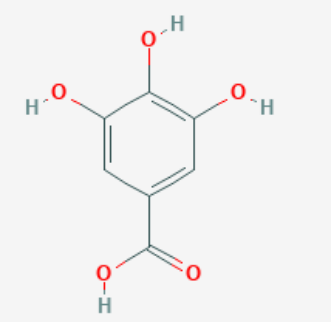


Figure 2.2: Showing the chemical structure of gallic acid

2.10 CITRIC ACID

Citric acid is a compound which has organic nature. And naturally found in citrus fruits. It is dissolved in water, DMSO, acetone, ethanol. But during the study, citric acid was easily dissolved in water. Therefore the solvent is water in case of citric acid. From the research, citric acid shows antibacterial activity against many pathogenic bacteria's and also against *Acinetobacter baumannii*.[16]

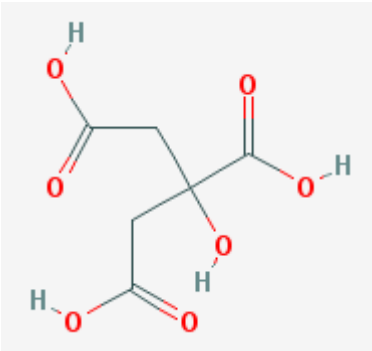


Figure 2.3: showing the chemical structure of citric acid

2.11 Quercetin

It is a 3 ring structure and an anti-oxidant. This is found in fruits and vegetables. Having an astringent aroma it is used as an ingredient to many food items and beverages also. It is studied that Quercetin show the anti-micribial activity against the bacteria's which are involved under the "ESKAPE" pathogen. So it is used as a study that weathers it shows activity of *A.baumannii* as well.

It is soluble in ethanol, acetone, methanol. So, it was being dissolved in absolute ethanol. [14]

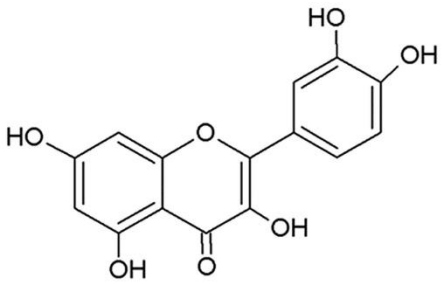


Figure 2.4: representing the structure of Quercetin

2.12 PLANT: *Juglan regia*

It is a kind of walnut, found in most parts of Himalayas. It shows nutritional values. Its leaves were taken. In the local area of Himachal Pradesh, people from that region its leaves are used as “datun” which helps in protecting the teeth and the plaque. Therefore it act as an antibiofilm.

Figure2.4: representing the tree of ***Juglan regia***

CHAPTER 3: AIMS AND OBJECTIVES

- To study the growth characteristics of strains of *Acinetobacter baumannii*.
- To determine antimicrobial activity of

