STUDIES ON BACTERIAL GROWTH INHIBITION USING ANTIMICROBIAL NUTRACEUTICAL COMPOUNDS

A thesis submitted towards partial fulfillment of the requirements for the degree of

Master of Engineering in Bioprocess Engineering

Submitted by

PALLAVI CHAKRABORTY

Registration No.140624 of 2017-2018
Class Roll No.001710303004
Examination Roll No. M4BPE19005

Under the Guidance of

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M.E. (Bioprocess Engineering)
Course affiliated to
Faculty of Engineering and Technology
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Kolkata, India

CERTIFICATE OF RECOMMENDATION

It is to certify that the thesis entitled ***"STUDIES ON BACTERIAL GROWTH INHIBITION USING ANTIMICROBIAL NUTRACEUTICAL COMPOUNDS"*** *is a bonafide work carried out by* ***Ms. Pallavi Chakraborty*** *(Examination Roll No.M4BPE19005., Registration No. 140624 of 2017-18, Class Roll No.001710303004), under my supervision and guidance for the partial fulfillment of the requirement of* ***Master of Engineering in Bioprocess Engineering*** *from Department of Chemical Engineering, Jadavpur University, Kolkata, during the academic session 2017-2019.*

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Committee of Final Examination for Evaluation of Thesis

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Declaration of Originality and Compliance of Academic Ethics

I hereby declare that this thesis contains literature survey and original research work by the undersigned candidate, as part of her Master of Engineering in Bioprocess Engineering course of Department of Chemical Engineering, Jadavpur University for the session 2017 – 2019.

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ABSTRACT

The present investigation illustrates the inhibitory effect of nutraceutical phytochemical allicin containing garlic extract on model laboratory strains i.e. *Bacillus subtilis* (*B.subtilis*) and *Escherichia coli* (*E.coli).* The development of the modern countermeasures of infectious diseases is a real life challenge as pathogens are prone to developing resistance against commercial antibiotics. In this study, quality parameters of freshly prepared garlic extract were studied and it indicated the presence of total protein, flavonoid and phenol content in the extract. The extract was applied at 3 different growth phases on *B.subtilis* and *E.coli*. The comparative study between normal growth at favorable condition and in presence of allicin at MIC and MBC value illustrated that fresh garlic extract (FGE) containing allicin inhibited the growth of both bacterial strains. The best result was obtained at mid exponential phase on *B.subtilis*. The death rate constant was determined by time kill kinetics for only FGE containing allicin and for synergistic combinations. 0.591 hr^{-1} and 0.595 hr^{-1} were the death rate constants for *E.coli* and *B.subtilis* at mid exponential phase respectively after treating them with allicin at MBC value. These were the highest death rate constants obtained by allicin at MBC. Synergistic study of allicin with rifaximin, oxytetracycline, levofloxacin and azithromycin were performed on the designated bacterial strains at lag and mid exponential phase and rifaximin had shown the best result for both strains at mid exponential phase. For synergistic combinations, 0.587 hr^{-1} and 0.378 hr^{-1} were the death rate constants for *E.coli* and *B.subtilis* at mid exponential phase respectively with rifaximin. E_{max} and EC_{50} values of FGE containing allicin were determined by maximum effect model. For *E.coli,* the E_{max} and EC_{50} values were 16% and 0.1 mg/ml respectively.47.6% and 0.14mg/ml were the values of E_{max} and EC_{50} respectively for *B.subtilis*. The maximum effect of allicin was higher against *B.subtilis* and indicated greater potency of allicin against gram-positive bacteria.

Keywords: Nutraceutical, allicin, antimicrobial, death rate constant, E_{max}.

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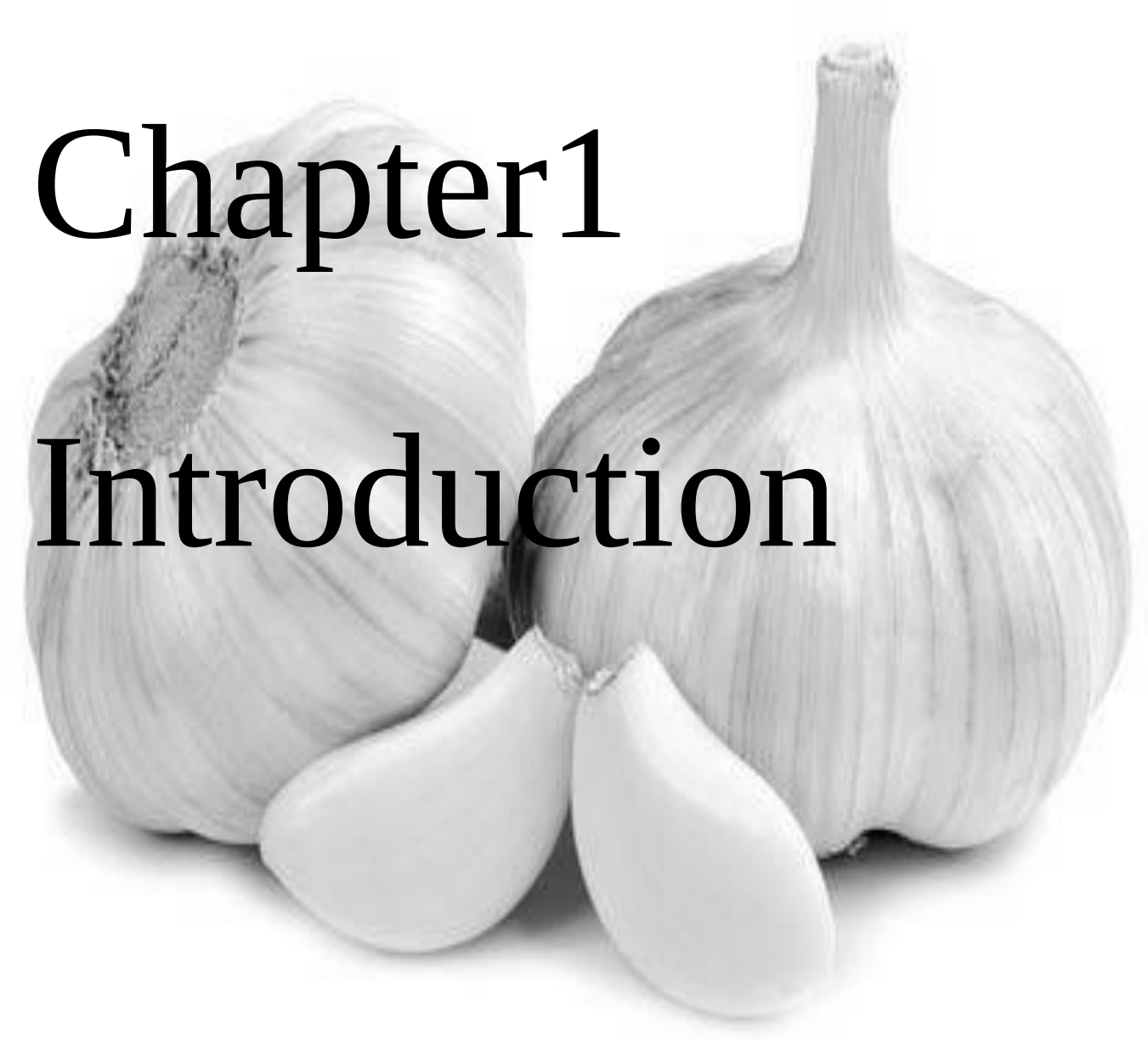
ABBREVIATIONS

CFU	Colony forming unit
MHB	Mueller Hinton broth
MHA	Mueller Hinton Agar
FGE	Fresh garlic extract
rpm	Rotations per minute
ppm	Parts per million
CV	Crystal violet
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
O.D	Optical density

Chapter1

Introduction



1.1General introduction

Over the years, humans are being affected by different diseases and with time the threat is increasing. The outbreak of infectious diseases has increased the urge of discovering new drugs. The emerging risk to human lives has put the continuous pressure of finding new antimicrobials. The reason behind such diseases is different microbes. From ancient time, human body is the shelter for various organisms which can't be detected by naked eyes. There are different bacteria living in the surroundings but not every species is harmful. Some bacteria are often considered infectious due to their capability of damaging the host. The ability of affecting the host is known as the virulence of microbes. They enter into the host's cell by adhesion, colonization or invasion, grow within the host cell and cause infection [1]. They produce toxins in the host's body which leads to different diseases by disrupting the cells of host. To prevent the spread out of a disease and to control the bacterial growth, antimicrobials play an important role. These chemotherapeutic agents are also known as antibiotics and hold a certain level of selective toxicity against pathogens. There are already different antibiotics available in the commercial market to prevent diseases but nowadays mutations in different pathogens are making them antibiotic resistant and increasing their virulence [2]; it causes outbreak of different diseases which is a threat to mankind.

Dreadful resurgence of antibiotic resistant bacteria has attracted attention on the need for aggressive antibiotic discovery. The increasing number of infectious diseases worldwide results in a dire need of alternative antibacterial therapies which are efficient, biocompatible and cost effective. The development of drug resistant bacteria due to mutations has also increased the risk thousand times. Researchers are putting enough efforts to find a potential drug which can treat diseases and has less toxicity and side effects. Considering all the above mentioned characteristics, nutraceutical compounds are suitable candidate as pharmaceuticals and have attracted attentions of researchers .Ancient Indian medicinal system (Ayurveda) provided concepts of pharmacotherapy to treat infectious diseases. Various medicinal plant parts contain numerous types of nutraceutical phytochemicals, like flavonoids,phenols,thiosulfinates, quinones, alkaloids, terpenes and plant antimicrobial peptides(pAMPs) which shows significant antimicrobial properties [3]. These compounds are capable enough to inhibit bacterial growths but due to their instability it's tough to use them commercially. Their mode of action includes

DNA inhibition, enzyme inactivation, cell wall and membrane damage, protein misfolding etc. Nutraceutical compounds are basically food or food ingredients which possess pharmaceutical properties. Allicin is a nutraceutical compound, found in garlic extract. It exhibits inhibition of bacterial growth, lowering of lipid level in blood; it has anti-oxidant properties and anti-carcinogenic in nature. Allicin is a prospective nutraceutical compound to be used as future drug. It is biocompatible, less toxic and easily available but the instability of allicin in adverse temperature, pH and in other solvents, prevents the commercial use of allicin as a drug. Even after having certain limitations, it's easy availability, less toxicity and effective antimicrobial properties have made allicin a suitable candidate for being used as commercial drug.

1.2 Pathogenicity of bacteria

Relationship of two organisms can be complex sometimes; the organism which supports the growth and survival of the other one is known as host. They share a symbiotic relation which includes mutualism, commensalism and parasitism. Parasitism often leads to infectious diseases; several types of parasitism are observed [4]. If an organism lives on the surface of a host it is ectoparasite, if it stays inside the body of the host then it is an endoparasite. Virulent bacteria which create infections are mostly endoparasite. The relation between the host and parasite is dynamic, when a parasite is growing and multiplying in a host body, it leads to infection. These parasites are known as pathogens and their ability to cause diseases is known as pathogenecity [5].

Bacterial pathogenesis is currently a threat to humans. They attack humans and spread the infections. Firstly, they enter the host cells by direct contact from host to host by means of coughing, sneezing and body contacts. There are also other ways, an infected host can shed bacteria into the environment and once they are deposited on any surface, from that they can be transmitted to host by indirect vehicles like soil, water, air and food. After entering into host's body they adhere to and colonize host cells [6]. They reproduce within the cells by invading into the host cell by using their special strategy. To invade the cells they break the cell surface by producing lytic substances that attack the extracellular matrix and basement membranes, break the carbohydrate protein complexes between cells or on the surface. Apart from these, wounds, scratch, lesions on the host's skin surface allow the bacteria to invade easily [7].

After invading cells or tissues, bacterial pathogen finds a suitable place within the cell where it produces and grows rapidly. They multiply and lead to infection.

Two distinct categories of diseases can be recognized based on the role bacteria; one is infections and another one is intoxications. Infections are mostly caused by the reproduction and growth bacteria. Intoxications are the diseases that are caused by the bacterial toxin production while they grow. Toxins are the metabolic products of bacteria which can alter the host cell metabolism and induce diseases. There are two types of toxins:

Exotoxins

Exotoxins are soluble, heat labile and mostly protein in nature. It is released by the gram positive bacteria into the surroundings while they grow [8.].Some gram-negative bacteria can also produce exotoxins. Exotoxins may also travel from the site of infection to other host tissues and can damage them.

Four types of exotoxins are present:

1. AB toxins: It is composed of an enzymatic subunit (A) that is responsible for the toxic effect and a binding subunit (B). A subunit lacks the binding and cell entry capability and B subunits bind to target cells but are nontoxic, biologically inactive.
2. Specific Host Site Exotoxins: They are categorized on the basis of the site affected; neurotoxins (nerve tissues), enterotoxins (intestinal mucosa) and cytotoxins (general tissues) [9].
3. Membrane Disrupting Toxins: This type of Exotoxins disrupts the cell membrane like hemolysins, leukocidins and phospholipase enzymes.
4. Superantigens: superantigens are bacterial proteins that can provoke as many as 30% of a person's T cells to release massive concentrations of cytokines. Excessive production of cytokines stimulates endothelial damage, circulatory shock and multiorgan failure.

Endotoxins

Gram-negative bacteria have lipopolysaccharide (LPS) in the outer membrane of their cell wall that is toxic to specific hosts. This LPS is known as endotoxins and it is attached to the bacterium and released when the microorganisms lyses [10].

Endotoxins are heat stable, toxic and generally capable of producing systematic effects like fever, shock blood coagulation, weakness, diarrhea, inflammation. Intestinal hemorrhage and fibrinolysis (enzymatic breakdown of fibrin).

Endotoxins induce fever in the host by causing macrophages to release endogenus pyrogens that reset the hypothalamic thermostat [11].

1.3 Host defense mechanism and evasion by bacteria

To prevent the invasion of bacteria, firstly the first line of defense of host gets activated. These are the physical and chemical barriers for the bacteria like multilayered skin, mucous membrane with cilia, pH regulations of stomach and others. But this defense mechanism is often unsuccessful to prevent the entry of pathogens into the host cell. After the invasion of bacteria into the cells, host activates the innate immune system; it is rapid and independent of antigens. It includes chemical mediators like cytokines, activation of complement cascade to identify bacteria and participation of white blood cells to kill the pathogens [12]. Complement system is composed with serum proteins and serves as a defense system against bacteria by activating phagocytosis through opsonization, chemotaxisand activation of leukocytes and lysis of bacterial cell wall [13].

Another type of immune response by the host is adaptive immunity; it takes time to be developed but it is an efficient mechanism to prevent the invaders. This specific immune system identifies the foreign element, develops immunity against it and remembers the invader.

Acquired immunity is one type of adaptive immunity; naturally acquired active immunity occurs when a person comes in a contact of foreign stimulus (antigen) and develops immune response against it [14]. Naturally acquired passive immunity happens when one host transfers the antibodies to the other host. Artificially acquired immunity is the process of developing immune

response against a specific pathogen through immunization. Adaptive immune system is composed of specialized cells like T cells and B cells and antibodies are the glycoprotein which is produced by the activated B cells [15].

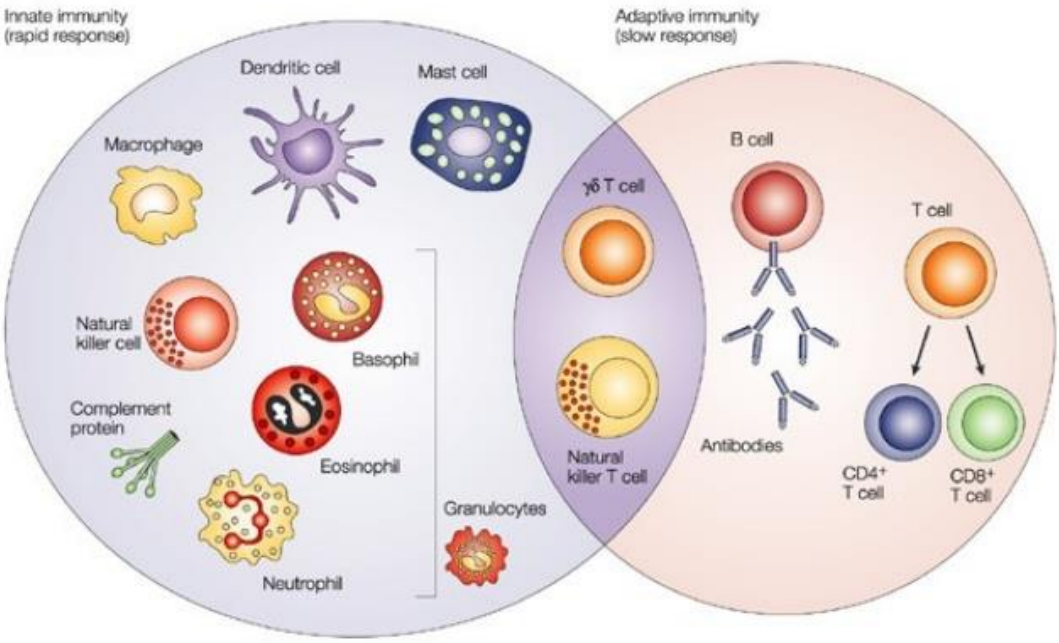


Figure 1.1: Host immunity system

Source: Nature Reviews Cancer, 4, 11-22, copyright 2004.

But even after so many host defense mechanisms, some bacteria are capable to invade in host cells and to create diseases. Bacteria also have evolved many mechanisms to evade the host defense.

To evade the activity of complement system, some bacteria have capsules that prevent complement activation. Some gram negative bacteria also lengthen O chains in their lipopolysaccharide which inhibits the formation of membrane attack complex.

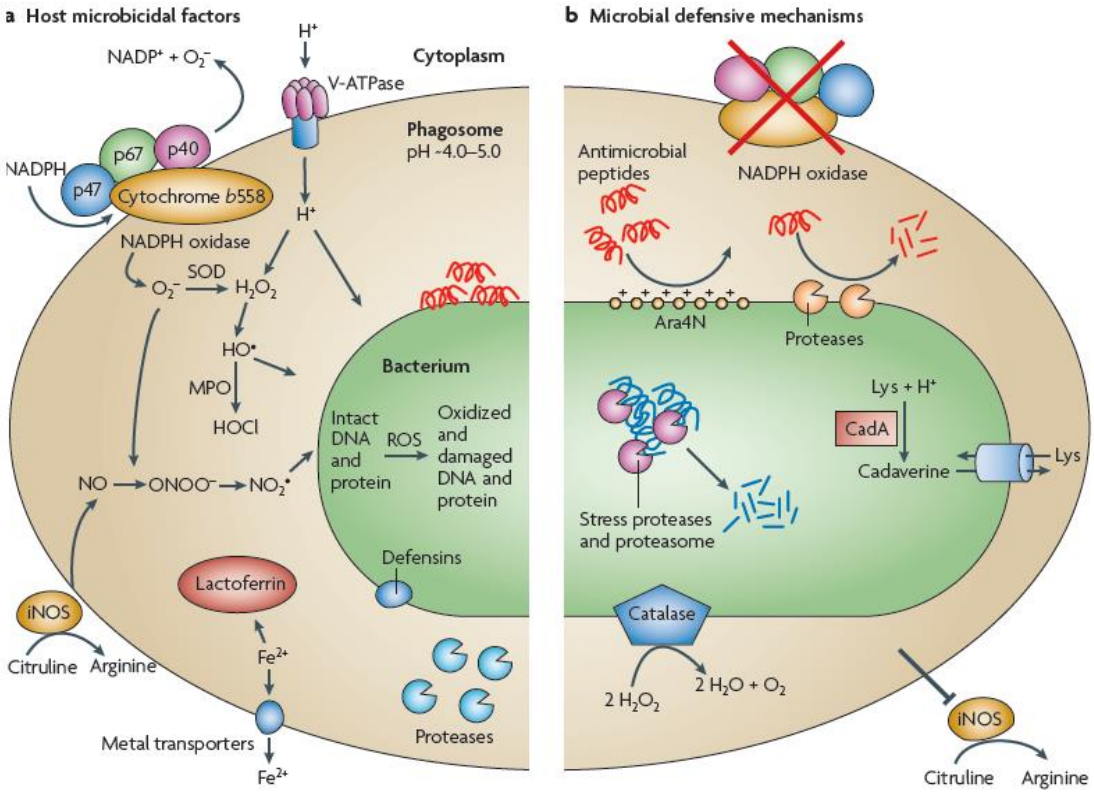


Figure 1.2: Host defense mechanism and microbial defensive mechanism

Source: Antimicrobial mechanisms of phagocytes and bacterial evasion strategies Ronald S. Flannagan, Gabriela Cosío* and Sergio Grinstein*

Bacterial defensive mechanisms also include modification of their surface to resist or break down antimicrobial peptides, and expression of enzymes, such as catalase, that convert reactive species to less harmful compounds or prevent recruitment of the protein complexes that synthesize reactive nitrogen species (RNS) or reactive oxygen species (ROS) [16] .They are also capable of resisting phagocytosis by inhibiting the contact of phagocytic cell to the surface of bacteria. Even some bacteria have evolved the ability to survive inside neutrophils, monocytes and macrophages. To avoid the specific immune response, they may undergo genetic variations so that specific antibodies are useless against it. Even they can also produce certain chemicals which can destroy the antibodies [17]. So, basically some microbes have evolved certain mechanisms to avoid host defense and sustain within the host cell, that's why invention of antibiotic was an essential move.