  a) Chemical compounds

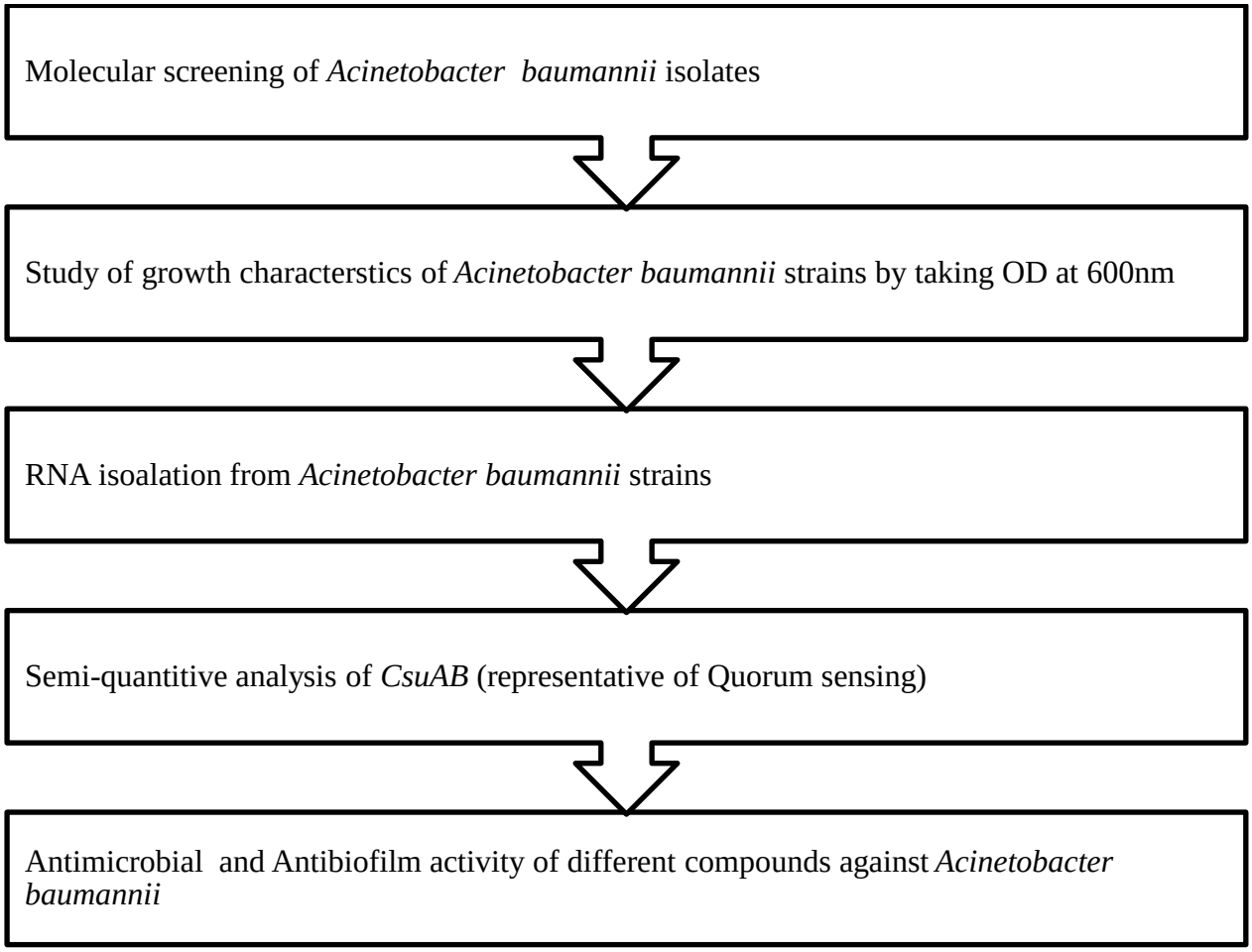
  b) Traditional compound

  , against *Acinetobacter baumannii*.

- To determine the Anti biofilm activity against *Acinetobacter baumannii* of the same.

CHAPTER 4: MATERIALS AND METHODS

WORK PLAN


Molecular screening of *Acinetobacter baumannii* isolates
Study of growth characterstics of *Acinetobacter baumannii* strains by taking OD at 600nm
RNA isoalation from *Acinetobacter baumannii* strains
Semi-quantitive analysis of *CsuAB* (representative of Quorum sensing)
Antimicrobial and Antibiofilm activity of different compounds against *Acinetobacter baumannii*

4.1 Materials

Luria broth (Hi-media), Macconkey agar, Nutrient agar, PBS, ethanol, Gallic acid, citric acid, Nuclease free water, Quercetin, autoclaved water, methanol, Agarose, crystal violet.PCR enzymes, acrylamide,glycerol.

PCR master mix was purchased from takara, japan and primers were used from Eurofins.

Chemicals were of Hi-media)

4.2 Procedure

4.2.1 Culturing and molecular confirmation of *Acinetobacter baumannii* strains

1. 10-20 µl from glycerol stocks of each stain of *Acinetobacter baumannii* was taken and inoculated into 10ml of LB.
2. Cultures were incubated at 37 ˚C for overnight.
3. After 24hrs each strain was streaked on MacConkey agar plates and was also incubated at 37for overnight so to ensure that cultures are pure.
4. Pure isolated colonies were picked and dissolved in 15µl nuclease free water.
5. Using the DNA boiling method, colonies were boiled for 5-10 minutes at 96degree.
6. It was then centrifuged for 4 minutes at 6000 rpm.
7. Pellet was discarded and the supernatant contained DNA.
8. PCR reaction mixture was prepared using following reaction conditions:

Forward primer	0.3 µM
Reverse primer	0.3 µM
DNA template	250 ng
Master mix	7.5 µl
Final volume makeup with Nuclease free water upto	15 µl

The primers were amalgamated by EUROFINS.