1.4 Overview of antibiotics

The term antibiotic is coined from Greek words anti and bios where anti stands for against and bios means life. Antibiotics are mostly secondary metabolites, produced by microbes during their stationary phase of growth. They can kill susceptible microorganisms or inhibit their growth.

Some bacteria and fungi are able to produce many of the employed antibiotics. Several important chemotherapeutical agents such as sulfon-amides, chloramphenicol, ciprofloxacin, isoniazide and dapson are synthetic antibiotics and manufactured by chemical processes independent of microbial activity. Some antibiotics are semisynthetic in nature; they are natural antibiotics which are modified structurally by addition of chemical groups. Semisynthetic antibiotics are less susceptible to inactivation by pathogens [18].

These drugs vary in their range of effectiveness; narrow spectrum drugs are effective only against a limited variety of pathogens. Others are broad spectrum in nature which attack different microbes.

Drugs can be classified also based on the general microbial group they act against: antibacterial, antifungal, antiprotozoan and antiviral. Also these agents can be classified as static or cidal agent. Static agents inhibit growth and cidal agents kill the target pathogen.

These drugs inhibit the growth of bacteria by inhibiting their cell wall synthesis, nucleic acid synthesis and protein formation.

To prevent cell wall formation of bacteria, antibiotics activate the lytic enzymes or inhibit transpeptidation enzymes involved in formation of bacterial cell wall. They prevent protein synthesis by binding with ribosomal subunits and by blocking DNA replication and transcription, antibiotic inhibits nucleic acid synthesis [19].

Table 1: Some popular antibiotic groups and their mechanism of action

Mechanism of Action	Antibiotic Group	Primary Effect	Specific Mode of Action
Cell Wall Synthesis Inhibition	Penicillins	Cidal	Inhibit transpeptidation enzymes involved in cross linking the polysaccharide chains of bacterial cell wall.
			Activate cell wall lytic enzymes.
	Cephalosporins	Cidal	Same as above.
	Vancomycin	Cidal	Prevent transpeptidation of peptidoglycan subunits by binding to D-Ala-D-Ala amino acids at the end of peptide cross bridges.
Protein Synthesis Inhibition	Aminoglycosides	Cidal	Bind to small ribosomal subunit (30S) and interfere with protein synthesis by directly inhibiting protein synthesis and by misreading mRNA.
	Tetracyclines	Static	Same as above.
	Macrolides	Static	Bind to 23s rRNA of large ribosomal subunit (50S) to inhibit peptide chain elongation during protein synthesis.
	Chloramphenicol	Static	Same as above.
	Quinolones and	Cidal	Inhibit DNA gyrase and

	Fluoroquinolones		topoisomerase IV for blocking DNA replication and transcription.
Inhibition of Nucleic Acid Synthesis	Rifampin	Cidal	Inhibit bacterial DNA dependent RNA polymerase.
	Polymyxin B	Cidal	Binds to plasma membrane and disrupts its structure and permeability properties.
Cell Membrane Disruption	Sulfonamides	Static	Inhibits folic acid synthesis.

The above mentioned antibiotic groups are widely used to treat bacterial infections but there are certain limitations on the usage of these antibiotics which have increased the need of developing new drugs.

Disadvantages

Drug resistance

The sales of antimicrobial drugs are a huge business in world market. The massive quantities of antibiotics are being prepared and used but due to spread out of new diseases and the bacterial strains are turning drug resistant which has decreased the value of commercial antibiotics. For example, earlier sulfonamide was used for treating gonorrhea but after a long time use the bacteria *Neisseria Gonorrhoeae* had gained resistance against sulfonamides. Then physicians turned to penicillins to treat this disease but within 16years a penicillin resistant strain emerged in Asia [20]. Thus, penicillin is no more in use to treat gonorrhea. In 1946, almost all strains of *Staphylococcus* were penicillin resistant. This situation is alarming, that all bacterial strains are gaining resistance against the widely used antibiotics.

The situation is made worse by using antibiotics almost in treating every situation, as the patients are not completing the course of antibiotics properly; it has increased the survival chance of drug

resistant strains. The use of antibiotics in animal feed is also another contributing factor in increasing the drug resistance; it has actually increased the number of drug resistant bacteria in animals like cattle, pig and chickens [21].

There are also other factors like mutations in the bacterial genes and also horizontal transfer of antibiotic resistance genes among strains from the same of different species. Two bacteria may use different mechanisms to withstand a drug. Some gram negative bacteria have gained resistance against penicillin as it cannot penetrate the outer membrane of bacteria's envelope. In *E.coli* there is an efflux system in their cells which can pump out the drug after it enters [22].
So, in reality different bacteria has developed different mechanism to fight the chemotherapeutical agents.

Side effects of drugs

The drugs we use are either a product of microbial growth or they are produced by chemical processes so, they can have harmful side effects on the hosts. The therapeutic index is the ratio of toxic dose to the therapeutic dose; it determines the drug level at which it becomes too toxic for a host [23]. Sometimes, a drug can inhibit the same process in a host cell and leads to undesirable effects. These side effects can be severe and can lead to the death of hosts even. So, drugs should be administered with care.

Table 2: Side effects of some popularly used antibiotics

Name of the antibiotics	**Members**	**Side Effects**
Penicillins	PenicillinG, penicillin v,methicillin,ampicillin ,carbenicillin	Allergic responses. Nausea, diarrhea, anemia, renal toxicity.
Cephalosporins	Cephalosporin,cefoxitin,cefaperazone	Allergic responses, thrombophelbitis, renal injury.
Vancomycin	Vancomycin	Ototoxic(tinnitus and deafness) , nephrotoxic, allergic reactions

Aminoglycosides	Neomycin,kanamycin, gentamycin, streptomycin	Deafness, renal damage, loss of balance, nausea, allergic responses
Tetracyclines	Oxytetracycline, chlorotetracycline	Deafness, renal damage
Macrolides	Erythromycin, Clindamycin	Gastrointestinal upset, teeth discoloration, renal, hepatic injury
Chloramphenicol	Chloramphenicol	Bone marrow malfunction, allergic reactions
Quinolones and Fluroquinolones	Norfloxacin, ciprofloxacin, levofloxacin	Tendonitis, headache, lightheadness,convulsions, allergic reactions
Rifampin	R-cin,rifacilin, rifamycin, rimactane,rimpin	Nausea,vomiting,diarrhea,fatigue,anemia,drowsiness,headache,mouth ulcer, liver damage
Polymyxin B	Polymyxin B, polymyxin topical oinment	Can cause severe kidney damage, drowsiness, dizziness
Sulfonamides	Sulfisoxazole, Sulfamethoxazole, Sulfanilamide, Silver sulfadiazine	Nausea, vomiting, diarrhea; hypersensitivity reactions.
Dapsone	Dapsone	Back leg or stomach pains; discolored fingernails, lips or skin; breathing difficulties, fever, loss of appetite, skin rash and fatigue.

The drugs which are widely used to treat diseases have certain disadvantages. Drug resistance of bacteria, toxicity and side effects of antibiotics has increased the need of developing new drugs.

1.5 Nutraceutical compounds

Nutraceutical compounds have emerged as an alternative of present commercially used antibiotics. The Hippocrates of Kos (a Greek physician) said "*let food be your medicine and medicine be your food*," in ancient Indian medicinal system (Ayurveda) lot of emphasis was given to plant extracts to cure diseases. The term nutraceutical was coined by Dr. Stephen De Felice, MD, USA; "nutraceutical" is any substance that may be considered a food or part of a food which provides medical or health benefits, encompassing, prevention and treatment of diseases [24]. Recently scientifically supported nutritional and medical evidence has allowed nutraceuticals to emerge as being potentially effective as drugs, though the use of nutraceutical by people has a long history [25]. Health Canada, a government authority of Canada, has defined nutraceutical as a compound which is produced from food but sold in the form of powder or pill, has medicinal properties and doesn't hold any direct relation with foods [26]. These compounds work as therapeutic agents and mostly help to treat different diseases from common cold and cough to cancer. It prevents certain cancer, blood pressure, sugar, cholesterol level, and osteoporosis [27]. Broadly, nutraceuticals can be classified into two groups' potential nutraceuticals and essential nutraceutical. Only after enough clinical evidence one potential nutraceutical can turn into an essential one.

The common ingredients in cooking which we use on a daily basis hold pharmaceutical properties. Spices like garlic, onion, curcumin and red pepper have potential health benefits. Various medicinal plant parts contain numerous types of nutraceutical phytochemicals, like flavonoids, phenols, thiosulfinate, quinones, alkaloids, terpenes, and plant antimicrobial peptides (pAMPs) which delivers significant antimicrobial properties. They kill or inhibit the growth of bacteria by DNA inhibition, cell wall and membrane damage, protein denaturation etc. These extracts have also shown promising results in synergy with modern antibiotics by increasing their effect against several pathogens which have become drug resistant. Several research works indicated that these extracts are very effective against pathogens and drug resistant bacteria like Methicillin resistant *Staphylococcus aureus* (MRSA), uropathogens, *Klebsiella*, *Escherichia coli* [28].

Table 3: Some plant compounds with antimicrobial activities

Compounds	Examples	Mechanisms
Simple phenols	Catechol,epicatechin	Substrate deprivation, membrane disruption
Quinones	Hypericin	Adhesin binding,complex with cell walls, enzyme inactivation
Flavonoids	Chrysin	Adhesin binding
Flavones	Abyssinone	Enzyme inactivation, HIV reverse transcriptase inactivation
Tannins	Ellagitannins	Protein binding, adhesin binding, membrane disruption, complex with cell walls, metal-ion complexation and others.
Terpenoids,essential oil	Capsaicin	Membrane disruption
Alkaloids	Berberine,piperine	Intercalation into cell wall or DNA
Thiosulfinates	Allicin	Inactivation of crucial metabolic enzymes through s-allylmercapto modification of cysteines.

Source: Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev. 1999 12(4):564-82.

Cardiovascular diseases (CVD) are one of the leading causes of mortality in worldwide and the reason behind premature deaths, so effective drugs are needed to prevent deaths by CVD. Nutraceuticals also serve as lipid lowering agents which help in preventing cardiovascular diseases by decreasing cholesterol levels. Plant sterols, garlic extract take active participation in cholesterol lowering. Plant sterols inhibit absorption of cholesterol in intestine and garlic extract

stops the absorption in liver results in excretion of cholesterol [29]. So, it is evident enough that nutraceuticals have potential health benefits.

To use widely, a drug needs to have certain qualities; Apart from being an efficient therapeutic agent to treat diseases, it should also be stable in nature, shelf life of a drug should be high enough and it needs to have less toxicity. Nutraceutical compounds have less toxicity and side effects, thus administering these compounds as drugs won't harm the hosts. Although, after having so many benefits over commercial drugs, it has certain limitations which is preventing its use .Nutraceutical compounds are unstable in nature. They degrade very quickly and sensitive to pH and temperature. It's tough to synthesize them chemically and possesses low stability which restricts their commercial use. But, in this modern era, when the urgency of alternative medicine is high, natural plant derived compounds can provide useful source of antimicrobials to mitigate the challenges which may provide prospective biopharmaceutical industries products [30].

1.6. Overview of allicin

Allicin is an organosulfur thiosulfinate compound. It is the main bioactive compound found in garlic extract and has potential health benefits. When intact garlic tissues are damaged by crushing or cutting, alliin is converted into allicin by the enzyme allinase [31].

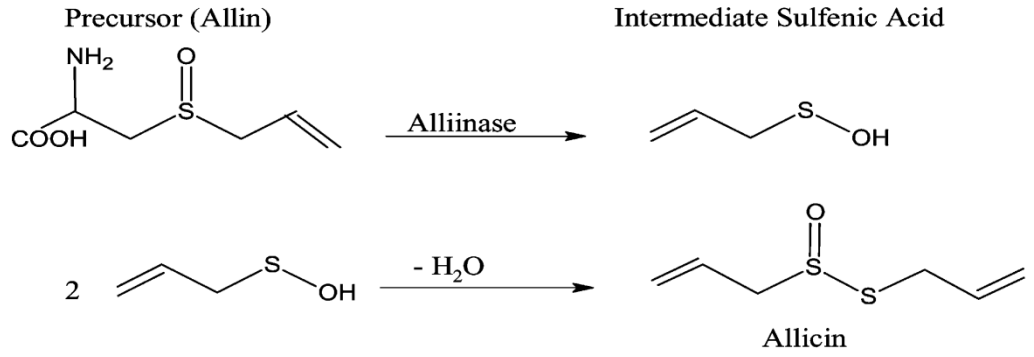


Figure 1.3: Formation of allicin from allin

Source: Bose, S., Laha, B. and Banerjee, S., 2014. Quantification of allicin by high performance liquid chromatography-ultraviolet analysis with effect of post-ultrasonic sound and microwave radiation on fresh garlic cloves

It exhibits antibacterial activities against a wide range of gram-positive and gram-negative bacteria. Apart from antibacterial activity it helps in lipid-lowering, it has anti-blood coagulation, anti-hypertension, anti-cancer, antioxidant and anti-microbial properties. It is also capable of inhibiting cancer promotion by inducing apoptosis in cancer cells.

Antimicrobial activity of allicin

Since ancient times mankind has had to face many different kinds of disease and there was much speculation about their causes. In World War I extracts of garlic were used in antibacterial and antiseptic therapeutics. Numerous scientific studies concerning the antibacterial potential of garlic have been published till date.

It was clearly shown by Cavallito and Bailey, in 1944, [32] that allicin is almost exclusively responsible for the antibiotic properties of garlic but the mode of action of allicin is not fully known. To achieve antibacterial activity, it must be able to get inside the microbial cell. In the case of bacteria, an antibiotic has to penetrate the bacterial cell wall and the cell membrane. In addition to these two boundaries, the slime layers or capsules of certain bacteria can constitute an extra layer of resistance. After entering the cell, it depends on the potentiality of allicin to reach and react with the target. Cavallito and coworkers investigated the chemistry of several plant-derived antimicrobial compounds. It was found that the active principles of these plants and even non plant derived antibiotics like penicillin react with cysteine. Scientists found that pretreatment of these antibiotics with cysteine led to a total loss of activity against bacteria [33].it was found that allicin reacts with every cysteine group as long as the –SH-group was freely available.

As allicin reacts readily with all free cysteine residues available, it is buffered by proteins and low molecular weight thiols, independently of their importance for cellular viability that's why it ends up with a lower efficiency than other antibiotics. Several case studies have also reported the reactivity of allicin with thiol-containing enzymes [34]. Some scientists used allicin to inhibit the activity of several enzymes in vitro. Among them were important enzymes for primary metabolism like hexokinase, triosephosphate dehydrogenase or alcohol dehydrogenase. Even allicin inhibits some enzymes which cannot contain thiol group.

It suggests that not only cysteine is a potential target for allicin. Also it can inhibit some important enzymes.

Apart from these, allicin also reduces lipid level by lowering serum cholesterol and triglyceride. It stops the absorption in liver results in excretion of cholesterol.

Being a thiosulfinate, allicin is a reactive sulfur species (RSS) and is physiologically active in microbial, plant and mammalian cells. In a dose-dependent manner allicin can inhibit the proliferation of both bacteria and fungi or kill cells outright, including antibiotic-resistant strains [35].

Allicin shows antimicrobial activity and even in combination with other antibiotics it decreases the minimum inhibitory concentration (MIC) against some specific bacteria. Research shows that the fresh garlic extract can also improve the efficiency of other antibiotics and can work in synergistic combinations. Spread out of different infectious diseases and the increased number of mutated, antibiotic resistant bacteria strain have emerged the need of developing new drugs. Looking at the potentiality of allicin, it can be stated that in future it has possibilities to be used as a drug.

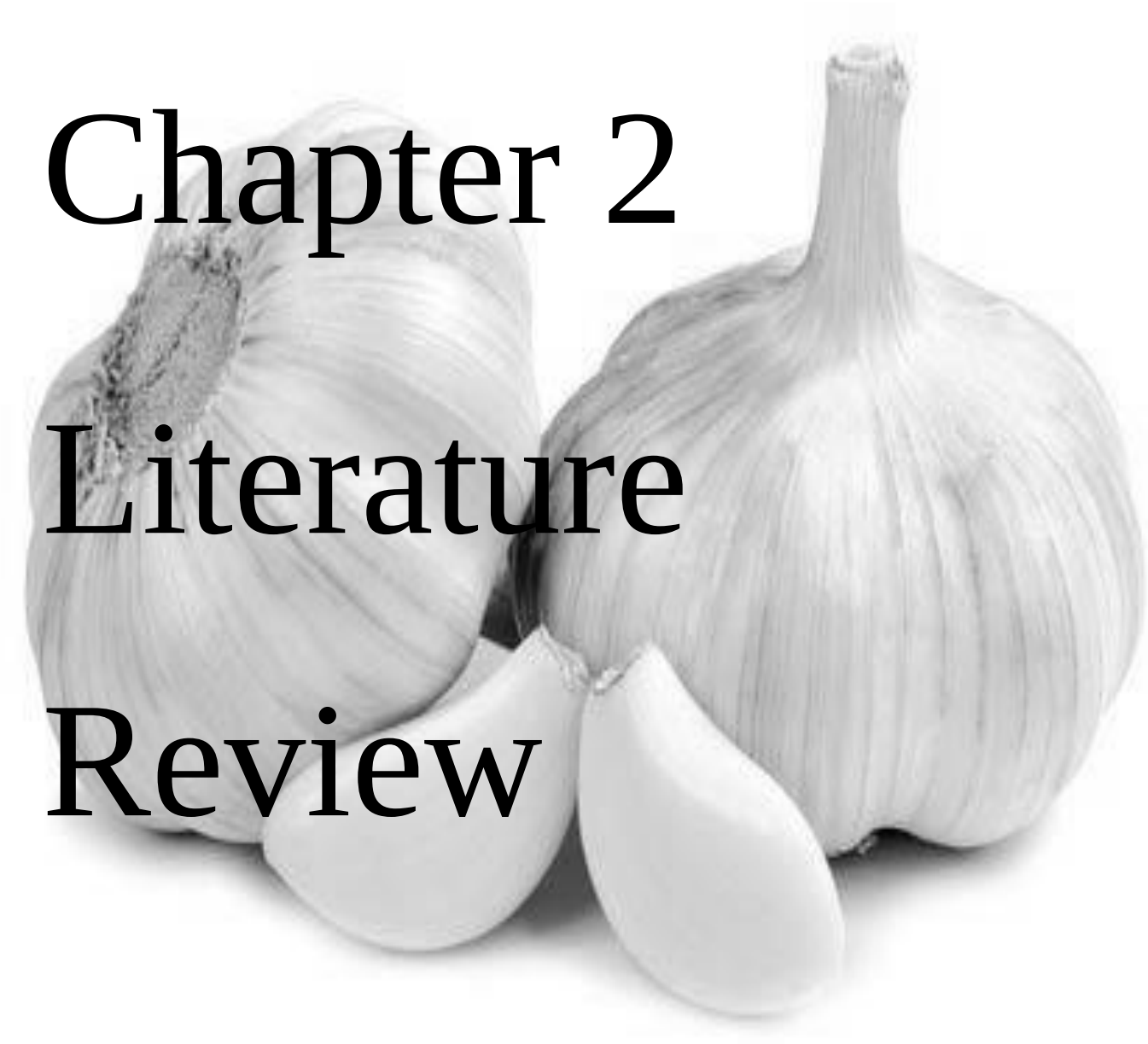
Nowadays, there is a continuous threat of infections caused by the multidrugresistant (MDR) strains of bacteria. To prevent such infections there are two ways either to form new drugs or to formulate new synergistic combinations using different commercially available antibiotics, or to combine an antibiotic with active phytochemicals that have antimicrobial properties. Several in vitro studies have reported synergistic effects with significant reduction in the minimum inhibitory concentrations (MIC) of the antibiotics, resulting from the combination of different antibiotics with different crude plant extracts against *Staphylococcus aureus (S. aureus)* strains[36], and emerge as the real sources of potential resistance modifying agents. In addition to that, synergistic effects of different phytochemicals have been reported against gram negative bacteria [37]. Allicin is a potential candidate to treat MDR. Even it is also proven that in combination with different antibiotics, it helps to increase the efficiency of other antimicrobial agents.The ability of plant extracts to potentiate antibiotics has not been well explained. It is predicted that inhibition of drug efflux and alternative mechanisms of action could be responsible for the synergistic behavior.

Allicin is easily available and cost efficient. Most antibiotics are synthesized chemically or they are the product of microbial growth, so they hold side effects and are toxic in nature. Misuses of antibiotics have increased the toxicity level in humans, thus, finding an alternative is important. Allicin is a compound formed in garlic and has negligible toxic effect. Its biocompatibility, less toxicity and almost zero side effects have escalated its importance.

However, in spite of having numerous positive properties, the use of allicin is limited because of its unstable nature. After generation of allicin it easily degrades in adverse temperature, pH or in presence of any other solvents. The lower stability of allicin is one of barriers in its commercial use as a drug [38]. It prevents the wide use of allicin as an antimicrobial agent though the efficiency of allicin makes it an important contributor as a future drug.

Chapter 2

Literature Review



2.1 Review on extraction, detection of allicin in garlic and stability of allicin

2.1.1 Extraction and detection of allicin

Allicin has attracted the attention of researchers due to its biocompatibility and easy availability. When garlic cloves are crushed or chopped by an external mechanical pressure, the enzyme allinase, present in garlic converts allin into allicin. Allicin degrades quickly in the environment after extraction due to change in pH, temperature and ion levels. There are other factors which also might take part in degradation of allicin. Researchers were studying the efficiency of allicin and observed varying results indicating the role of different parameters involved in allicin extraction and stability. This literature review is about the extraction and detention procedures of allicin by different methods and stability of allicin with varying parameters.

In the year 2000, Bocchini *et al.* reported [39] an analytical method for determination of allicin (diallyl thiosulfinate) in garlic (Allium sativum L.) samples using reversed-phase HPLC with both UV and electrochemical detection (ED) and on-line post-column photochemical reaction. They synthesized standard allicin and its behavior at the chosen analytical condition was verified. They observed that the post-column irradiation at 254nm decreased the response of allicin to UV detector but it also allowed the determination of this compound in electrochemical detector though the compound was electrochemically inactive. They obtained the detection limits of 0.1 and of 0.01mg/l using UV and ED detectors respectively and found the linearity of response in the range of 1–8mg/l.

In the year 2004, Rybak *et al.* described [40] a quantitative method of determining allicin quantity in garlic. They used standard additions of allin and converted to allicin in situ by endogenous allinase (L-(+) - S-alk(en)ylcysteine sulfoxide lyase). They achieved complete conversion of allin to allicin by making additions after homogenization-induced conversion the naturally occurring cysteine sulfoxides to thiosulfinates had taken place, thus eliminating the likelihood of competing reactions. They determined the value of allicin in samples of fresh garlic and commercially available garlic powders by supercritical fluid extraction (SFE) and high performance liquid chromatography extraction with UV-VIS absorbance detection. They observed optimum CO2-SFE conditions provided 96% recovery for allicin with precision of 3%

(RSD) for repeat samples, incorporation of an internal standard (allyl phenyl sulfone) in the SFE resulted in an improvement of recovery (99%) and precision was 2%.

In 2012, Olech and Zaborska proposed [41] a spectrophotometric method to determine the thiosulfinate concentration in fresh garlic extract based on the reactions between thiosulfinatesand selected chromogenic thiol compounds: 2-mercaptopyridine (2-MP), 4-mercaptopyridine (4-MP), 1-oxide-2-mercaptopyridine (MPO), 2-mercaptopyrimidine (MPM) and 2-nitro-5-thiobenzoic acid (NTB). They studied the kinetics of the reaction and determined the order and rate constant. They determined that all reactions were of first order with respect to the thiol and 1st order with respect to thiosulfinates. As a result they run all tested thiols with garlic thiosulfinates in accordance with second order kinetics. They found varying reaction rates of thiol compounds: for 2-MP the constant rate was 6.7 (mol/L)-1.min-1 and 1129 (mol/L)-1.min-1 for NTB.

In the year 2014, Campomanes *et al.* performed [42] pressurized liquid extraction (PLE) in presence of ethanol as a solvent at 313K and 6MPa to extract allicin. The global yield of garlic PLE was 1.3 ± 0.3% on a wet basis. The garlic PLE extract had the highest allicin concentration (332 ± 5 μg of allicin.g-1 of sample) and they also detected presence of allicin in garlic powder and fresh garlic but not in garlic oil.

2.1.2 Stability of allicin

In 1989, Yu *et al.* studied [43] effects of pH adjustment after blending with 30-min standing and heat treatment of garlic cloves on the stability and the formation of volatile compounds of garlic by gas chromatography. They found that the amount of the two isomeric cyclic compounds 3-vinyl-4H-1,2-dithiin and 2-vinyl-4H- 1,3-dithiin, which were artifacts from allicin, decreased with increasing pH values though the amount of diallyl disulfide, propenethiol, propyl allyl disulfide, and diallyl sulfide increased with increasing pH values. They also observed that formation of diallyl trisulfide, methyl allyl trisulfide, 1, 3-dithiane, 2, 4-dimethylfuran, aniline, and trans-l-propenyl methyl disulfide were favored in neutral conditions, whereas formation of methyl allyl disulfide, methyl allyl sulfide, 1, 2-epithiopropane, and methyl propyl disulfide were favored around pH 9.

In 1989, His-Yu and May-Wu showed [44] Stability of allicin in garlic juice. It was evaluated by gas chromatography (GC) and non-heating HPLC. They observed that allicin in garlic juice decreased to a non-detectable amount after 144 hr at 40°C. They reported ,due to heating effects, allicin, in GC analysis, was converted to 2-vinyl-[4H]-1,3-dithiin and 3-vinyl-[4H]-1,2-dithiin but not 3-vinyl-l,2-dithi-4-ene as reported by other investigators too. By HPLC analysis it was shown that unheated garlic juice did not contain detectable amounts of 2-vinyl-[4H]-1, 3-dithiin and 3-vinyl-[4H]-1, 2-dithiin.

In the year 2002, Shen *et al.* used [45] model reaction system to generate pure thiosulfinates from S-alk(en)yl-L-cysteine sulfoxides to facilitate studies on the intrinsic pH and thermal sensitivities of individual thiosulfinate species. Thiosulfinate decay could be characterized as first-order processes over the pH range of 1.2-9.0 and at 20-80 °C. The stability of thiosulfinates was greatest at pH 4.5-5.5, followed by pH 1.2, pH 6.5-7.5, and pH 8.0-9.0. Thiosulfinates with longer and saturated alk(en)yl groups were generally more stable than those with shorter and unsaturated alk(en)yl groups. Thiosulfinates underwent thioalkyl-exchange reactions at pH 8-9 without loss of total thiosulfinate levels within 60-90 min at 20 °C.

In 2005, Lawson and Gardner, designed [46] an experiment to compare the effects of crushed fresh garlic and two types of garlic supplement tablets on serum lipids and characterized on the basis of composition ,stability of suspected active compounds, and availability of allylthiosulfinates (mainly allicin) under both simulated gastrointestinal conditions and in vivo. They measured the bioavailability of allylthiosulfinates from the tablets and found it to be complete and equivalent to fresh garlic extract.

In the year 2008, Fujisawa *et al.* cited [47] instability of garlic derived allicin in various aqueous and ethanolic solutions as well as in vegetable oil through chemical and biological analysis. They observed that crushed fresh garlic cloves generated antibacterial activity and allicin. It declined on a daily basis in aqueous and ethanolic solutions at room temperature showing biological and chemical half lives of 6 and 11days respectively and more stable in 20% alcohol than water but extremely unstable in vegetable oil (biological and chemical half lives of 0.8hr and 0.31hr).

In the year 2014, lee *et al.* investigated [48] factors influencing the stability of thiosulfinates in garlic to improve understanding of the antimicrobial activity of garlic in food preservation. They observed as temperature, pH, the garlic concentration and the liquid oil content increased, the stability of thiosulfinates decreased. They also reported that thiosulfinates were more unstable as the degree of unsaturation of fatty acids increased. They found that solid fatty acid did not affect the stability of thiosulfinates and also other major food components, such as free amino acids, proteins, and carbohydrates did not influence the stability of thiosulfinates. They concluded that garlic can be used as a natural preservative for food items with low pH values and with low oil contents stored at low temperatures.

In the year 2014, Wang et al. displayed [49] that allicin is sensitive to temperature and pH of the storage. They showed that at room temperature, allicin in aqueous extract was most stable at pH5–6 but degraded quickly at lower or higher pH. It began to degrade within 0.5 h and was not detectable after 2 h when the pH was higher than 11 or lower than 1.5. It started degrading at a temperature of more than 40 °C and mostly in higher 70 °C but at room temperature allicin in water could be stored up to 5 days.

2.2 Antimicrobial activity of allicin

The antimicrobial activity of allicin has long been recognized; even other thiosulfinates and their transformation products also have shown antimicrobial activity. Allicin is inhibitory against almost all tested microorganisms such as bacteria, fungi, viruses, and parasites. Even it inhibits multidrug-resistant microorganisms and can often work in synergy with common antibiotics. Allicin has shown promising results to be utilized as drugs.

In 1988, Fedelberg *et al.* showed [50] Diallylthiosulfinate (allicin), found in garlicand was responsible for the antibacterial and antifungal activity. They observed the effect of bacteriostatic concentrations of allicin (0.2 to 0.5 mM) on the growth of *Salmonella typhimurium* revealed a pattern of inhibition characterized by a lag of 15 min between addition of allicin and onset of inhibition, a transitory inhibition phase whose duration was proportional to allicin concentration and inversely proportional to culture density and an entry into stationary phase at lower culture density.

In 1993, Rees *et al.* used [51] an aqueous extract of freeze-dried garlic (Allium sativum) and observed the inhibition of many bacteria, yeasts, fungi and a virus. They determined minimum inhibitory concentrations for bacteria and yeasts and the values were ranging from 0.8 to 40.0 mg garlic/ ml. They observed fungal radial colony growth was inhibited by at least 25% at concentrations as low as 2.0 mg garlic/ml. They found the 50% endpoint neutralization titre for rotavirus was 2.4 to 2.8 µg /ml and lactic acid bacteria were the least sensitive microorganisms to the inhibitory effects of garlic. In mixed culture studies of *Lactobacillus acidophilus* and *Escherichia coli*, garlic prevented the establishment of *E. coli*& although the final outcome of competition was not affected.

In 2000, Elsom *et al.* determined [52] minimum bactericidal concentration and minimum inhibitory concentration of aqueous garlic extract against nine species of bacteria of which *Helicobactor pylori* proved to be extremely sensitive and others including *Bacteroidesfragilis*, *Clostridium perfringens*, *Enterobacter cloacae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonellatyphimuriumn*and*Staphylococcus aureus* were moderately sensitive.

In 2002, Canizares *et al.* optimized [53] the Allium sativum solvent extraction for growth inhibition of *Helicobactor pylori*. They used two different extraction process (soxhlet, stirred tank reactor) and four different solvents (ethanol ,acetone, water & hexane) and extracts that showed the highest bacteriostatic activities were selected to evaluate the influence of the most important operation variables on the extraction yield: stirring speed, operation time, garlic conditioning, and garlic storage time. They obtained the best results using ethanol and acetone as solvents in a stirred tank.

In 2004, Curtis *et al.* investigated [54] the effectiveness of garlic extract against a range of plant pathogenic organisms in vitro and in diseased tissues. They quantified allicin in garlic extracts by spectrophotometer and developed a rapid bioassay for routine use. They compared the in vitro activity of allicin against a prototrophic *E. coli* isolate with the conventional antibiotics ampicillin and kanamycin. They reported activity of allicin in vitro against different plant pathogenic bacteria such as *Agrobacterium tumefaciens, Erwinia carotovora, Pseudomonas syringae,* the fungi *Alternaria brassisicola, Botrytis cinerea, Plectosphaerella cucumerina, Magnaporthe grisea,* and the Oomycete *Phytophthora infestans*. They studied

disease reduction effect by allicin in planta for *Magnaporthe grisea*-infected rice, *Hyaloperonospora parasitica*-infected *Arabidopsis thaliana* and *Phytophthora infestans*-infected potato tubers. They studied that the active principle was effective in reducing *P. infestans* spore germination in vitro and disease in blighted tubers via the fumigation as well as by the direct application at the inoculation site. They concluded that for *Arabidopsis* the reduction in disease was apparently due to a direct action against the pathogen since no accumulation of salicylic acid was observed after application. They also discussed the potential for developing preparations of garlic to use as an alternative to synthetic fungicides in case of organic food productions.

In the year 2004, Aeronstein and Hayes [55] tested the antimicrobial activity of allicin against a number of bacterial and fungal pathogens (*Paenibacillus larvae larvae, P. l. pulvifaciens, Ascosphaera api*s and *Ascosphaera aggregata*) associated with social (*Apis mellifera*) and solitary bees. They determined the minimum inhibitory concentrations (MIC) of allicin by using a broth micro dilution method in the range of 1000 ppm to 25 ppm. It was observed that allicin liquid showed activity against gram-positive bacteria isolates (MIC 350 ppm) and fungal isolates (MIC 250 ppm). They also tested the antimicrobial activity of allicin in an agar diffusion test using 250 µg of allicin per disk. Bacterial isolates (*P. l. pulvifaciens* and *P. l. larvae)* were associated with zones of inhibition in the range of 24–26 mm and 45–50 mm respectively. They found that the fungal isolates were associated with zones of inhibition in the range of 31–35 mm (*A. apis*) and 35–37 mm (*A. aggregata*). They used macrolide class antibiotic tylosin as a control in both the MIC assay and in the agar diffusion test. They concluded from this study that allicin is potential to inhibit growth of bee pathogens and reduce occurrence of at least two major bee diseases.

In 2004, Cutler and Wilson tested [56] a new, stable, aqueous extract of allicin (extracted from garlic) on 30 clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) that show a range of susceptibilities to mupirocin They tested Strains using agar diffusion tests, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. From Diffusion tests they got that allicin liquids produced zone diameters >33 mm when the proposed therapeutic concentration was of 500 µg/mL (0.0005% w/v). They selected this concentration based on evidence from the MIC, MBC and agar diffusion tests in this study. They found that among the tested strains, 88% had MICs for allicin liquids of 16µg/mL, and all

strains were inhibited at 32µg/mL. Also, they reported that 88% of clinical isolates had MBCs of 128µg/mL, and all were killed at 256µg/mL. They reported that 82% of these strains showed intermediate or full resistance to mupirocin(used to treat MRSA). Also from this study they indicated that a concentration of 500 µg/mL in an aqueous cream base was required to produce an activity equivalent to 256 µg/mL allicin liquid.

In 2007, Cai *et al.* determined [57] the minimum inhibitory concentration (MIC) of allicin alone or in combination with cefazolin/oxacillin against *Staphylococcus* spp. or with cefoperazone against *Pseudomonas aeruginosa* and they observed that allicin did not have good antibacterial activity alone but it is capable to decrease the MIC values of other antibiotics. In the presence of 1/8, 1/2 of the MIC of allicin, the MIC90 values of cefazolin, oxicillin, and cefoperazone were reduced by 4 to 128, 32 to 64 and 8 to 16 fold.

In 2013, Booyens and Thantsha designed[58] an experiment to investigate the antimicrobial effects of different garlic preparations on five strains of *bifidobacteria* by disk diffusion assay characterized by zone of inhibition ranging from13.0 ± 1.7 to 36.7 ± 1.2 mm. Minimum inhibitory concentration (MIC) values for garlic clove extract ranged from 75.9 to 303.5 mg/ml. they observed that *Bifidobacteriumlactis*Bi-07 300B was the most resistant strain to garlic, followed by *B. lactis*Bb12, *B. longum*LMG 13197, *B. longum*Bb356 and *B. bifidum*11041.

In 2014, Li *et al.* investigated [59] the antibacterial activity of fresh garlic extract(FGE) only and incombination with other antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*and*Candida albicans*and observed Fresh garlic extract displayed evident inhibition properties against *C. albicans*and MRSA, but less inhibition *P. aeruginosa*. FGE showed the potential to improve the effect of antibiotics on antibiotic resistant pathogens. The synergism of fluconazole and itraconazole with FGE on *C. albicans*yielded larger sized inhibition zones compared with fluconazole and itraconazole without FGE. The synergism of cefotaxime and ceftriaxone with FGE on *P. aeruginosa* yielded larger sized inhibition zones than cefotaxime and ceftriaxone without fresh garlic extract.

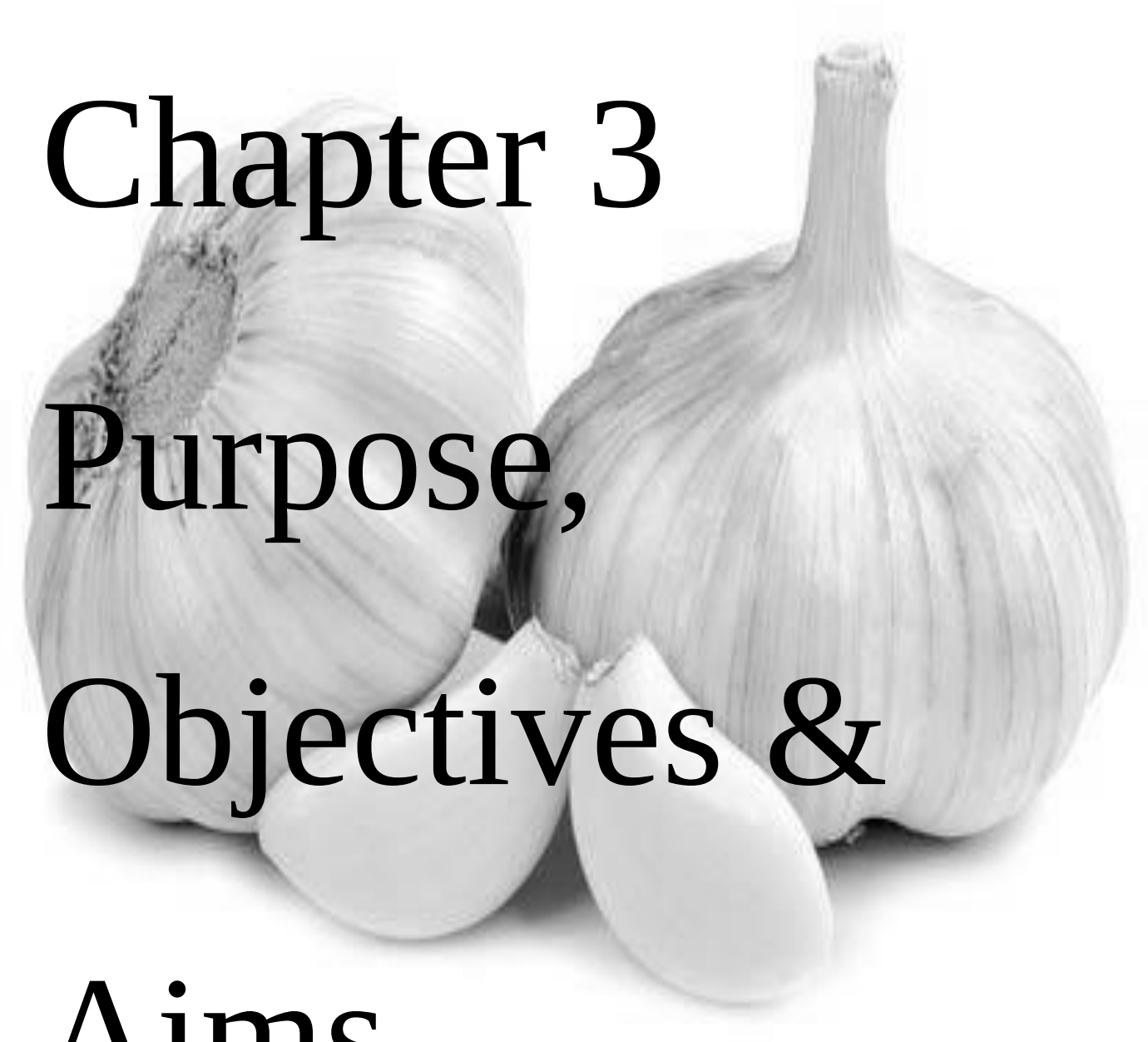
In the year 2017, Reiter *et al.* showed [60] that allicin (diallylthiosulfinate) was responsible for garlic odoranditsvolatility was potential enough to treat lung infections. They

synthesized allicin(>98%pure)by oxidation ofdiallyldisulfidebyH2O2.They observed thegrowth inhibitory effect of allicin vapor and allicin in solution against clinical isolates of lung pathogenic bacteria from the genera *Pseudomonas, Streptococcus*and*Staphylococcus*including multi-drug resistant (MDR) strains. They determined minimal inhibitory (MIC) and minimal bactericidal concentrations (MBC) and compared to clinical antibiotics using standard European Committee on Antimicrobial Susceptibility Testing (EUCAST) procedures. They tested the cytotoxicity of allicin to human lung and colon epithelial and murine fibroblast cells in vitro and shown to be ameliorated by glutathione (GSH).They also reported that sensitivity of rat precision-cut lung slices (PCLS) to allicin was decreased by raising the [GSH] to the approximate blood plasma level of 1 mM. They concluded that allicin inhibited bacterial growth as a vapor; it could be used to combat bacterial lung infections via direct inhalation.