9. The PCR reaction was run in the Applied Biosystem thermocycler.

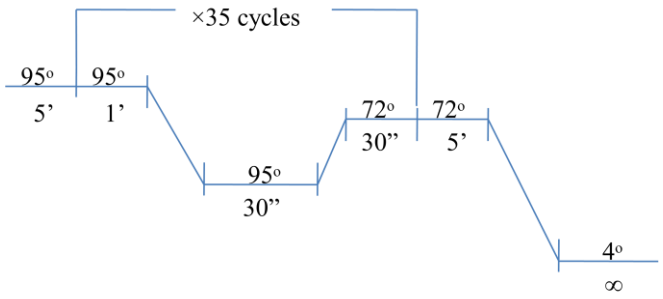
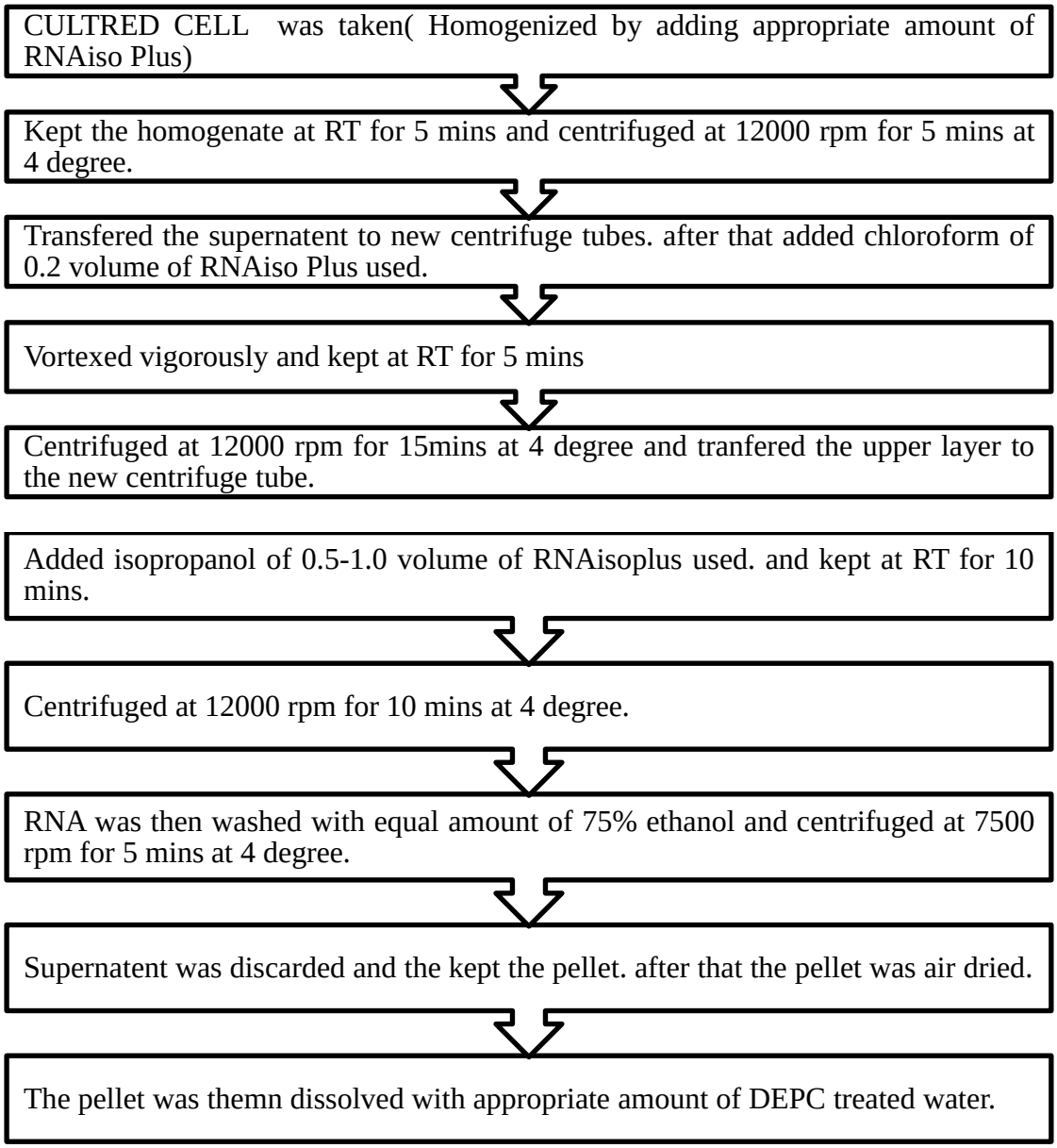


Figure 4.1: PCR reaction for the amplification of ITS region in which the reaction allowed to run 35 cycles.

4.2.2 Growth Curve Analysis

1. Culture was inoculated and kept in Incubator for 24hrs at *37 °C* .
2. 2-3 ml of culture was taken in 10ml lb flask.
3. From that 10 ml 3ml of the culture was taken from the flask and the OD was taken at 600 nm in ThermoScientific spectrophotometer at 0^{th} hour.
4. The step was repeated after every 2 hr for 50hrs.
5. Readings were taken in Spectrophotometer for 50 hours at a gap of 2hr.
6. The blank used in the experiment was Lb.
7. Graph of growth curve was then plotted.

4.2.3 RNA ISOLATION



- Then the cDNA synthesis was performed using Bio-Rad iScript cDNA kit.

4.2.4 Semiquantitive analysis of *CsuAB* gene responsible for Quorum sensing using RT PCR

- *CsuAB* gene which is responsible for Quorum sensing in *Acinetobacter baumannii* was amplified using RT PCR.