In 2018, Dwivedi *et al.* have shown [61] that allicin reduced the bacterial burden in the lungs of mice infected with *Mycobacterium tuberculosis* (M.tb), and also induced strong anti-tubercular immunity. They reported that allicin/garlic extract exhibits strong anti-mycobacterial responses in vitro and in vivo against drug-sensitive, multiple drug resistant and extremely drug resistant (MDR and XDR) strains of TB. They observed that allicin also induced pro-inflammatory cytokines in macrophages and allicin/garlic extract treatment in murine models of infection resulted in induction of strong protective Th1 response and it lead to drastic reduction in mycobacterial burden. They so reported from the studies that allicin/garlic extract had both antibacterial and immunomodulatory activity and also garlic extract reversed the immune dampening effects of frontline anti-TB drugs.

Chapter 3

Purpose, Objectives & Aims



3.1 Purpose of the Study

In this modern world, there has been an alarming increase in the number of infectious diseases and outrage of different bacteria strains. Antibiotic resistant pathogens have increased the risk as the commercial drugs are not capable to treat the outbreak of new diseases. The emerging threat to the civilization by multidrug resistant bacteria strain has attracted the attention of researchers. Side effects of different antibiotics are also a reason of major concern. Such current situation has forced scientists to take actions to control the spread out of diseases, to maintain the use of antibiotics and to develop new drugs. Phytochemicals have emerged as an alternative source to treat bacterial infections. Studies have shown that nutraceutical compounds can prevent the spread out of pathogens. They are easily available, less toxic and cost efficient. This study aimed to check the potentiality of allicin, a nutraceutical compound found in garlic extract, against gram positive and gram negative bacteria. It focused on the efficiency of allicin to inhibit the growth of bacteria and also to check the synergistic action of allicin with other antibiotics. Nowadays misuse of antibiotics is high and it leads to increased level of toxicity. So, synergistic combinations of antibiotics with nutraceuticals should be found to formulate newer drugs with less toxicity and side effects.

3.2 Aim of the study

The primary aim was to observe the growth inhibition kinetics of gram-positive and gram-negative bacteria in presence of fresh garlic extract containing allicin and also in synergistic combinations with different commercial antibiotics. The secondary aim was to study the quality parameters of fresh garlic extract and to find the maximum antibacterial effect of fresh garlic extract containing allicin against the respective bacterial strains.

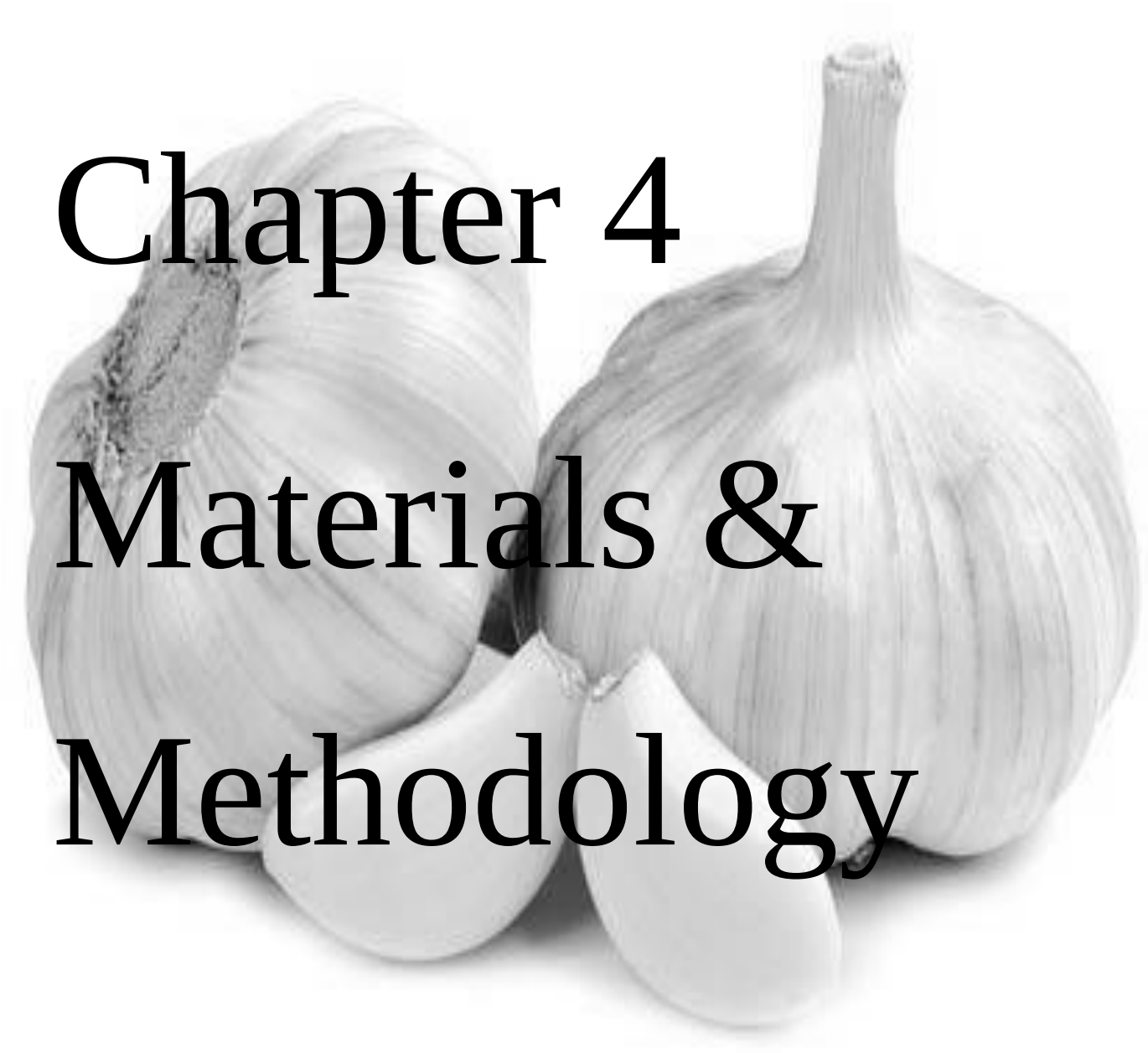
3.3 Specific objective of the study

- To prepare garlic extract containing allicin and to find the concentration of allicin.
- To study the parameters of garlic extract containing allicin to determine the presence of protein, flavonoid and phenol by different assays.
- Applications of garlic extract containing allicin in the lag, exponential and stationary phase of designated bacterial strains.

- To study the effect of the antimicrobial activity on growth kinetics.
- Determination of bacterial death kinetics constant.
- Studies on synergistic effect of garlic containing allicin with modern antibiotics such as rifaximin, oxytetracycline, levofloxacin and azithromycin.
- Determining the maximum effect of allicin against designated bacterial strains.

Chapter 4

Materials & Methodology



4.1 Chemical reagents

Table 4: List of the used reagents

Serial Number	Name Of The Reagent	Purpose Of Use	CAS Number
1.	Muller Hinton broth (MHB)	Liquid media used for growth of bacteria	NA
2.	Muller Hinton agar (MHA)	Solid media used for determining MIC,MBC and CFU/ml	NA
3.	4- Mercaptopyridine (4-MP)	To determine the concentration of allicin in fresh harlic extract	4556-23-4
4.	Bovine serum albumin (BSA)	Standard protein. It was used to prepare standard curve for Bradford assay.	9048-46-8
5.	Bradford reagent	Used in Bradford assay.	NA
6.	2,2-diphenyl-1-picrylhydrazyl(DPPH)	Used in DPPH radical scavenging assay.	1898-66-4
7.	Folin-Ciocalteu phenol reagent	Used for phenol assay.	NA
8.	Potassium dihydrogen phosphate	Used to prepare phosphate buffer.	7778-77-0
9.	Dipotassium phosphate	Used to prepare phosphate	7758-11-4

		buffer.	
10.	Potassium acetate	Used in total flavonoid content determination.	4251-29-0
11.	Aluminum chloride	Used as a reagent in total flavonoid content assay	7446-70-0
12.	Gallic acid	Used to prepare standard curve for total phenol content assay.	149-91-7 5995-86-8
13.	Quercetin	Used to prepare standard curve for total flavonoid content assay.	849061-97-8
14.	Ethylenediaminetetraacetic acid (EDTA)	Used as a chelating agent in 4-MP solution prepration.	6381-92-6
15.	Ethanol	Used as a solvent in total flavonoid content assay and also used as a sterilizing agent.	64-17-5
16.	Sodium carbonate	Used as a reagent in total phenol assay.	497-19-8
17.	Hydrochloric acid	Used to prepare solvent.	7647-01-0
18.	Phosphoric acid	Used to adjust pH.	7664-38-2
19.	Sodium hydroxide	Used to adjust pH.	1310-73-2

All chemicals were bought from HiMedia and Sigma-Aldrich and supplied by local vendors.

4.2 Extraction

4.2.1 Preparation of fresh garlic extracts (FGE) containing allicin

Fresh garlic bulbs were purchased from the local market at Jadavpur in Kolkata, West Bengal. It was mixed with water in 1:1 ratio and crushed in a mixer-grinder. The extract was made debris free by using a cheese cloth as a filter medium, followed by a centrifugation at 6000 rpm for 25mins. The extract was then filtered using Whatman filter paper of pore size 1.25 mm to form a debris free extract.

4.2.2 Determination of allicin concentration in fresh garlic extract

In 2001, Miron *et al.* developed [62] a method to detect the amount of allicin in fresh garlic extract (FGE). It wasstated that allicin reacts with 4-mercaptopyridine (4-MP). It forms 4-allylmercaptothiopyridine after 60minutes incubation time. The decrease in the absorbance at 324 nm is directly proportional to the amount of allicin present in the extract [].We used this method to determine the concentration of allicin. The extract was formed by the above mentioned process. 1 mM 4-MP solution was prepared as the stock solution in phosphate buffer (pH 7.2) with 2mM EDTA as a chelating agent. Garlic extract was diluted 200 times and the solution of garlic extract was incubated with 4-MP for 60mins. The absorbance of each sample was measured at 324nm.

4.3 Quality parameters study of the extract

To understand the characteristics of fresh garlic extract containing allicin properly, parameter study is an important aspect. The amount of flavonoid, phenol and protein present in the garlic extract, gives us a basic idea about the properties of the extract.

4.3.1 DPPH radical scavenging assay

DPPH is an abbreviated form of a common known organic compound 2,2-diphenyl-1-picrylhydrazyl. It is mostly used to monitor chemical reactions involving free radicals. It is a preferred method to determine the radical scavenging effect of antioxidants. Shimamura *et al.* had used DPPH assay to evaluate the antioxidant capacity of food additives. We followed the

principle he utilized in his experiments to find the inhibition ratio of garlic extract. 0.2mM DPPH solution was prepared in 99.5% ethanol solution [63]. As the solution took time to stabilize, so it was kept in dark for 2 hours after preparing the DPPH solution. 0.1 M Tris HCl buffer of pH 7.4 was prepared. 1ml DPPH with 200µl ethanol and 800 µl Tris HCl were used as control. 200 µl garlic extract was added with 800 µl 0.1M Tris HCl buffer , after that 1ml DPPH solution was mixed with it. The solution with ethanol and the other one with analytical sample were kept in dark for 30mins. 1.2ml ethanol with 800 µl Tris HCl buffer was used as blank. The absorbance of solution was measured at 517 nm against the reagent blank.

To calculate the inhibition ratio, a simple mathematical formula used by Shimamura *et al.,* was utilized. The inhibition ratio (%) was calculated from the following equation:

Inhibition ratio (%) = {(Ac-As)/Ac} ×100 ------- (1)

Where, Ac was used to denote the absorbance of the control solution which was formed by adding ethanol with DPPH and Tris HCL. As was the absorbance of the solution containing garlic extract, DPPH and Tris HCl.

4.3.2 Bradford assay

In 1976, Bradford developed a method to determine protein quantity [64]. He showed that protein binds with the dye Coomassie Brilliant Blue G 250 and results in a shift of the absorption maxima of the dye from 465nm to 595nm. We used this principle of Bradford assay to measure the quantity of protein present in garlic extract.

To prepare the standard curve Bovine serum albumin (BSA) was used as a standard protein. Different dilutions of BSA were prepared ranging from 1 to 50 ppm. 0.1ml of each dilution was added with 1ml of Bradford reagent and kept in dark for 15minutes at room temperature. Water in place of the protein solution was used to prepare the blank. The absorbance of each standard solution was measured at 595 nm against the reagent blank and a standard curve was plotted.

The garlic extract was prepared and diluted to different ratios. 0.1 ml of each diluted extracts was added with 1ml of Bradford reagent and kept in dark for 15 minutes at room temperature. Blank was prepared by using water with Bradford reagent. Absorbencies of different solutions were

measured at 595nm. By correlating the absorbance values of unknown solutions with the standard curve, the amount of protein was determined.

4.3.3 Determination of total phenol content

Phenol is an organic compound which contains a phenyl group bonded to a hydroxyl group. In 1965, Singleton *et al.* determined phenol content in a sample by using Folin-ciocalteu reagent. We determined the total phenol content in garlic extract by using Folin-ciocalteu assay [65].

Gallic acid was used to prepare the standard curve. Different dilutions of gallic acid solution were prepared with varying concentration of gallic acid from 100 to 400 µg/ml. 1 ml of each solution was added with 9 ml of distilled water , after that 1 ml of Folin-ciocalteu phenol reagent was mixed with each solution and shaken well. After 5minutes incubation 10 ml of 7 % Na_2CO_3solution was added with each mixture and the volume was made up to 25ml. Water was used as blank. After 90 minutes incubation at room temperature the absorbance against the reagent blank was measured at 550nm by using UV-visible spectrophotometer. The standard curve was plotted.

The garlic extract was prepared and diluted to different ratios. 1ml of each diluted extract was added with 9ml of distilled water, followed by the addition of 1ml Folin-ciocalteu phenol reagent. After 5 minutes incubation, 10 ml of 7% Na_2CO_3 solution was added with the solution and the volume was made up to 25ml. Water was used as blank. After incubating the solution for 90minutes at room temperature, the absorbance against the reagent blank was measured of the analytical sample. By correlating the absorbance value with the standard curve, total phenol content in the sample was obtained.

4.3.4 Determination of total flavonoid content

Flavonoids are the most commonly found polyphenolic group in plants. They generally have two phenyl rings and one heterocyclic ring. In 1997, Woisky and Salantino reported a method to find the flavonoid quantity by a colorimetric method using aluminum chloride ($AlCl_3$, $6H_2O$) [66]. We used the modified version of their proposed method [67].

10 mg quercetin was dissolved in 80% ethanol to make a calibration curve , the solution was then diluted to different concentrations of flavonoid ranging from 10 µg/ml to 100µg/ml. 0.5 ml of each diluted solution was added with 1.5ml of 95% ethanol solution, followed by the addition of 0.1 ml 10% aluminum chloride solution. 0.1 ml of 1 M potassium acetate was added to each mixture of solution; later 2.8 ml of distilled water was mixed with the solutions and incubated for 30 minutes at the room temperature. To prepare the blank solution, 10% aluminum chloride was substituted by the same amount of distilled water; other compounds were added to the blank solution in same proportion. The absorbance of the mixtures was measured against the blank at 415 nm and a calibration curve was plotted.

The above mentioned procedure was duly performed again only quercetin was substituted by the garlic extract of different dilutions. The blank was also prepared by the same procedure as earlier. Absorbance of the analytical solutions were measured at 415nm and correlated with the standard curve to measure the quantity of total flavonoid.

4.4 Selection of model bacterial strains

Escherichia coli (ATCC 25922) and *Bacillus subtilis* (ATCC 6051) were chosen as model bacteria strains to study the effects of fresh garlic extract containing allicin and in synergistic combination with other antibiotics. A model organism is a species that has been widely studied because it is easily maintained and can grow in laboratory and have many advantages. *Escherichia coli and Bacillus subtilis* have turned into "model organism" for studying many of life's essential processes [68]. *Escherichia coli* and *Bacillus subtilis* are gram-negative and gram-positive bacteria respectively. They have higher growth rate, simple nutritional requirement and completed genomic sequence [69]. It gives them advantages over any other organism. These organisms are currently being used for different experiments worldwide. *Escherichia coli* has a cell division rate higher than most of the organisms. It grows rapidly and a model organism for gram-negative pathogens. As mentioned above *Bacillus subtilis* is one of the best characterized bacterium among gram-positives. Considering their advantages over any other bacteria, they were chosen as model organisms to conduct the study. Some basic biochemical tests were done to test the viability of strains.

4.4.1 Gram staining

Gram staining method was discovered by the Danish scientist and physician Hans Christian Joachim Gram in 1884 [70]. Gram staining is a differential staining technique that differentiates bacteria into two major groups: gram-positives and gram-negatives. It is based on the ability of microorganisms to retain color of the stains used during the gram stain reaction. Gram-negative bacteria are decolorized by the alcohol, losing the color of the primary stain crystal violet (purple). Gram-positive bacteria are not decolorized by alcohol and will remain purple. After decolorization step, a counterstain, safranin is used to impart a pink color to the decolorized gram-negative organisms.

Steps:

1) Application of the primary stain, Crystal Violet (CV) to a heat-fixed smear of bacterial culture: A drop of the liquid culture to be examined was transferred on a glass slide with an inoculation loop. The culture was air dried and fixed over a gentle flame avoiding overheating. A few drops of crystal violet stain were added over the fixed culture. The sample was kept with the stain for 1 minute. The stain was washed off with water then.

2) Addition of Gram's Iodine: Iodine solution was added on the smear. After 1 minute iodine was rinsed with running water. Iodine (I – or I3 –) acts as a mordant and also as a trapping agent. It increases the affinity of the cell wall with a stain by binding to the primary stain. It forms an insoluble complex which gets trapped in the cell wall. In the Gram stain reaction, the crystal violet and iodine form an insoluble complex (CV-I) and helps to trap the stain into cell walls.

3) Decolorization with ethanol: A few drops of ethanol were added. It was rinsed off with water after 5 seconds. Alcohol or acetone dissolves the lipid outer membrane of Gram negative bacteria, thus leaving the peptidoglycan layer exposed and increases the porosity of the cell wall. The CV-I complex is then washed away from the thin peptidoglycan layer, leaving Gram negative bacteria colorless. On the other hand, alcohol has a dehydrating effect on the cell walls of Gram positive bacteria which causes the pores of the cell wall to shrink. The CV-I complex gets tightly bound into the multi-layered, highly cross-linked Gram positive cell wall thus staining the cells purple.

4) Counterstain with Safranin: The conterstain safranin was added at the last. It was washed off with water after 40 to 60 seconds. The decolorized Gram negative cells can be rendered visible with safranin which is usually positively charged safranin, which stains them pink.

The slide was then dried under open air & observed under microscope at 100X zoom.

4.4.2 Catalase test

Catalase mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. To find out if a particular bacterial isolate is able to produce catalase enzyme, small inoculums of bacterial isolate is mixed into hydrogen peroxide solution (3%) and the rapid elaboration of oxygen bubbles occurs [71]. The lack of catalase is evident by a lack of or weak bubble production. A small amount of bacterial colony was transferred to a surface of clean, dry glass slide using inoculation loop. A drop of 3% H_2O_2 was placed on to the slide and mixed. A positive result is the rapid evolution of oxygen as evidenced by bubbling. A negative result indicates no bubble formation.

4.4.3. Starch hydrolysis test

Starch agar is a differential medium that tests the ability of an organism to produce certain exoenzymes including a-amylase and oligo-1, 6-glucosidase, that hydrolyze starch. Starch molecules are too large to enter the bacterial cell, so some bacteria can secrete exoenzymes to degrade starch into subunits that can then be utilized by the organism. Depending on their ability to degrade starch, organisms can be differentiated [72].Starch agar was prepared & sterilized. It is a simple nutritive medium with only starch as carbon source. After incubation at 37^{o}C for 24 - 48 hours iodine was added to the plate. Iodine turns blue, purple, or black depending on the concentration of iodine in the presence of starch. A clearing zone around the bacterial growth forms for the organism which has hydrolyzed the starch and indicates a positive result. No formation of clearing zone means negative result.

4.5 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Determination of antimicrobial effectiveness against specific pathogens is required to check the antimicrobial activity of any drug. From MIC value we can determine the effectiveness of a chemotherapeutic agent against a pathogen. It's the lowest concentration needed to inhibit the growth of a pathogen. MBC value is determined to find the minimum concentration of an antibacterial agent to kill bacteria.

4.5.1 Determination of MIC and MBC values of fresh garlic extract containing allicin against *Escherichia coli* and *Bacillus subtilis*

MIC values of fresh garlic extract containing allicin against *Escherichia coli and Bacillus subtilis* were determined by dilution susceptibility test. Muller Hinton broth(MHB) was prepared and distributed equally in test tubes. These were sterilized using autoclave. Inoculums were prepared by isolating bacterial colony of each designated bacteria and diluting them in water.100 µl of each inoculums was added to the sterilized tubes containing media.

Fresh garlic extract containing allicin was extracted from garlic and the concentration of allicin was determined using 4-MP by the method of Miron *et al.* Two fold dilutions were done and fresh garlic extract containing allicin of each dilution was added to the test tubes uniformly. After that test tubes were kept in incubator at 37^{o}C for 16 to 20 hours. The lowest concentration which doesn't show any visible growth of bacteria is the MIC value of the fresh garlic extract containing allicin.

Agar well diffusion method was used to determine the MBC values. In this method Mueller Hinton agar was prepared and autoclaved for sterilization. The liquid media was poured over the petri plates and allowed to solidify. Standardized solutions of the inoculums were prepared for *Escherichia coli* and *Bacillus subtilis*, respectively. Afterwards, 100µl of inoculums, were added to the agar plates and spread all over uniformly with the help of a spreader/cotton buds. Wells were made (about 6mm in diameter) on the plates .Standardized Fresh garlic extract containing allicin was extracted. Twofold dilutions of fresh garlic extract containing allicin extract were done and each dilution was added into the wells. After almost 24hours incubation at 37^{o}C, the

concentration of fresh garlic extract containing allicin which created a clear inhibition zone and had the highest inhibitory zone diameter was considered as the MBC value.

4.5.2 Determination of MIC and MBC values of antibiotics against *Escherichia coli and Bacillus subtilis*

Rifaximin, oxytetracycline, levofloxacin, azithromycin and metronidazole these antibiotics were used to study antibacterial effects against *Escherichia coli* and *Bacillus subtilis*. MIC values were determined by dilution susceptibility test. MHB was prepared and equally distributed in test tubes. Broth tubes were sterilized. Inoculums were prepared by isolating one bacterial colony of *Escherichia coli* and *Bacillus subtilis* respectively and diluting each into 1ml water. 100 µl of each inoculum was added to the broth tubes. 0.5 ml of different antibiotics at various concentrations was added into different broth tubes.

For Escherichia coli, concentrations of different antibiotics were:

Rifaximin was added ranging from 32µg/ml to 2µg/ml with two fold dilutions. Oxytetracycline was added ranging from 4µg/ml to 0.25µg/ml with two fold dilutions. Levofloxacin and azithromycin were added ranging from 32µg/ml to 2µg/ml and 256µg/ml to 16µg/ml respectively with two fold dilutions. Metronidazole was also added ranging from 512µg/ml to 32µg/ml with two fold dilutions.

Similarly, for Bacillus subtilis, concentrations of different antibiotics were:

Rifaximin was added ranging from 8µg/ml to 0.5µg/ml with two fold dilutions. Oxytetracycline was added ranging from 1µg/ml to 0.06µg/ml with two fold dilutions. Levofloxacin and azithromycin were added ranging from 0.4µg/ml to 0.025µg/ml and 32µg/ml to 2µg/ml respectively with two fold dilutions. Metronidazole was also added ranging from 128µg/ml to 8µg/ml with two fold dilutions. Antibiotics at these varying concentrations were added to different broth tubes in a standardized amount and kept in incubator for 16 to 20 hours at 37^{o}C.The lowest concentration of each antibiotic which doesn't show any visible growth of bacteria is the MIC value of that respective antibiotic.

Agar well diffusion method was used to determine the MBC values. In this method MHA was prepared and autoclaved for sterilization. The liquid media was poured over the petri plates and allowed to solidify. Standardized solutions of the inoculums were prepared for *Escherichia coli* and *Bacillus subtilis,* respectively. Afterwards, 100µl of inoculums, were added to the agar plates and spread all over uniformly with the help of a spreader/cotton buds. Wells were made (about 6mm in diameter) on the plates .Antibiotics (rifaximin, oxytetracycline, levofloxacin, azithromycin and metronidazole) were added to the wells at above mentioned concentrations. After almost 24 hours incubation at 37ºC, the concentration of each antibiotic which created a clear inhibition zone and had the highest inhibitory zone diameter was considered as the MBC value of that antibiotic.

4.6 Growth curve of *Escherichia coli* and *Bacillus subtilis*

Growth curve shows changes in bacterial population size with time. When a liquid nutrient medium is inoculated with seed culture, the organism takes up the dissolved nutrients and grows in number. In a typical batch growth, the organism mainly undergoes lag phase, log or exponential phase, stationary phase and death phase. Limitations of nutrients or adding some antimicrobial agent can cause the organism to reach the death phase. Usually, in lag phase the organism synthesizes new enzymes to sustain in the new medium and in log phase, they grow exponentially as they have adapted the new environment already. They grow rapidly in numbers and after certain time due to limitations of nutrients they undergo the stationary phase where the growth ceases. The reason of termination of growth can be an inhibitory factor or exhaustion of essential nutrients. To study the inhibitory effect of an antimicrobial agent against some bacterial strains, the growth pattern of those designated bacterial strains should be known.

4.6.1 Measurement of optical density (O.D) of the culture

MHB was prepared and sterilized using autoclave. A loop of single colony of bacterial cultures was transferred to the stock media respectively in laminar air flow chamber and the flasks were kept in incubator shaker under aerobic condition at 100 rpm, 37ºC for 24 h. Then 1% (v/v) of the freshly pre-cultured were inoculated into respective conical flasks containing 150ml MHB media and kept in the incubator shaker at 37ºC and at 100rpm. Aliquots of the cultures were collected at every 2 hour interval unless the growth of bacteria reached stationary phase and were kept in

vials. Absorbance of each one was measured at 600nm using spectrophotometer by Lambert-Beer's law. Muller Hinton broth media was used as the blank.

The law stated:

A = εbc ---- (2)

Where, A is absorbance (no units, since $A = \log_{10} P_0 / P$).

ε is the molar absorptivity with units of L mol^{-1} cm^{-1}

b is the path length of the sample. **c** is the concentration of the compound in solution, expressed in mol L^{-1}

P_0 is the intensity of incident light. **P** is the intensity of transmitted light.

4.6.2 Determination of colony forming unit (CFU)

Mueller Hinton agar (MHA) was prepared and autoclaved for sterilization. The liquid media was poured over the petri plates and allowed to solidify. Afterwards, from each of the samples which were collected during the growth of bacteria, 0.1ml was taken and spread uniformly over the solidified media with a spreader. Dilutions were done as required. The plates were kept in the incubator-shaker for 24hrs at 37°C. The number of colonies was counted for each plate with relevant dilutions. The experiment was done in triplicate.

To calculate the CFU/ml, the equation was mentioned below:

$$CFU/ml = \frac{Numberofcoloniesformed}{Amountofthesampleplated \times Degreeofdilution} \quad \text{---- (3)}$$

CFU/ml for collected samples for both strains were plotted with respective times to obtain the growth curve of both bacterial strains.

4.7 Time kill curve experiment with fresh garlic extract containing allicin against *Escherichia coli* and *Bacillus subtilis*

Time kill curve is essential to analyze the effect of antimicrobial agent on bacterial growths and also to find the death kinetics parameters. The antimicrobial agent is usually dosed at a specific phase during the growth of the designated bacteria and change in population size of bacteria with time is observed. The curve is prepared by plotting the change in CFU of bacteria with time.

4.7.1 Separation of biomass

Mueller Hinton broth was prepared and sterilized using autoclave. A loop of single colony of both bacterial cultures was transferred to the stock media respectively in laminar air flow chamber and the flasks were kept in incubator shaker at 100 rpm, 37^{o}C for 24 h. Then 1% (v/v) of the freshly pre-cultured were inoculated into respective conical flasks containing 150ml MHB media and kept in the incubator shaker under aerobic condition at 37^{o}C and 100rpm. Biomass was separated from the inoculums at lag, log and stationary phase. For that, at required growth phase the bacterial samples were collected in vials. Afterwards, the vials were centrifuged at 8000 rpm for 5minutes. Then the supernatant was discarded carefully. The cell mass left in the vials was the required amount of biomass which was resuspended in water for further use.

4.7.2 Time kill study with fresh garlic extract containing allicin

MHB was prepared and equally distributed in conical flasks. These flasks were sterilized using autoclave. The isolated biomass of designated bacterial strains was added to the flasks. Fresh garlic extract containing allicin was extracted from garlic and added to the flasks at its required MIC and MBC values. The flasks containing biomass and allicin kept in the incubator shaker under aerobic conditions at 37^{o}C and 100rpm. Aliquots of the cultures were collected at every 2 hour interval and kept in vials. The experiment was done in duplicate.

4.7.3 CFU count and preparation of time kill curve

Afterwards, from each of the samples which were collected during the time kill assay of bacteria with fresh garlic extract containing allicin only, 0.1ml was taken and spread uniformly over the solidified, sterilized MHA media with a spreader. Dilutions were done as required. The plates

were kept in the incubator-shaker for 24hrs at 37^{o}C. The number of colonies was counted for each plate with relevant dilutions. CFU/ml for each collected sample was determined by using the equation (3) mentioned in 4.6.2. CFU/ml for each collected sample for both strains was plotted with respective times to obtain the time kill curve for both bacterial strains.

4.8 Synergy of fresh garlic extracts containing allicin with antibiotics (rifaximin, oxytetracycline, levofloxacin, azithromycin and metronidazole) by checkerboard method

Nowadays, there has been an alarming increase in the incidence of new and re-emerging infectious diseases. So, combinations of new drugs are needed to evaluate. Synergistic studies give the idea of the additive effect of two different drugs together against a specific microbe. This study was conducted to check the synergistic effect of fresh garlic extract containing allicin with different antibiotics [73].

4.8.1 Physicochemical characterization of antibacterial agents

SwissADME, an online free web tool was used to check the properties of different antibacterial agents. It evaluates pharmacokinetics, Drug likeliness and medicinal chemistry of small molecules. To be effective as a drug, a potent molecule must reach its target in the body in sufficient concentration, and stay there in a bioactive form long enough for the expected biological events to occur. This web tool helped to analyze the drug likeliness of one molecule, its water solubility and lipophilicity. It gave us the idea of the physiochemical properties like molecular weight, molecular refractivity of a particular molecule. It also analyzed the medicinal chemistry of that molecule [74].

4.8.2 Synergistic study

Muller Hinton broth was prepared and sterilized using autoclave. It was distributed uniformly into eppendorf tubes. Stock solutions of fresh garlic extract containing allicin and each antibiotic (rifaximin, oxytetracycline, levofloxacin and azithromycin) were prepared.

Serial two fold dilutions of fresh garlic extract containing allicin and antibiotics were done from their respective MIC values. Fresh garlic extract containing allicin was added along the ordinate in respective dilutions gradually from higher to lower values while rifaximin was diluted along the abscissa. Inoculums were prepared by isolating one bacterial colony of *Escherichia coli* and *Bacillus subtilis* respectively and diluting each into 1ml water. Each eppendorf tube was inoculated with 100µl of each bacterial inoculum. The plates were incubated at 37°C for 24 h under aerobic conditions. The process was repeated for each above mentioned antibiotics.

The checkerboard method contained the highest concentration of each antibiotic at opposite corners. After the incubation time no growth was observed in some well. The MIC values of these wells were used to calculate the fractional inhibitory concentrations (FIC) of each combination of drugs.The FIC for a drug in a given tube was derived by dividing the drug concentration in the given well by the control MIC of the test organism.

$$\textbf{FIC Index} = \textbf{FIC}_Z + \textbf{FIC}_W = \frac{\mathbf{Z}}{(\mathbf{MIC})\mathbf{Z}} + \frac{\mathbf{W}}{(\mathbf{MIC})\mathbf{W}} \qquad \text{---- (4)}$$

Where the**(Z)** is the concentration of drug Z in a given well and **(MIC)Z** is the control MIC of the organism to drug Z alone; $\textbf{FIC}_Z$is the fractional inhibitory concentration of drug Z. $\textbf{FIC}_W$ and **W** and **(MIC)W** are defined similarly for drug W (Krogstad and Moellering, 1986). The combination is considered synergistic when the FIC is ≤ 0.5, indifferent when the FIC is 0.5 to 2 and antagonistic when the FIC is 2 [75].

4.9 Time kill curve experiment with fresh garlic extract containing allicin in synergy with rifaximin, oxytetracycline, levofloxacin and azithromycin against *Escherichia coli* and *Bacillus subtilis*

Time kill curve is essential to analyze the effect of antimicrobial agent on bacterial growths and also to find the death kinetics parameters. This study was conducted to understand the additive effect of garlic extract containing allicin with the antibiotics against the selected bacterial strains [76].

4.9.1 Separation of biomass

A loop of single colony of both bacterial cultures was transferred to the stock media respectively in laminar air flow chamber and the flasks were kept in incubator shaker at 100 rpm, 37ºC for 24 h. Then 1% (v/v) of the freshly pre-cultured were inoculated into respective conical flasks containing 150 ml MHB media and kept in the incubator shaker at 37ºC and at 100rpm under aerobic conditions. It was allowed to grow unless the growth of bacterial strains reached the stationary phase. Biomass were separated from the flasks at lag and log phase. At required growth phase, bacterial samples were collected in different vials the vials containing the biomass were centrifuged at 8000 rpm for 5minutes. Then the supernatant was discarded. The cell mass left in the vials was the required amount of biomass which was resuspended in water for further use.

4.9.2 Time kill curve study with synergistic combinations of fresh garlic extract containing allicin and antibiotics

MHB media was prepared for each combination of fresh garlic extract containing allicin and different antibiotics. The biomass isolated at lag and exponential phases of both bacterial strains was added to the prepared media for synergistic study. Fresh garlic extract containing allicin was extracted from garlic and diluted to its MIC values for respective bacterial strains. Four fold dilutions were done and diluted fresh garlic extract containing allicin was added to the flasks containing respective bacterial strains.

Rifaximin solution was prepared at its MIC values for respective bacterial strains. Four fold dilutions were done for each prepared rifaximin solution. With the fresh garlic extract containing allicin, 5% (v/v) diluted rifaximin was added in both conical flasks containing biomass at lag and exponential phase of each bacterial strain *Escherichia coli* and *Bacillus subtilis.*

Same procedure was replicated for azithromycin, levofloxacin and oxytetracycline respectively.

Conical flasks containing synergistic combinations of fresh garlic extract containing allicin and other antibiotics with the both bacterial strains in lag and exponential phases were kept in the incubator-shaker at 37ºC and 100 rpm under aerobic conditions. Aliquots of the cultures were collected at every 2 hour interval. The experiment was done in duplicate.

4.9.3 CFU count and preparation of time kill curve with synergistic combinations

From each of the collected samples during the synergistic study, 0.1ml was taken and spread uniformly over the solidified, sterilized MHA media with a spreader. Dilutions were done as required. The plates were kept in the incubator-shaker for 24 hrs at 37^{o}C. The number of colonies was counted for each plate with relevant dilutions. CFU/ml for each collected sample was determined by using the equation mentioned earlier. A graph was plotted between CFU/ml determined for each synergistic combination of fresh garlic extract containing allicin with the antibiotics with the respective time.

4.10 Growth kinetic study and time kill kinetics modeling

Growth kinetic study of the bacterial strains will allow us to understand their growth pattern and will help in further studies. It helps us to determine the growth rate and doubling time of the cell mass. After the lag phase, the cell gets adjusted to the new environment and grows rapidly during the exponential phase. This period is of balanced growth in which all components of the cell grow at same rate. In log phase, the cell grows exponentially and the growth kinetics of the cell follows the 1st order reaction kinetics [77].

The net specific growth rate is denoted by μ and the for log phase,

$$\frac{dx}{dt} = \mu x \qquad \text{---- (5)}$$

By integrating the above mentioned equation,

$$ln\frac{x2}{x1} = \mu t \qquad \text{---- (6)}$$

Where **x2** and **x1** are the cell concentration at time **t** and at **t = 0** respectively and **μ** is the net specific growth rate.

The doubling time of the cell mass is given by the equation

$$T = \frac{ln\,2}{\mu} = \frac{0.693}{\mu} \qquad \text{---- (7)}$$

Time kill kinetics:

When the antibacterial agents are added to the bacterial growth phases, it ceases the growth. Antibacterial agents kill the bacteria or inhibit the growth of bacteria by certain mechanism like DNA binding, inhibiting the cell wall synthesis, cell lysis and others. To study the inhibition kinetics, an equation was developed. It helps to determine the death rate constant and allows us to find the lethal rate for specific bacteria against a specific antibiotic.

The equation is:

$$\frac{dN}{dt} = -Kt \quad \text{---- (8)}$$

By integrating the above mentioned equation,

$$ln\frac{N1}{N2} = Kt \quad \text{---- (9)}$$

Where, **N1**is the initial cell concentration at **t=0.**

N2 is the final cell concentration obtained after time **t** and **K** is the first order death rate constant.

Where, **K** is the death rate constant.

4.11 Determination of E_{max} value by maximum effect model of fresh garlic extract containing allicin for *Escherichia coli* and *Bacillus subtilis*

The E_{max} curve often follows a hyperbolic shape and it helps to determine the maximum effect of a drug. It shows that increase in drug concentration near the maximum pharmacological effect of that drug triggers a very low response.

4.11.1 Separation of biomass and study with fresh garlic extract containing allicin at different concentrations

Mueller Hinton broth was prepared and sterilized using autoclave. A loop of single colony of both bacterial cultures was transferred to the stock media respectively in laminar air flow chamber and the flasks were kept in incubator shaker at 100 rpm, 37ºC for 24 h. Then 1% (v/v) of the freshly pre-cultured were inoculated into respective conical flasks containing 150ml MHB media and kept in the incubator shaker under aerobic condition at 37ºC and 100rpm. Biomass was separated from the inoculums at lag phase. Samples were collected in vials and vials were centrifuged at 8000 rpm for 5minutes. Then the supernatant was discarded carefully. The cell mass left in the vials was the required amount of biomass which was resuspended in water for further use.

Fresh garlic extract containing allicin was prepared and it was diluted to different concentration.MHB was prepared and sterilized and equally distributed into different conicals. Isolated biomasses of designated bacterial strains were added to the broths. Fresh garlic extract containing allicin at different concentrations were added to the media for each strain. The flasks containing biomass and fresh garlic extract containing allicin kept in the incubator shaker under aerobic conditions at 37ºC and 100rpm. Aliquots of the cultures were collected after 2 hour intervals and kept in vials. All above mentioned experiments were done in triplicate.