1. Took 2-3 fresh colonies of ATCC 19606and Strain 2 and assorted in 20 microlitre of nuclease free water.
2. Spin it for a few micro seconds so that it is mixed accurately.
3. Heat boil it for 10 minutes at 95 degree.
4. Centrifuged it for 3 minutes at 7000 rpm.
5. Took the supernatant in new vials and surplus the pellet.
6. According to the PCR conditions following components were added:

Forward primer	0.3 µM
Reverse primer	0.3 µM
DNA template	250 ng
Master mix	7.5 µl
Final volume makeup with Nuclease free water up to	15 µl

The PCR conditions were:

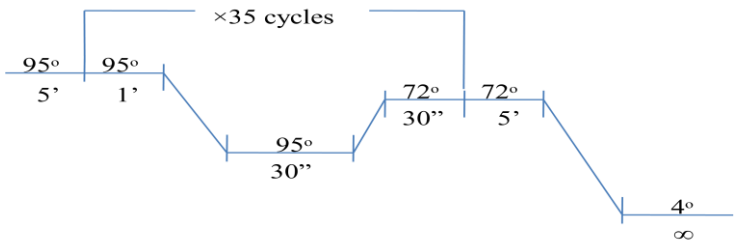


Figure 4.2- RT PCR reaction for amplification of *CsuAB* gene responsible for Quorum sensing.

4.2.5 Antimicrobial activity:

- Antimicrobial activity of following compounds against *Acinetobacter baumannii* ATCC 19606and ATCC 25922 (*E.coli*) which was taken as control.

a) Chemical compounds:

4.2.5.1 Gallic acid

1. Took 20.4 microgram Gallic acid which was dissolved in 1ml 30% ethanol so that it becomes 20.4 µg/ml.
2. It was then diluted till 2µg :
3. The activity was performed in 96 well-Plate as triplicates.

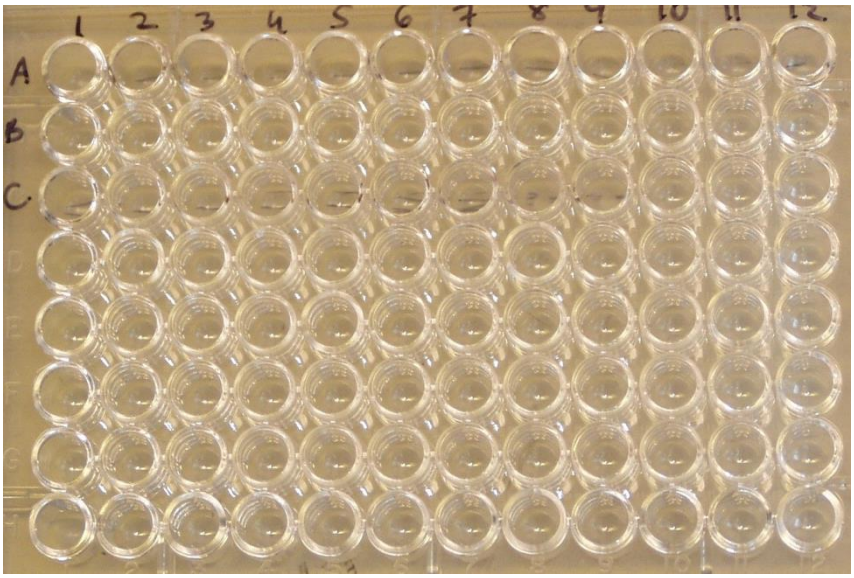


Figure 4.3: representing the 96 well plate

- B1-G1 -Blank i.e. lb media (200µl)
- B2-G2 –Culture + solvent (190µl +10µl)
- B2-D2- ATCC 19606
- E2-G2- ATCC 25922
- B3-B12 (C3-C12, D3-D12)– ATCC 19606 + Dilutions of Gallic acid in decreasing order according to the table.
- E3-E12 (F3-F12,G3-G12)- ATCC 25922+ Dilutions of Gallic acid in decreasing order according to the table.

4. The plate was incubated overnight at 37 °C and then the OD was taken from Thermoscientific spectrophotometer.

4.2.5.2 Citric acid

1. Took 20.4 mg citric acid and was dissolved in 1ml water.
2. It was then diluted till 2µg :
3. The activity was performed in 96 well-Plate as triplicates.

- B1-G1 -Blank i.e. lb media (200µl)
- B2-G2 –Culture + solvent (190µl +10µl)
- B2-D2- ATCC 19606
- E2-G2- ATCC 25922
- B3-B12 (C3-C12, D3-D12)– ATCC 19606 + Dilutions of Citric acid in decreasing order according to the table.
- E3-E12 (F3-F12,G3-G12)- ATCC 25922 + Dilutions of Citric acid in decreasing order according to the table.
-

4. The plate was incubated overnight at 37 °C and then the OD was taken from Thermoscientific spectrophotometer.

4.2.5.3 Quercetin

1. Took 20.4 mg citric acid and was dissolved in 1ml of Absolute ethanol.
2. It was then diluted till 2µg :
3. The activity was performed in 96 well-Plate as triplicates.

- B1-G1 -Blank i.e. lb media (200µl)
- B2-G2 –Culture + solvent (190µl +10µl)
- B2-D2- ATCC 19606
- E2-G2- Strain 3
- B3-B12 (C3-C12, D3-D12)– ATCC 19606 + Dilutions of Quercetin in decreasing order according to the table.

- E3-E12 (F3-F12,G3-G12)- ATCC 25922 + Dilutions of Quercetin in decreasing order according to the table.