4.11.2 CFU count and preparation of E_{max} curve

From each of the collected samples during the study, 0.1ml was taken and spread uniformly over the solidified, sterilized MHA media with a spreader. Dilutions were done as required. The plates were kept in the incubator-shaker for 24 hrs at 37ºC. The number of colonies was counted for each plate with relevant dilutions. CFU/ml for each collected sample was determined by using the equation mentioned earlier. The effect of fresh garlic extract containing allicin at different concentration was determined by the change in CFU/ml from initial stage and by converting the values to percentages.

$$\boldsymbol{Effect\ of\ FGE\ containing\ allicin}(\%) = \frac{\boldsymbol{Initial\ colony\ count\ per\ ml - final\ colony\ count\ per\ ml}}{\boldsymbol{Initial\ colony\ count\ \ per\ ml}} \times \mathbf{100}$$

---- (10)

A graph was plotted for effect of fresh garlic extract containing allicin against selective bacteria with its respective concentration.

4.11.3 Determination of E_{max} and EC_{50} values

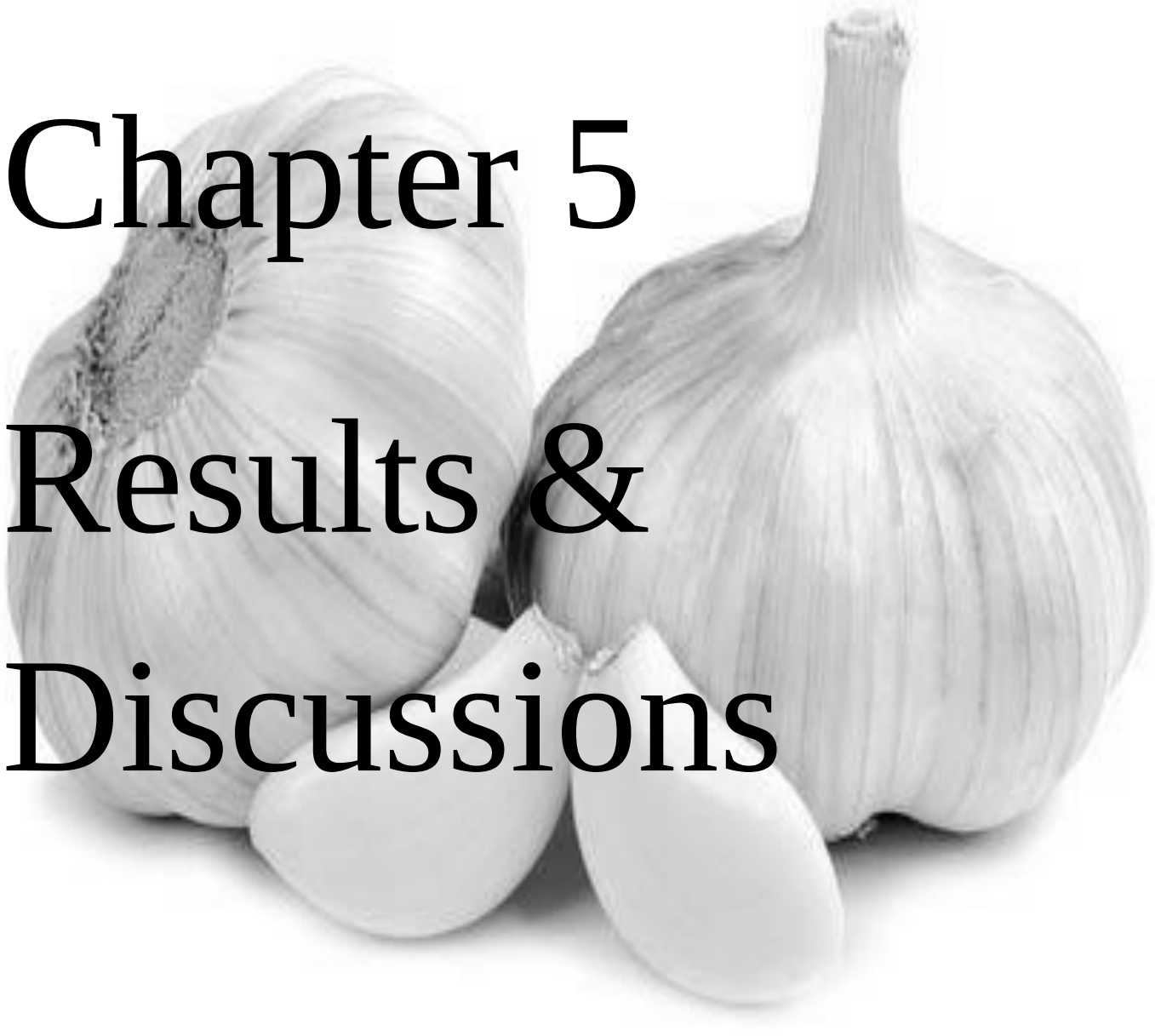
E_{max} model is a basic model which helps to determine the effect of a drug against a specific pathogen. This equation has a mechanistic basis in bimolecular interaction. The equation states,

$$\boldsymbol{E = \frac{E_{max}C}{EC_{50} + C}} \text{ ---- (11)}$$

E_{max} is the maximum effect of a specific drug and EC_{50} is the concentration of a drug which triggers half of the maximum response [78]. This equation was used to determine E_{max} and EC_{50} of fresh garlic extract containing allicin from graph obtained.

Chapter 5

Results & Discussions



5.1 Concentration of allicin in fresh garlic extract

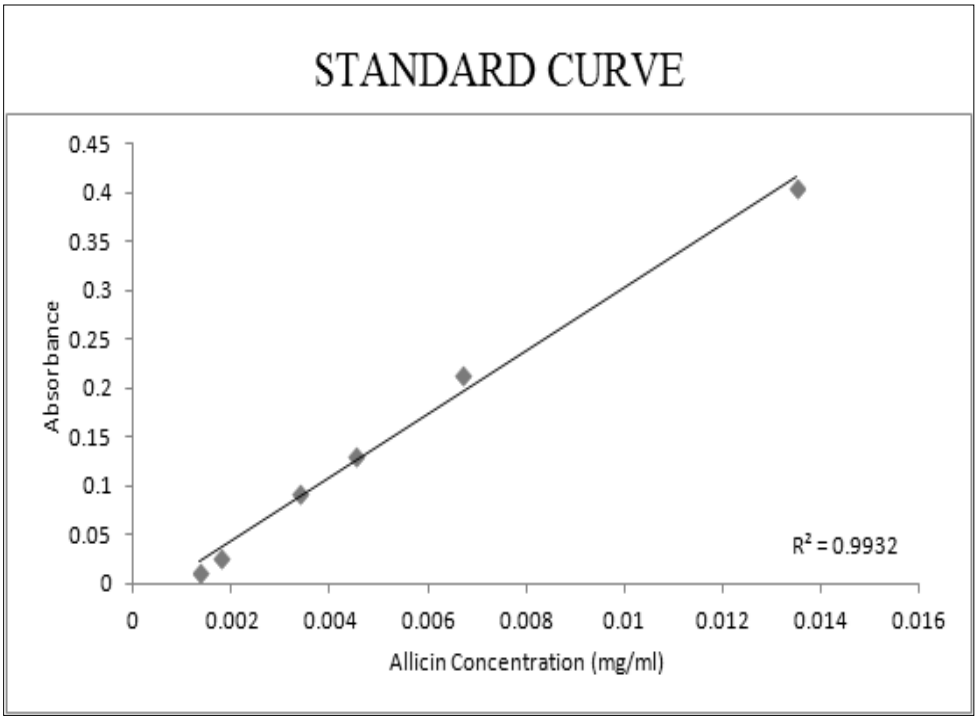


Figure 5.1: Standard Curve for allicin

Allicin concentration was determined by using the method developed by Miron *et al.* From the standard curve, concentration of allicin in fresh garlic extract was found to be 1.6 mg/ml.

5.2 Quality Parameters Study of the Extract

Some basic studies were conducted to get a general overview about fresh garlic extract containing allicin in fresh garlic extract.

5.2.1 DPPH radical scavenging assay

DPPH method is a preferred method to determine the antioxidant activity of a compound because it is fast, reliable and easy. The free radical scavenging activities of extracts depend on the ability of antioxidant compounds to lose hydrogen [79]. The ability of DPPH radical to bind hydrogen is considered to have a radical scavenging property. A solution of DPPH radicals prepared in ethanol is converted into DPPH-H (diphenylhydrazine) molecules in the presence of an antioxidant agent. Discoloration occurs due to the decreasing quantity of DPPH radicals in the

environment. The discoloration of the DPPHtherefore reflects the radical scavenging activity of the analyzedextract[80].When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced and as a result change of color is observed from violet to light yellow. Figure Ain appendix shows the discoloration of DPPH in presence of garlic extract.

Inhibition ratio was measured to be 62 % by the method prescribed by Shimamura *et al.* it shows that fresh garlic extract containing allicin is partially a good radical scavenger as it inhibited DPPH absorption to 62% which proves that it is an antioxidant in nature. It donates the hydrogen to DPPH, which binds to it and forms a compound resulting into the color change.

5.2.2 Determination of protein quantity by Bradford assay

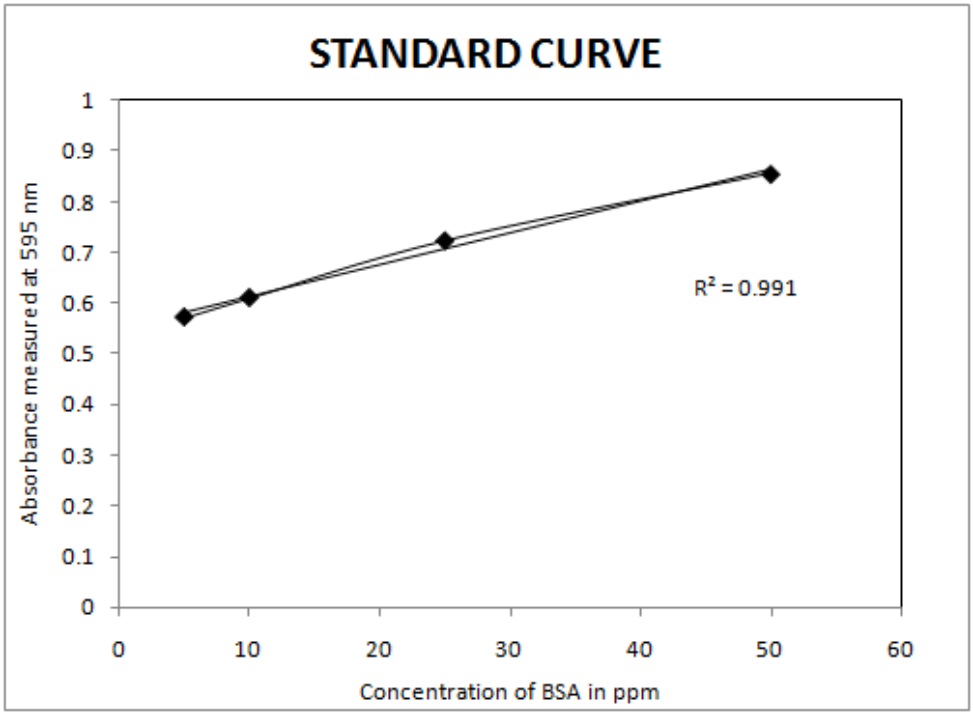


Figure 5.2: Standard curve for Bradford assay

Protein quantity in fresh garlic extract was determined by Bradford assay. The standard curve was plotted by using BSA as a known standard protein. Equation was obtained from the generated standard curve through the experiment and by correlating the absorbance value of the unknown sample with the standard curve, the protein quantity was measured.

From the standard curve, the protein quantity present in fresh garlic extract was 386 mg/L.

5.2.3 Determination of total phenol content

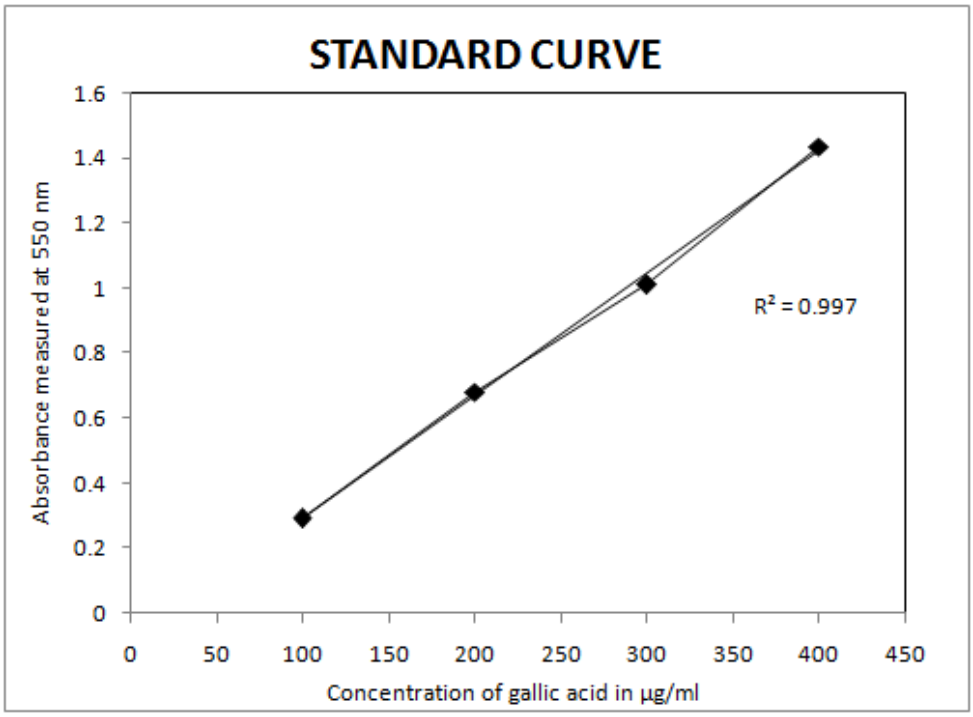


Figure 5.3: Standard curve for total phenol content assay

Total Phenol content in fresh garlic extract was determined by using Folin-ciocalteu phenol reagent. The standard curve was plotted by using Gallic acid as a known phenol substance. The absorbance value of the half diluted fresh garlic extract was measured. The equation was obtained from the generated standard curve through the experiment and by correlating the absorbance value of the unknown sample with the equation, the total phenol content was measured.

From the standard curve, the total phenol content was measured and was found to be 580µg/ml in fresh garlic extract.

5.2.4 Determination of total flavonoid content

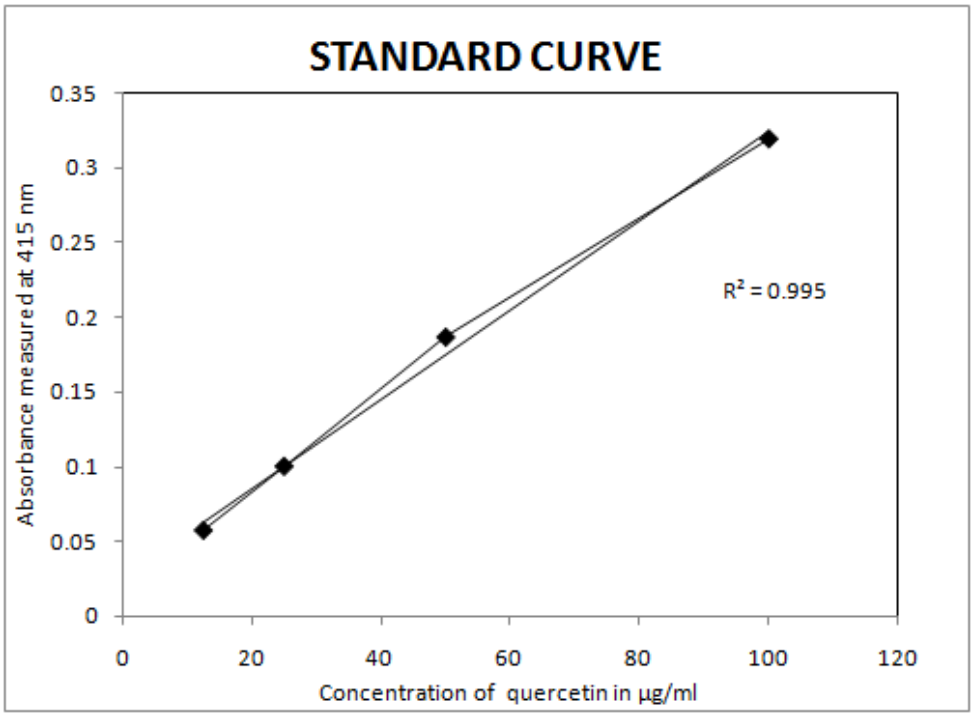


Figure 5.4: Standard curve for the total flavonoid content

Total flavonoid content in fresh garlic extract was determined by a colorimetric method using aluminum chloride ($AlCl_3$, $6H_2O$). The standard curve was plotted by using quercetin as a known flavonoid compound. The absorbance value of the 10 fold diluted fresh garlic extract was measured. From the standard curve, by correlating the absorbance value, the total flavonoid content was measured and was found to be 86µg/ml for fresh garlic extract.

5.3 Biochemical study

Table 5: Result of the biochemical studies

Name of the strains	Gram Staining	Catalase Test	Starch Hydrolysis Test
Escherichia coli (ATCC 25922)	Negative	Positive	Negative
Bacillus subtilis (ATCC 6051)	Positive	Positive	Positive

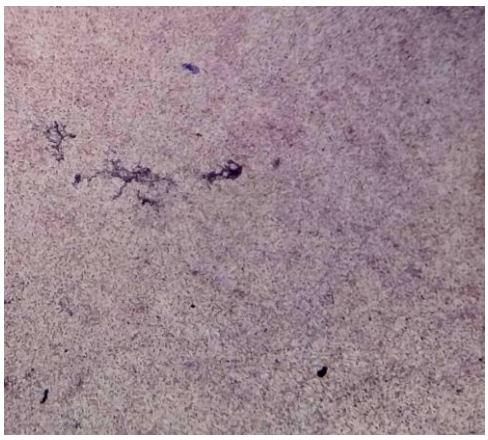


Figure 5.5: Gram staining of *Bacillus subtilis*

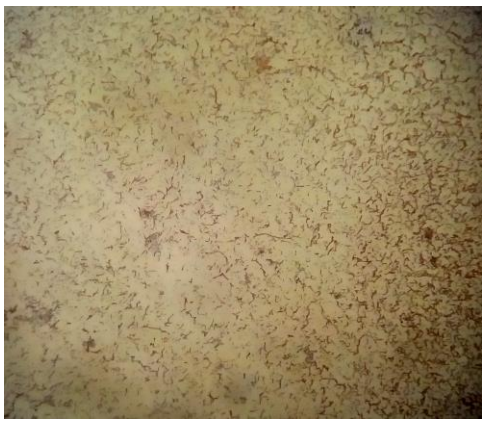


Figure 5.6: Gram staining of *Escherichia coli*

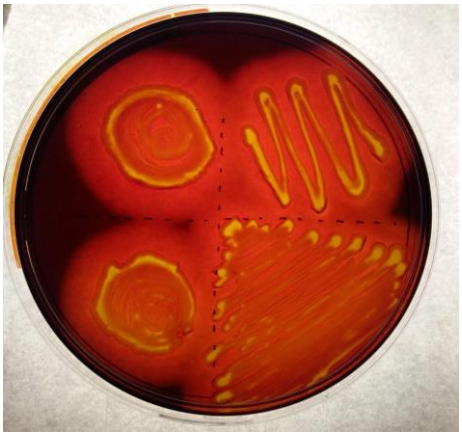


Figure 5.7: Starch hydrolysis test of *Bacillus subtilis*

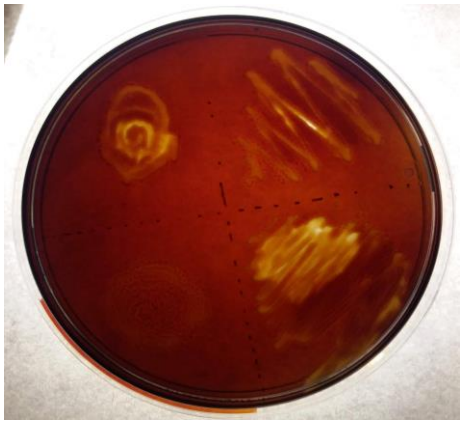


Figure 5.8: Starch hydrolysis test of *Escherichia coli*

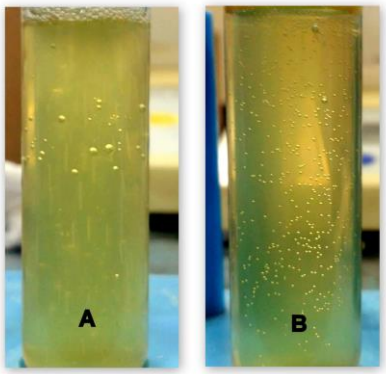


Figure 5.9: Catalase test of

A. *Escherichia coli* and B. *Bacillus subtilis*

Escherichia coli gave a negative result in Gram staining and starch hydrolysis test, which indicates that this strain is unable to degrade the starch present in the media and is gram-negative in nature. During catalase test it indicated bubble formation, so we can conclude it is capable of producing catalase enzyme.

On the other hand, *Bacillus subtilis* gave a positive result in gram staining and starch hydrolysis test which indicates that it is capable to secrete exoenzymes which can degrade the starch and it is gram-positive in nature. During catalase test it indicated bubble formation, so we can conclude it is capable of producing catalase enzyme.

5.4 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Table 6: MIC and MBC values of fresh garlic extract containing allicin

Antibacterial Agent	Name of the Strains	MIC values (mg/ml)	MBC values(mg/ml)
Fresh garlic extract containing allicin	*Escherichia coli*	0.1	0.8
	Bacillus subtilis	0.05	0.4

Table 7: MIC and MBC values of antibiotics

Name of Antibiotics	Name of the Strains	MIC Values (µg/ml)	MBC Values (µg/ml)
Rifaximin	*Escherichia coli*	8	16
	Bacillus subtilis	4	8
Oxytetracycline	*Escherichia coli*	1	2
	Bacillus subtilis	0.5	1
Levofloxacin	*Escherichia coli*	8	16
	Bacillus subtilis	0.1	0.2
Azithromycin	*Escherichia coli*	64	128
	Bacillus subtilis	8	16
Metronidazole	*Escherichia coli*	128	256
	Bacillus subtilis	32	64

5.5 Growth curve

5.5.1 Growth curve for *Escherichia coli*

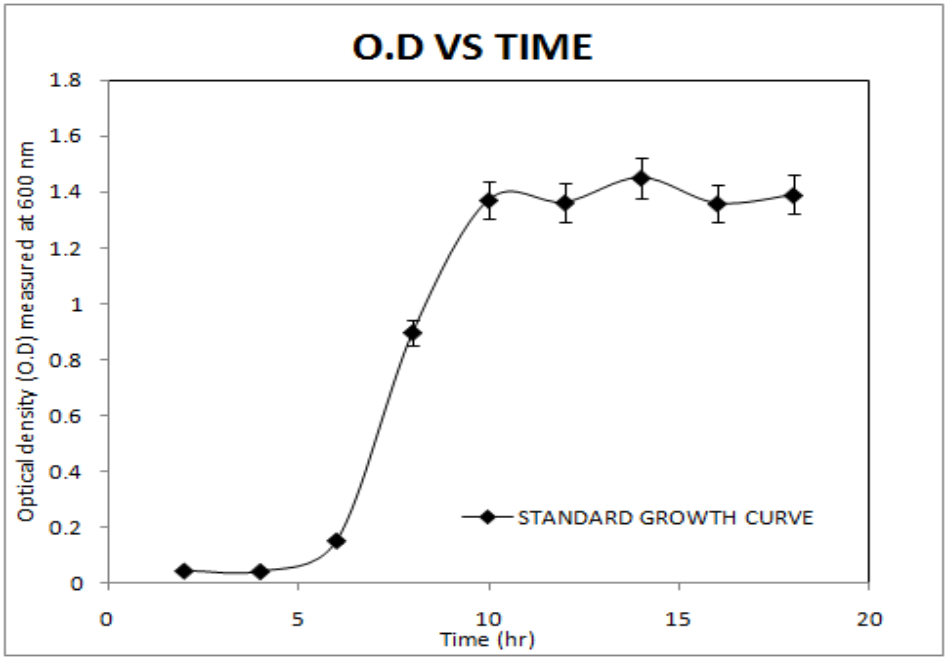


Figure 5.10: Standard growth curve of *Escherichia coli* using absorbance values

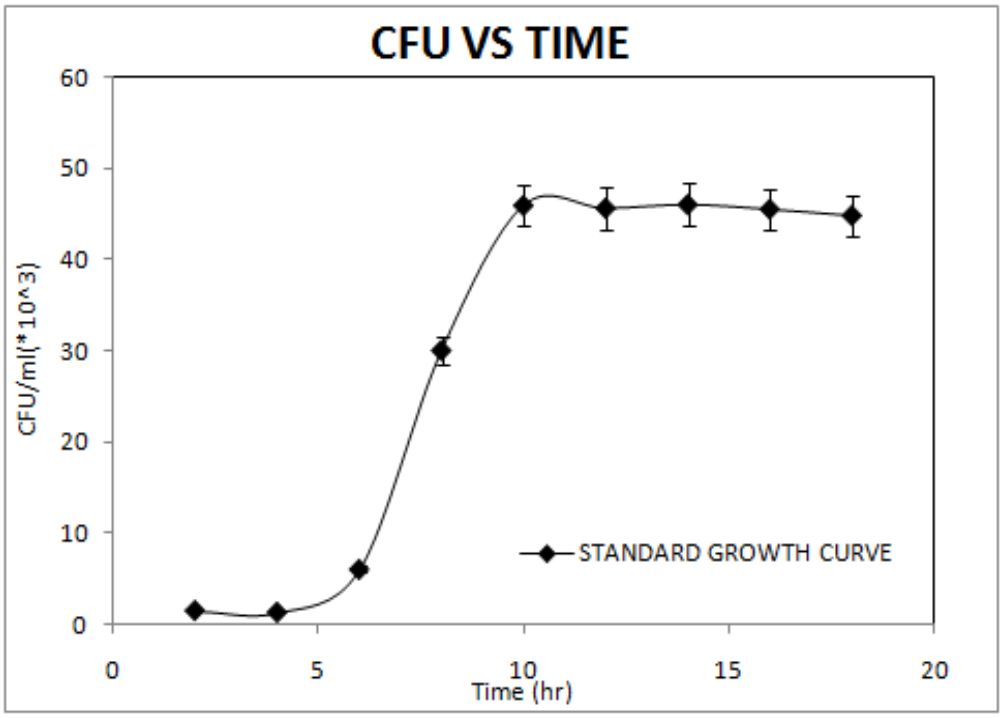


Figure 5.11: Standard growth curve of *Escherichia coli* using CFU values

Figure 5.10 and Figure 5.11 show the growth curve of *Escherichia coli.* The curve trend is sigmoid in nature. The first standard growth curve was formed using the optical density measured at different time intervals. The second curve was plotted using the CFU values obtained at different time intervals.

From both of the graph, it is clearly visible that the first 2 hours was the lag phase. This time was taken by the bacteria to get adjusted to the new environment. After that there was a steep increase in the absorbance value with respective time intervals until 10 hours. For the curve using the CFU values also shows almost similar nature. It was the log phase when the bacteria grow exponentially. The duration of the log phase was almost 8 hours.

From 10 hours the growth of bacteria ceased and almost no changes were observed in the CFU values. The stationary phase was continued till 18 hours. But standard growth curve using absorbance values shows fluctuations during stationary phase from 10^{th} hour onwards.

5.5.2 Growth curve for *Bacillus subtilis*

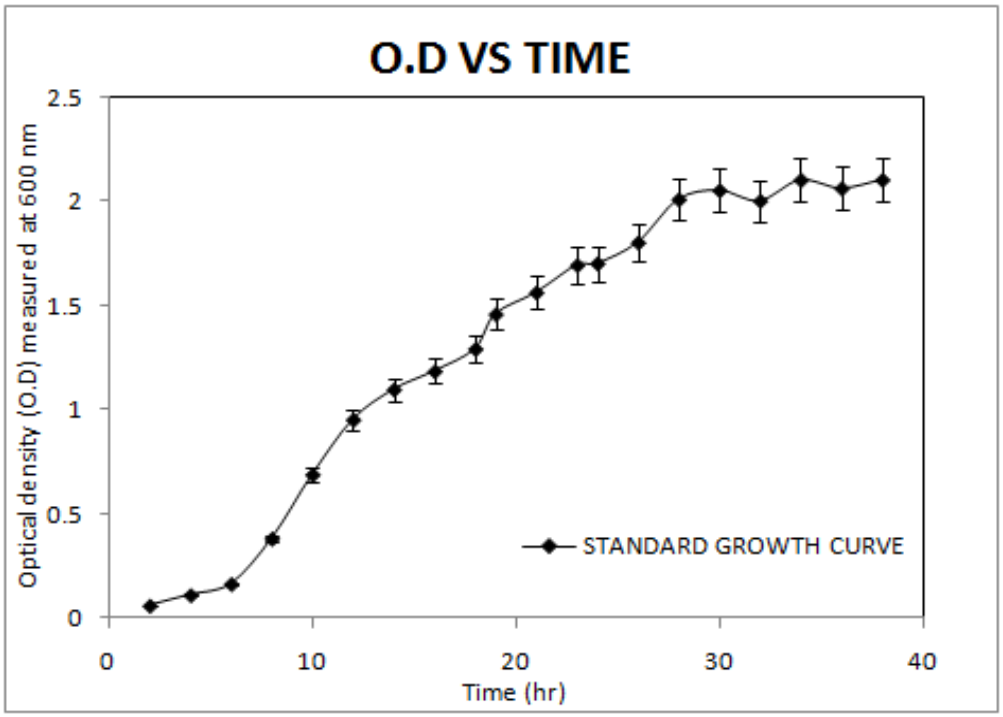


Figure 5.12: Standard growth curve of *Bacillus subtilis* using absorbance values

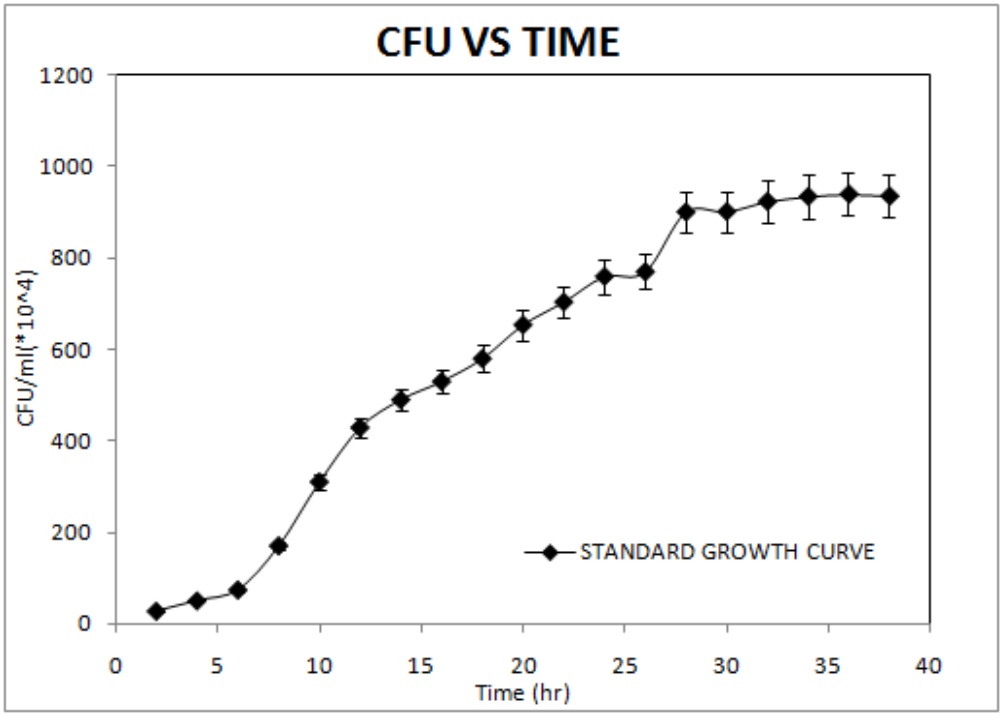


Figure 5.13: Standard growth curve of *Bacillus subtilis* using CFU values

Figure 5.12 and Figure 5.13 show the growth curve of *Bacillus subtilis*. This growth curve follows the trend of sigmoid nature. The first standard growth curve was formed using the optical density measured at different time intervals. The second curve was plotted using the CFU values obtained at different time intervals.

From both of the graph, it is clearly visible that the first 2 hours was the lag phase. This time was taken by the bacteria to get adjusted to the new environment. After that there was an increase in the absorbance value with respective time intervals until 28 hours. For the curve using the CFU values it shows continuous increase in CFU counts. It was the log phase when the bacteria grow exponentially. It stayed almost for 26 hours.

From 28 hours the growth rate of bacteria was almost equivalent to zero. No changes were in the CFU values. But for absorbance values, fluctuations were observed during this phase. The stationary phase was continued till 38 hours.

5.6 Time kill curve using only fresh garlic extract containing allicin against *Escherichia coli* and *Bacillus subtilis*

To check the antibacterial activity of fresh garlic extract containing allicin, it was applied against the designated bacterial strains at its specific MIC and MBC values. The graphs are attached below which depicts the effect of fresh garlic extract containing allicin at lag,log and stationary phase against the above mentioned bacterial strains. It clearly shows the inhibitory effect of fresh garlic extract containing allicin against the bacteria at different stages. It proves that fresh garlic extract containing allicin is capable of ceasing the growth of bacteria.

Time kill curve was obtained by using the CFU values of the bacterial strains after applying fresh garlic extract containing allicin at various growth stages to find its affectivity against a specific bacteria at a particular growth stage.

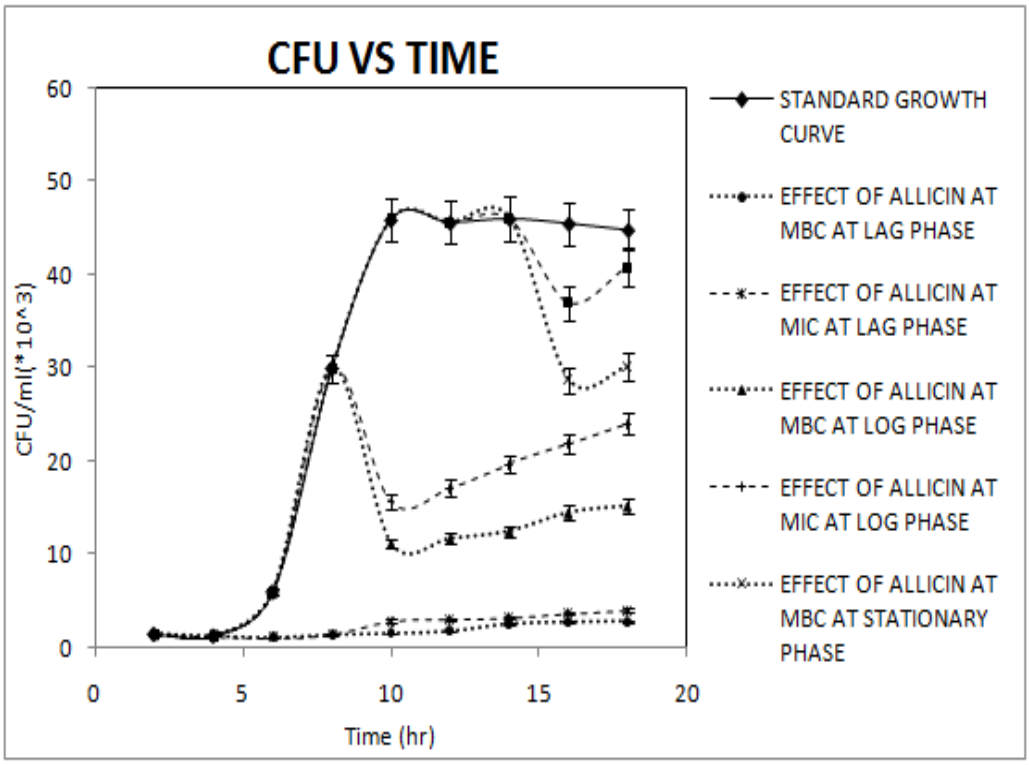


Figure 5.14: Time kill curve of *Escherichia coli* using fresh garlic extract containing allicin at different growth stages

The graph depicts the effect of fresh garlic extract containing allicin at MIC and MBC values against *Escherichia coli* at lag, mid exponential and stationary phase. It is visible from the graph that the MBC value of fresh garlic extract containing allicin was inhibitory than the MIC value of fresh garlic extract containing allicin at any stage. In lag phase, the initial effect of both MBC and MIC values were almost same, a gradual decrease in the growth was visible till 6 hours but from 8thhour onwards slight increase in the CFU values were obtained. Finally, for lag phase, the growth of bacteria was very minimal as the initial amount of inoculated biomass was comparatively lesser than log and stationary phase. In lag phase, the growth of bacteria was less for the MBC value of fresh garlic extract containing allicin. For the mid exponential phase, there was a steep decrease in the CFU values for both MBC and MIC. The sharp decrease indicated the affectivity of fresh garlic extract containing allicin. After few hours, gradual increase in CFU values of bacteria was obtained till 18th hour. Though, it was much lesser than the original growth of bacteria. In this phase, MBC value of fresh garlic extract containing allicin showed more potential than MIC value of fresh garlic extract containing allicin. For stationary phase, it

was observed that effect of fresh garlic extract containing allicin initially was good against the bacterial strain but within an hour it started growing again. For MIC value of fresh garlic extract containing allicin against *Escherichia coli* at stationary phase, the graph clearly indicated that it had a very less impact on the bacterial strain.

It can be assumed that due to the poor stability of fresh garlic extract containing allicin, it degrades quickly and the bacteria start growing after certain time. Also, the amount of inoculated biomass was higher in stationary phase than log and lag phase. Fresh garlic extract containing allicin can't completely kill the inoculated bacteria at log and stationary phase. So, as it degrades quickly, it can cease the growth only for a certain time resulting in a poor impact on stationary phase.

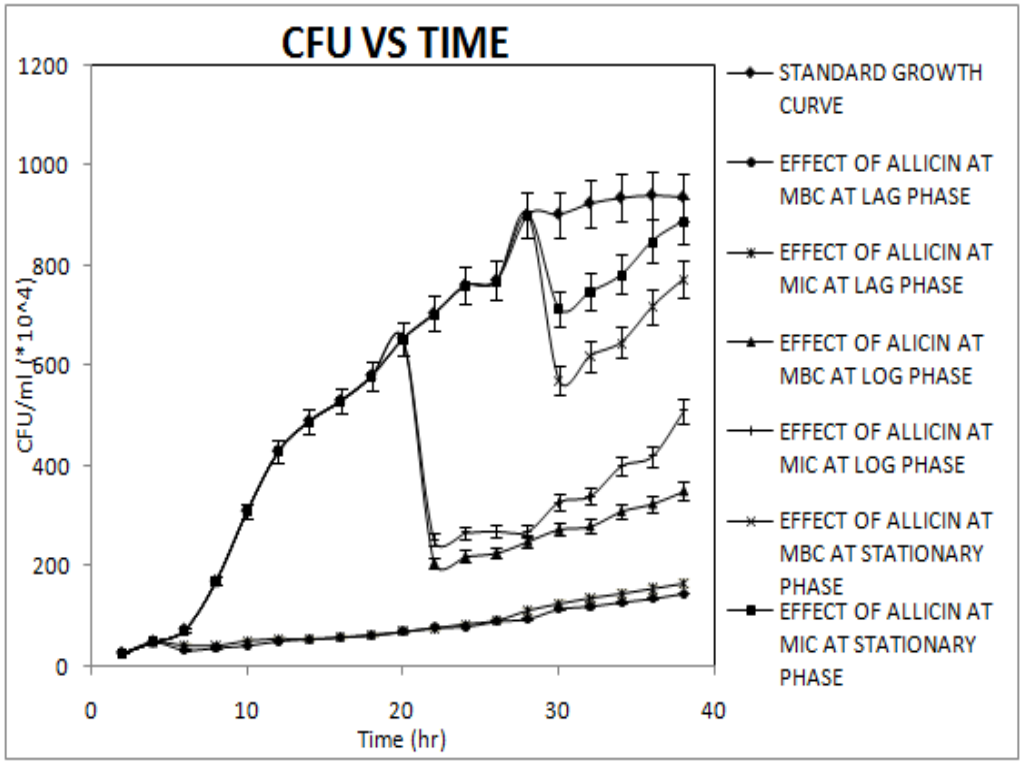


Figure 5.15: Time kill curve of *Bacillus subtilis* using fresh garlic extract containing allicin at different growth stages

The graph describes the effect of fresh garlic extract containing allicin at MIC and MBC values against *Bacillus subtilis* at lag, mid exponential and stationary phase. It is visible from the graph that the MBC value of fresh garlic extract containing allicin was more potential than the MIC

value of fresh garlic extract containing allicin at any stage. In lag phase, the initial effect of both MBC and MIC values were almost same, decrease in the CFU count was visible till 8th hour but from 10th hour onwards slight increase in the CFU values were obtained. For both MIC and MBC values of the extract containing allicin, till 28th hour growth rates were almost similar, after that at MIC value growth rate increased slightly than MBC. For lag phase, the growth of bacteria was very minimal as the initial amount of inoculated biomass was comparatively lesser than log and stationary phase.