4. The plate was covered (Quercetin is light sensitive) and incubated overnight at 37 °C and then the OD was taken from Sckan 2 Thermoscientific spectrophotometer.

b) Traditional compound: *Juglan regia*

1. Plant leaves were washed.
2. It was dries in oven at 160 °C for 1 hour.
3. Then it was put in aerated place for the complete drying.
4. And was then grinded in fine powdered form.(1;10 w/v) of plant powder was soaked and dissolved in water, ethanol and methanol for 24hr at 150rpm.
5. The filterate extract was dried at 40 degree and was resuspended in PBS as a final concentration of 500mg/ml.
6. The extract was weighed which was
    i. Methanol- 0.0823g i.e. 82mg/ml in PBS
    ii. Ethanol -0.0626g i.e. 62mg/ml in PBS
7. The dilutions were made.
8. The activity was conducted in 96 well plate .
    - B1-G1 -Blank i.e. lb media (200µl)
    - B2-G2 –Culture + solvent (190µl +10µl)
    - B2-D2- ATCC 19606
    - E2-G2- ATCC 25922
    - B3-B12 (C3-C12, D3-D12)– ATCC 19606 + Dilutions of water in decreasing order according to the table.
    - E3-E12 (F3-F12,G3-G12)- ATCC 25922+ Dilutions of ethanol in decreasing order according to the table.

5. The plate was incubated overnight at 37 °C and then the OD was taken from Thermoscientific spectrophotometer.

4.2.6 ANTI-BIOFILM Activity:

- For each compound the antibiofilm activity was performed.
- The experiment was performed in round bottom 96 well plate. Culture was prepared at .5 OD and then experiment was started.

  B1-B3- Blank (lb media)

  B4-B6- Culture

  B7-B9- Solvent + Culture

  C1-G12- Compound+Culture in decreasing dilutions of each compound.

1. The plate was kept for incubation at 37 ^{o}C .
2. After the incubation for overnight, readings were taken in spectrophotometer.
3. The media and culture were discarded and the adherent cells were washed thrice by PBS and it was stained with .02 % crystal violet for 20 mins.
4. The satin was then removed from adherent cells and ehanol:acetone in the ratio of (1:5) was used.
5. And then the absorbance was measured at 580nm and as well as at 600 nm using Thermoscientific spectrophotometer.

CHAPTER 5: RESULTS AND DISCUSSION

5.1 Culturing of *Acinetobacter baumannii* cells

Total 4 strains of *Acinetobacter baumannii* were taken and grown in the Luria broth. For the pure colonies, MacConkey agar plates were used to streak the strains. MacConkey agar was used to differentiate between the Lactose fermenting and Non-fermenting microorganisms.

Figure 5.1: Pure colonies of *Acinetobacter baumannii* strains when streaked on MacConkey agar plate.

Pure isolated colonies were seen which indicated that the bacteria grown is gram negative.

5.2 Verification of *Acinetobacter baumannii* strains with PCR

Its species recognition was done by a spacer region called ITS region. Primers ITS-forward and ITS- reverse were amplified and the size of product was 208 base pair.

PCR product was run on 1.2% agarose gel along with 100bp ladder confirms that the used culture is of *Acinetobacterbaumanni.*

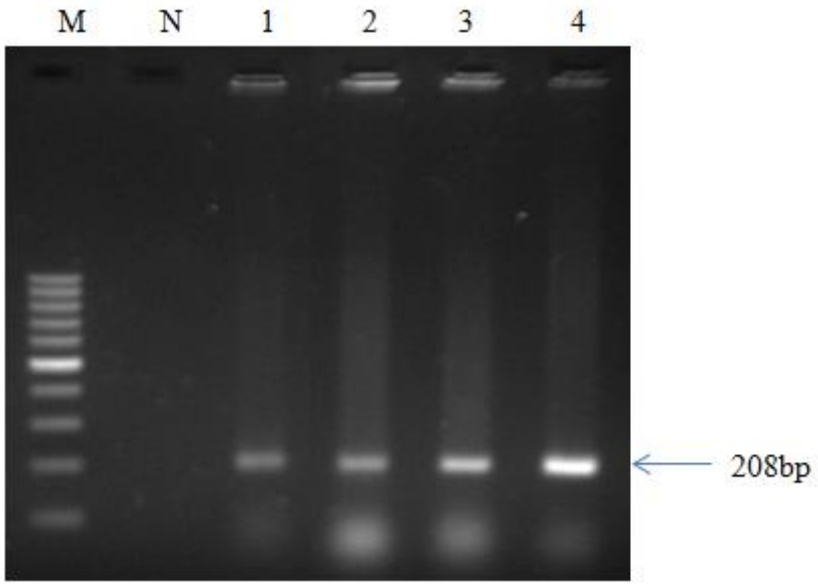


Figure 5.2: PCR product of the ITS region between 16s and 23s rRNA using 1.2% agarose gel

Lane 1 represents ladder 100bp , Lane 2 represents negative control, Lane 3 represents Strain1, Lane 4 represents strain2 and the Product size is 208bp

5.3 Growth characteristics of *Acinetobacter baumannii* strains

A.baumannii gives the maximum absorbance at 600nm which is taken as the standard for determining the growth cells. OD will increase when the cell division increases. According to the study OD of 0.3 has 0.9×10^{7}cells/ml and OD of 1.0 corresponds to 8×10^{6}cells/ml.

Optical density was taken at 600nm for all the strains that were cultured. The graph was then plotted between the OD(y-axis) and the time(x-axis).

The graph expresses the different phases of bacterial growth i.e. Lag, Log and staintionary phases.

Though the initial inoculums taken was more therefore there is negligible lag phase in the graph below. So the graph started from the lag phase and at the 23rd hr it entered the stationary phase.

TABLE 5.1

Sr.No	Time	Strain1	Strain2	Strain3	Strain4
1	0hr	.036	.030	.017	.033
2	2hr	.322	.231	.239	.267
3	4hr	.759	.858	.844	.764

4	6hr	.941	1.088	1.035	.944
5	8hr	1.216	1.438	1.256	1.352
6	12hr	1.380	1.587	1.522	1.489
7	20hr	1.495	1.731	1.672	1.566
8	23hr	1.734	2.144	2.090	1.981
9	27hr	1.808	2.144	2.073	2.005
10	31hr	1.838	2.145	2.071	2.010
11	34hr	1.811	2.111	2.039	1.967
12	44hr	1.772	2.045	1.978	1.921
13	48hr	1.695	1.922	1.867	1.875
14	50hr	1.677	1.897	1.798	1.801

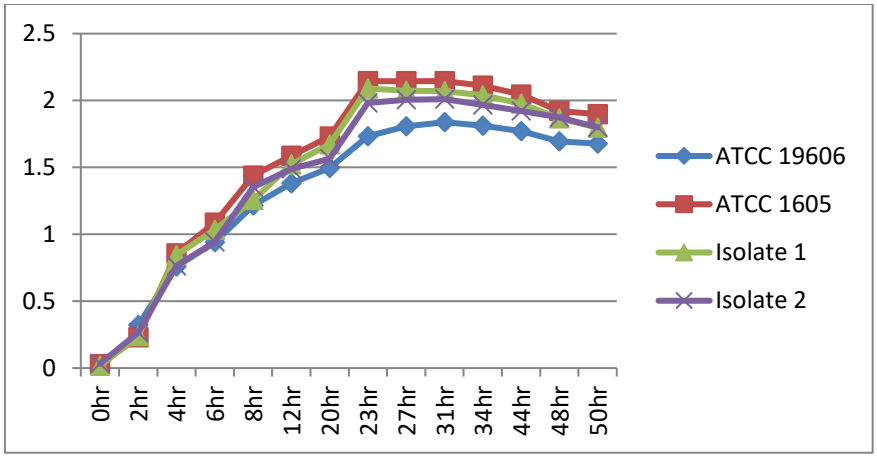


Figure 5.3: Comparative analysis of Growth characterstics of the *Acinetobacter baumannii* strains.

X-axis represents the OD at 600nm whereas y-axis represents the time in hrs. Blue line is showing the growth pattern of ATCC19606, while the red line shows the growth curve of ATCC 1605. The green and purple lines shows the growth pattern of strain 3 and strain 4.

5.4 Amplification of gene responsible for Quorum sensing using PCR

CsuAB gene which is responsible for Quorum sensing in *Acinetobacter baumannii* was amplified using RT PCR. There are many other genes like LUX-I and LUX-r genes responsible for the Quorum sensing in the *Acinetobacter baumannii*.

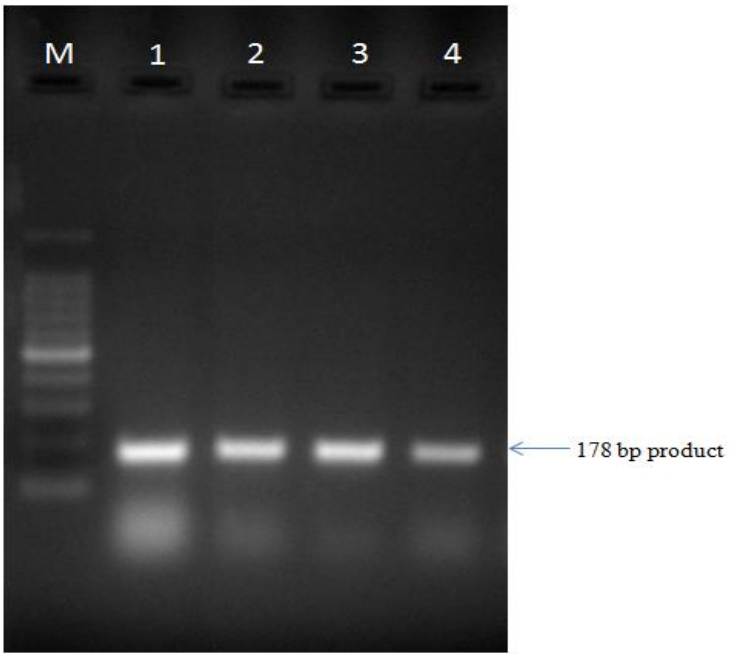


Figure 5.4: RT PCR amplification of *csuAB* in isolates of *Acinetobacter baumannii*

separated on 1.2% agarose where lane M is 100, 1 is ATCC 19606, 2 ATCC 1605, 3 is ATCC 19606 and 4 is strain2.

5.5 Antimicrobial activity of different chemicals against ATCCV25922

5.5.1 Gallic acid

No inhibition was observed using Gallic acid against strain 2 which was taken as control.

5.5.2 Citric acid

No inhibition was observed using Gallic acid against strain 2 which was taken as control.

5.5.3 Plant: *Juglan regia*

No inhibition was observed using Gallic acid against strain 2 which was taken as control.

5.5.4 Quercetin

Quercetin was dissolved in absolute ethanol. Though ethanol has its own antimicrobial activity. The control was already taken. Inhibition was observed against the strain 2 taken as control.

A graph was plotted in between the OD of Quercetin at 600nm (y-axis) and concentration of Quercetin. (x-axis)

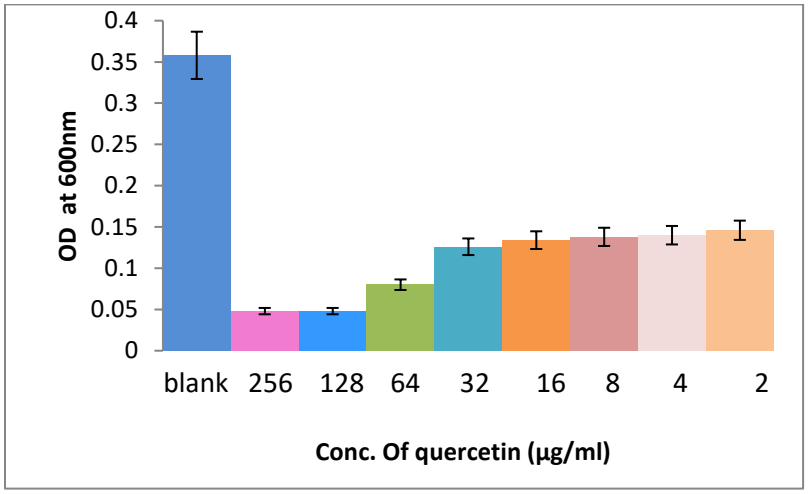


Figure5.5: graph representing inhibition of ATCC25922 with different conc. of Quercetin.

5.6 Antimicrobial activity of different compounds against *Acinetobacter baumannii* strain ATCC 19606

5.6.1 Gallic acid

No inhibition was observed using Gallic acid against ATCC 19606

5.6.2 Citric acid

No inhibition was observed using Gallic acid against ATCC 19606

5.6.3 Plant:*Juglan regia*

No inhibition was observed using Gallic acid against ATCC 19606

5.6.4 Quercetin

Inhibition was observed against the ATCC 19606

A graph was plotted in between the OD of Quercetin at 600nm (y-axis) and concentration of Quercetin.

Qurcetin showed inhibition against *Acinetobacter baumannii* strain.

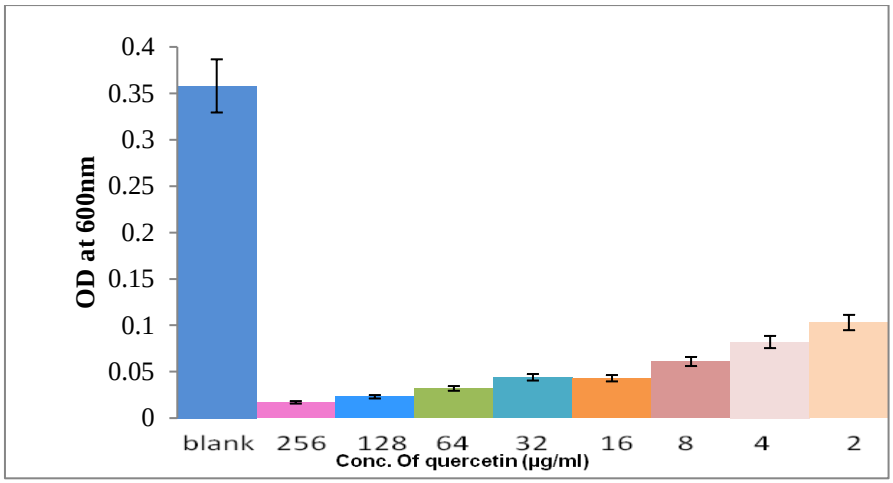


Figure 5.6: Graph representing inhibition of *A. baumannii* ATCC 19606 with different concentration of Quercetin

5.7 Antibiofilm activity

5.8.1 Plant: *Juglan regia* against ATCC 19606

From the antibiofilm activity it was concluded that there is no inhibition observed in *Juglan regia* against ATCC 19606

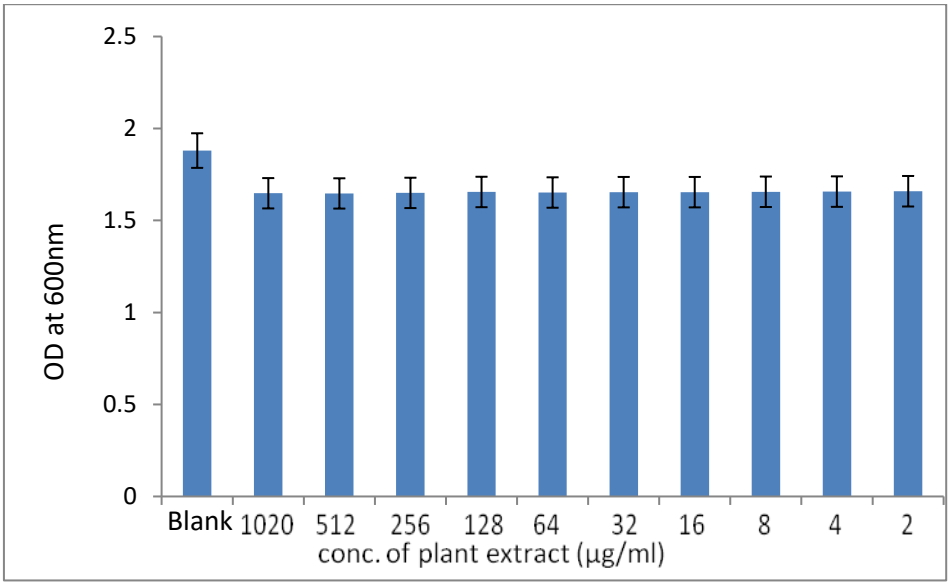


Figure 5.7: Shows the antibiofilm activity of *Juglan regia against* ATCC19606

5.7.2 Quercetin against ATCC 19606

There is no inhibition observed in case of Quercetin against ATCC 19606

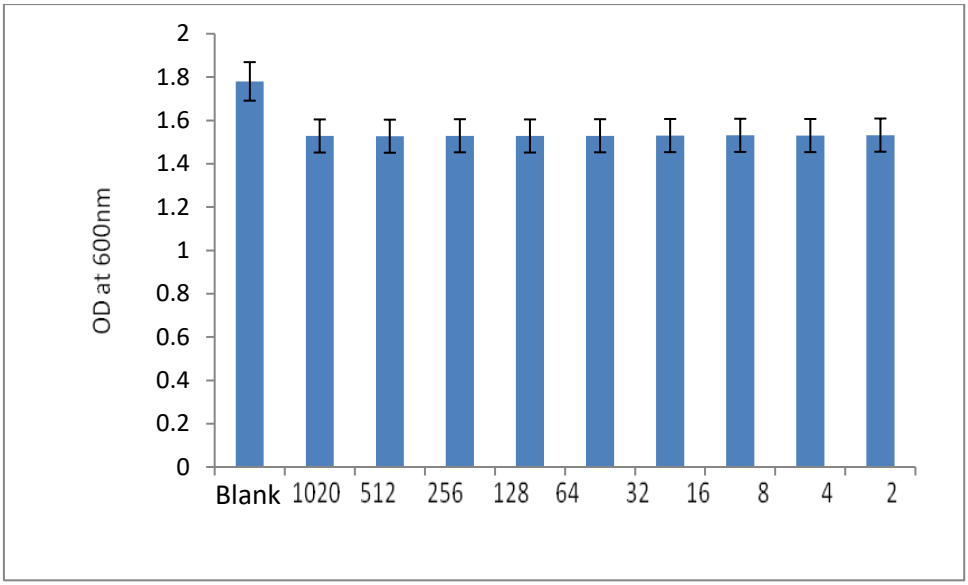


Figure 5.8 : Shows the antibiofilm activity og Quercetin against ATCC 19606

CHAPTER 6: CONCLUSION

There were total 4 strains of *Acinetobacter baumannii* were taken. The molecular screening was done firstly. Then the RNA analysis was done. After that semi Quantitative analysis if *CsuAB* gene was done and the results were shown.

To check the antibacterial activity , the antimicrobial activity was performed. It is concluded that Quercetin showed the inhibition against *Acinetobacter baumannii.* Therefore, it can be anticipated that Quercetin can show the antimicrobial activity against most of the multi-drug resistant bacteria too.

After checking the antibiofilm activity of the compounds. It is concluded that no compound which was taken show the antibiofim activity against *Acinetobacter baumannii.*

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APPENDIX I

1. MULLER-HINTON AGAR

COMPOSITION: (1 litre) ph-7 at 25 degree

Amount (grams)	Compound
2gm	Beef extract
17.5gm	Casein hydrolysate
1.5gm	Starch
17.0gm	Agar

2. MacConkey agar

COMPOSITION: (1 litre) pH - 7.1 +/− 0.2

Compound	Amount (grams)
Peptone	17 g
Proteose peptone	3 g
Lactose	10 g
Sodium chloride	5 g
Bile salts	1.5 g
Neutral red	0.03 g