For the mid exponential phase, there was a sharp decrease in the CFU values for MBC within 2hours. The sharp decrease indicated the affectivity of fresh garlic extract containing allicin at MBC. After that, slight increase in CFU values of bacteria was obtained till 38th hour. Though, it was much lesser than the original growth of bacteria. At MIC value of fresh garlic extract containing allicin, there was a gradual decrease in CFU counts till 8thhour. Then there was an increase in CFU counts at a higher rate than the strains treated with fresh garlic extract containing allicin at MBC value.

For stationary phase, it was observed that effect of fresh garlic extract containing allicin initially was good against the bacterial strain but after 2 hours bacteria started growing again. For MIC value of FGE containing allicin against *Bacillus subtilis* at stationary phase, the graph clearly indicated that it had a very less impact on the bacterial strain. Though at MBC value it had a better initial impact but it couldn't cease the growth for a longer time. It can be assumed that due to the poor stability of allicin, it degrades quickly and the bacteria start growing after certain time. Also, the amount of inoculated biomass was higher in stationary phase than log and lag phase. Fresh garlic extract containing allicin can't completely kill the inoculated bacteria at log and stationary phase. So, as it degrades quickly, it can cease the growth only for a limited time period resulting in a poor impact on stationary phase. This could be the probable reason of bacterial growth even after treating bacteria with fresh garlic extract containing allicin.

5.7 Synergy of fresh garlic extract containing allicin with antibiotics (rifaximin, oxytetracycline, levofloxacin, azithromycin and metronidazole) by checkerboard method

5.7.1 Physicochemical characterization of antibacterial agents

SwissADME web tool was used to get an analytical overview of these antibacterial agents. It analyzed the molecular weight, physiochemical properties, lipophilicity, and water solubility. Depending on this characteristics drug likeness of every molecule was found.

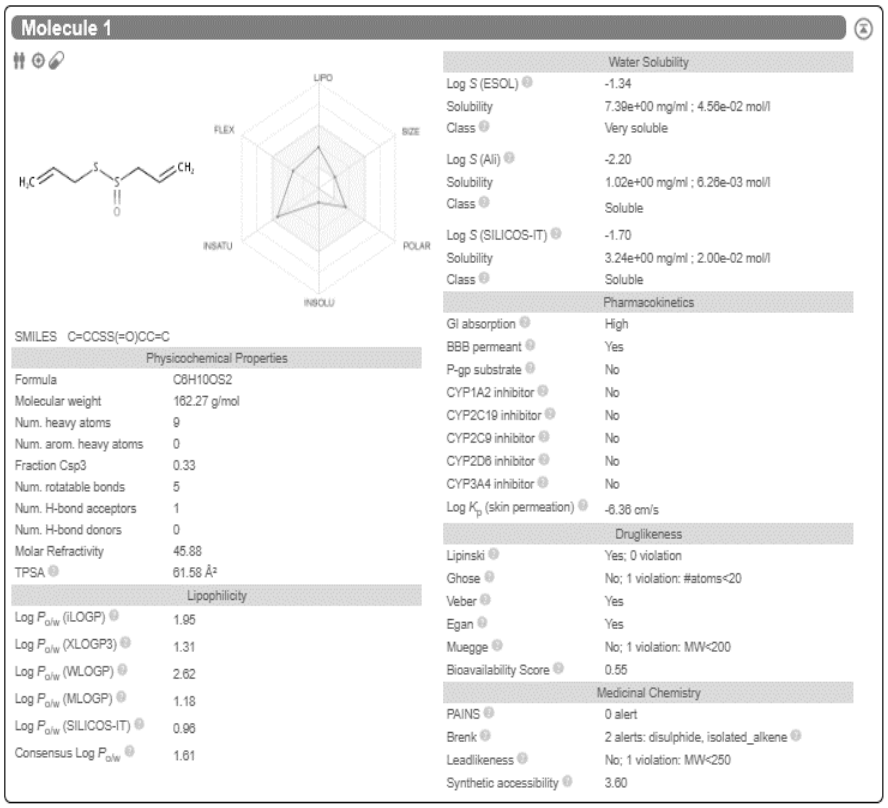


Figure 5.16: Physicochemical characterization of allicin

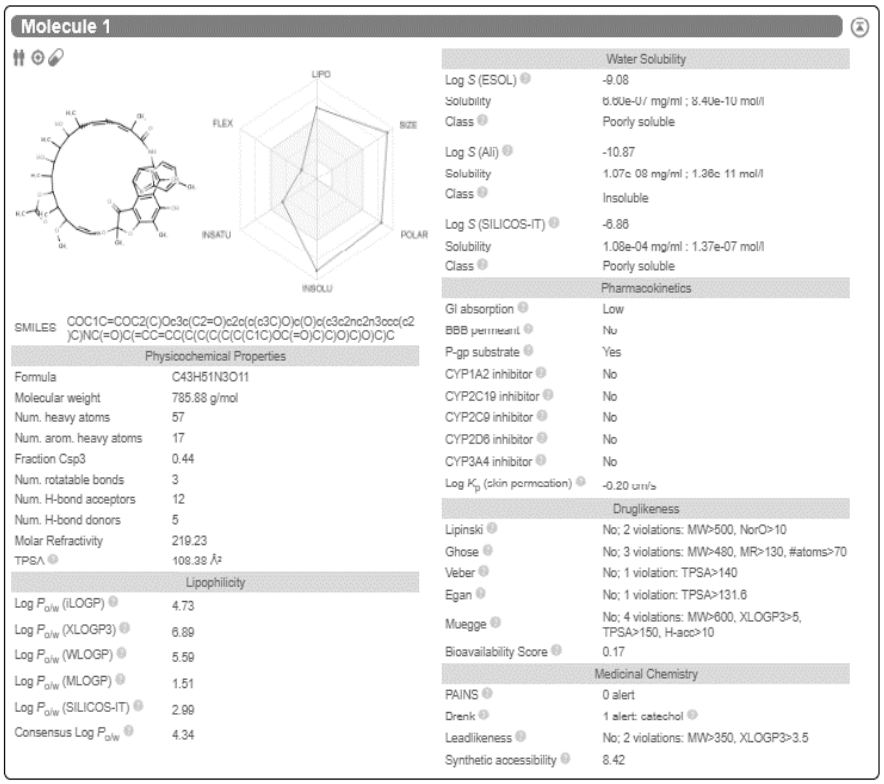


Figure 5.17: Physicochemical characterization of rifaximin

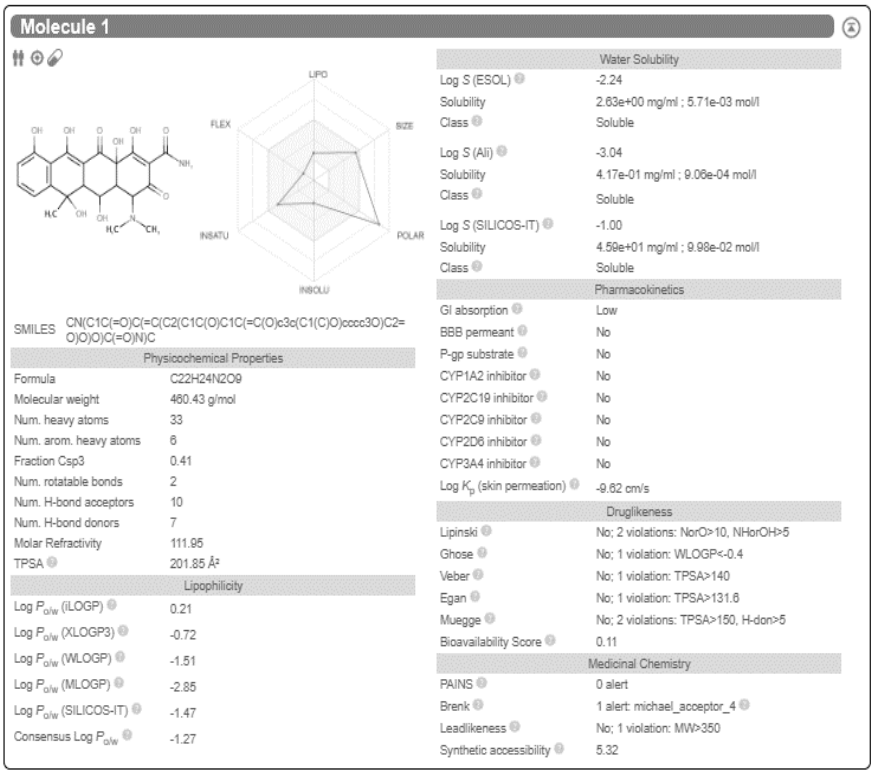


Figure 5.18: Physicochemical characterization of oxytetracycline

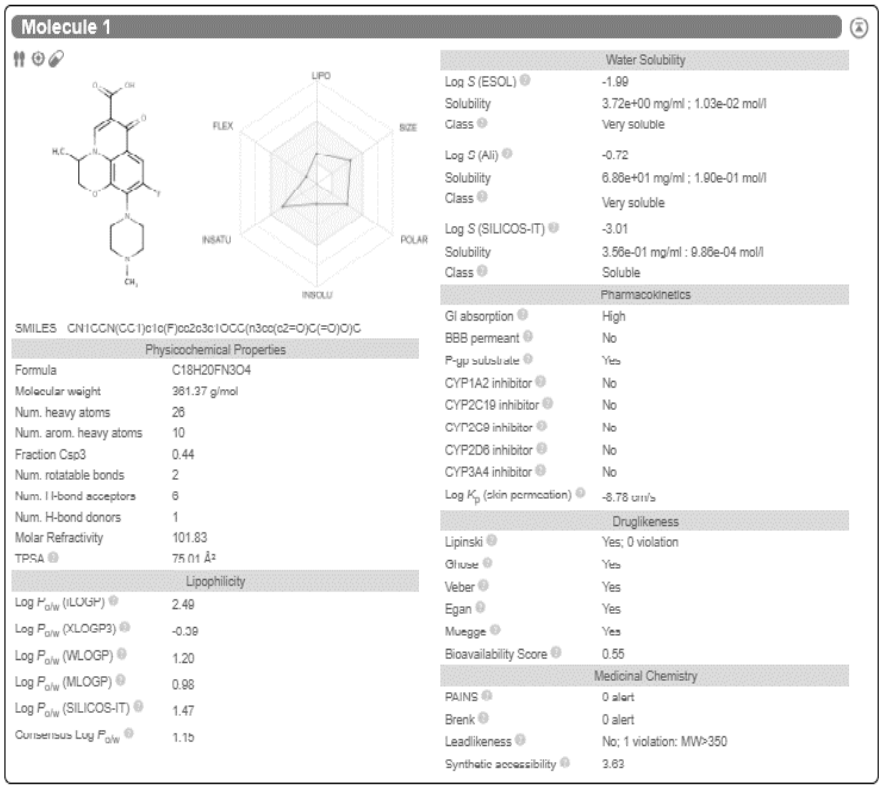


Figure 5.19: Physicochemical characterization of levofloxacin

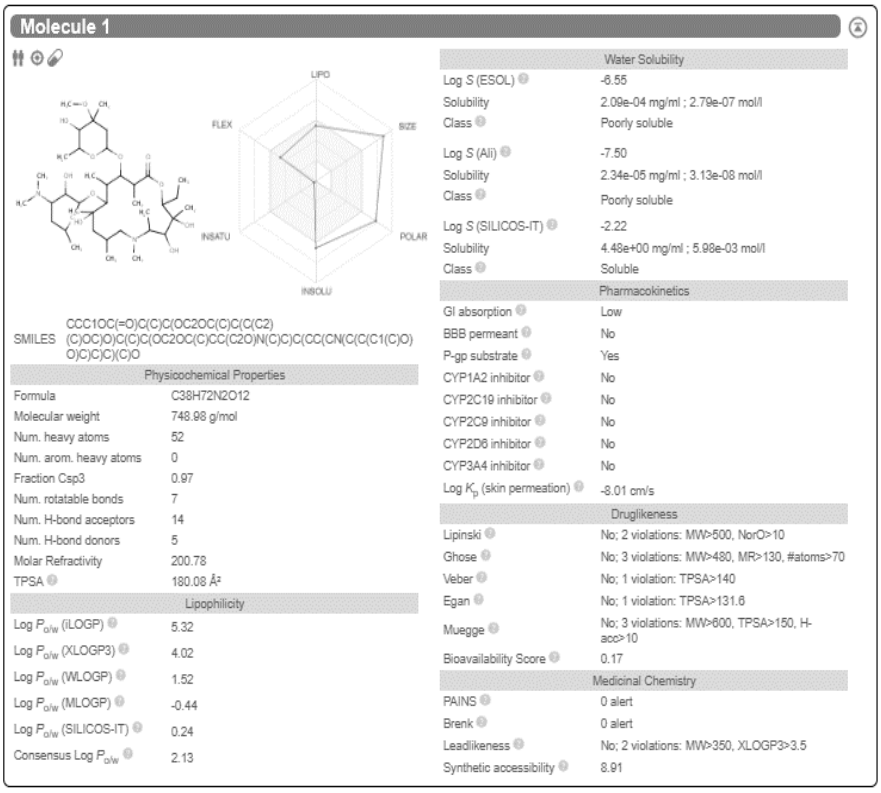


Figure 5.20: Physicochemical characterization of azithromycin

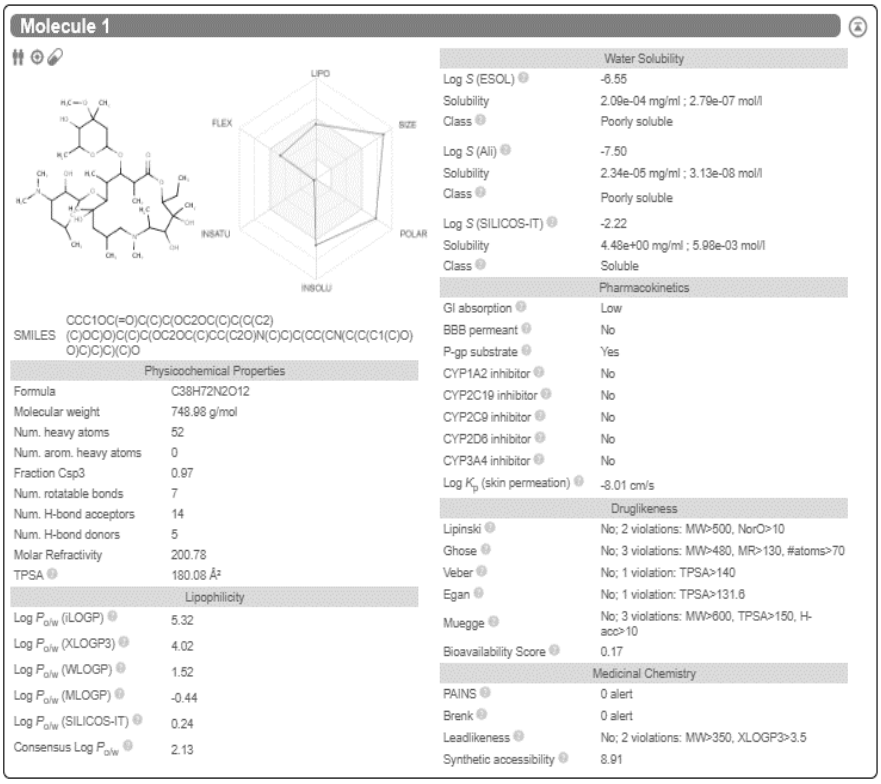


Figure 5.21: Physicochemical characterization of metronidazole

Lipinski rule of 5 helps in distinguishing between drug like and non-drug like molecules. It predicts high probability of success or failure due to drug likeness for molecules complying with 2 or more characters. It states that the molecular mass should be less than 500, should contain high lipophilicity and less hydrogen donors (<5). It should have less hydrogen acceptor (<10) and a moderate molar refractivity (40-130) [81]. Violations of this rule for any molecule make it a less drug like compound. SwissADME analyzed the properties for each molecule and produced the result for drug likeness.

From the general overview of antibacterial agents, it is evident that allicin and levofloxacin have zero violation of Lipinski's rule. Rifaximin, azithromycin and oxytetracycline each have 2 violations. It shows allicin and levofloxacin are more ideal antibacterial agent than rifaximin, azithromycin and oxytetracycline according to Lipinski's rule.

5.7.2 Synergistic study

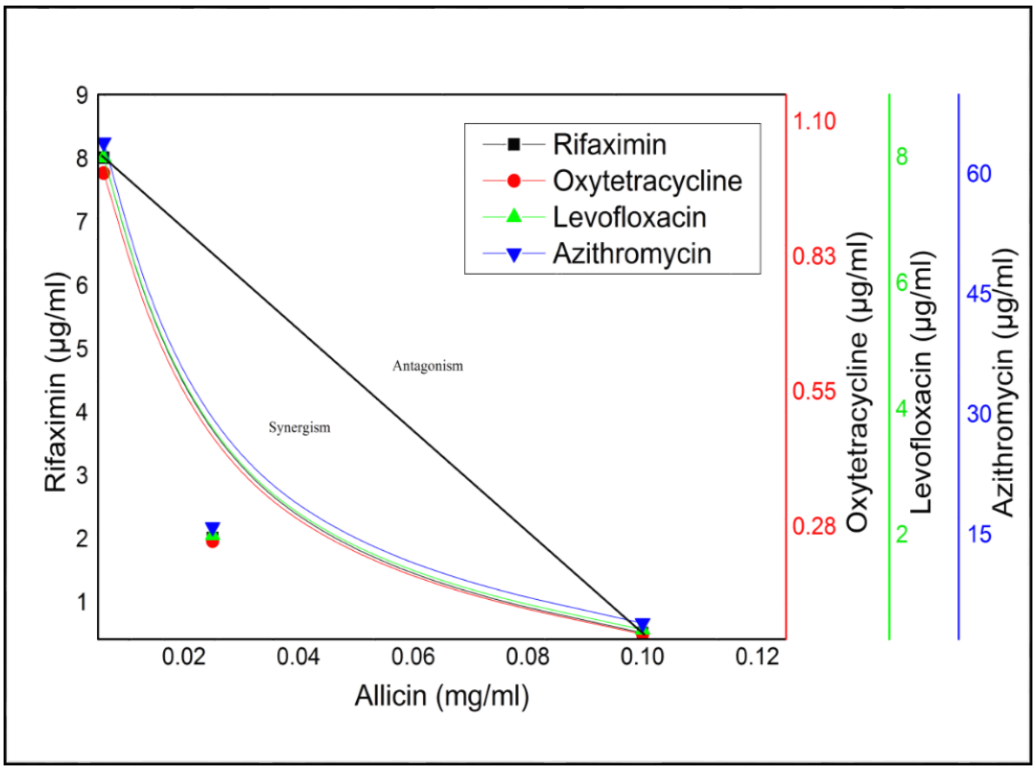


Figure 5.22: Isobologram of antibacterial agents against *Escherichia coli*

Figure 5.22 shows the synergistic combination of FGE containing allicin with rifaximin, levofloxacin, oxytetracycline and azithromycin. No synergy was obtained with metronidazole. The diagonal line is the additive line which differentiates the area of synergism and antagonism.

Usually two drugs have different mode of actions. For example rifaximin inhibits the DNA directed RNA polymerase subunit beta of the selected bacterial strain where allicin mostly attaches with cystein residues. So, in combination they work better than they do individually. Similarly for oxytetracycline, it inhibits the cell growth by ceasing translation. It attaches with 30S ribosomal unit and prevent tRNA binding with the site. Levofloxacin also inhibits topoisomerase and DNA gyrase enzyme. It is mostly effective against *Haemophilus influenza*e but synergistic effect was observed against *Escherichia coli* in combination with FGE containing allicin. Azithromycin inhibits 23S RNA and bacterial 50S ribosomal subunit. *Escherichia coli* are usually resistant to azithromycin but synergistic effect was obtained against this specific strain in combination with FGE containing allicin. So, it is evident that even if the antibiotics

aren't capable enough to inhibit the growth of the designated bacteria, they have shown synergistic effect with FGE containing allicin.

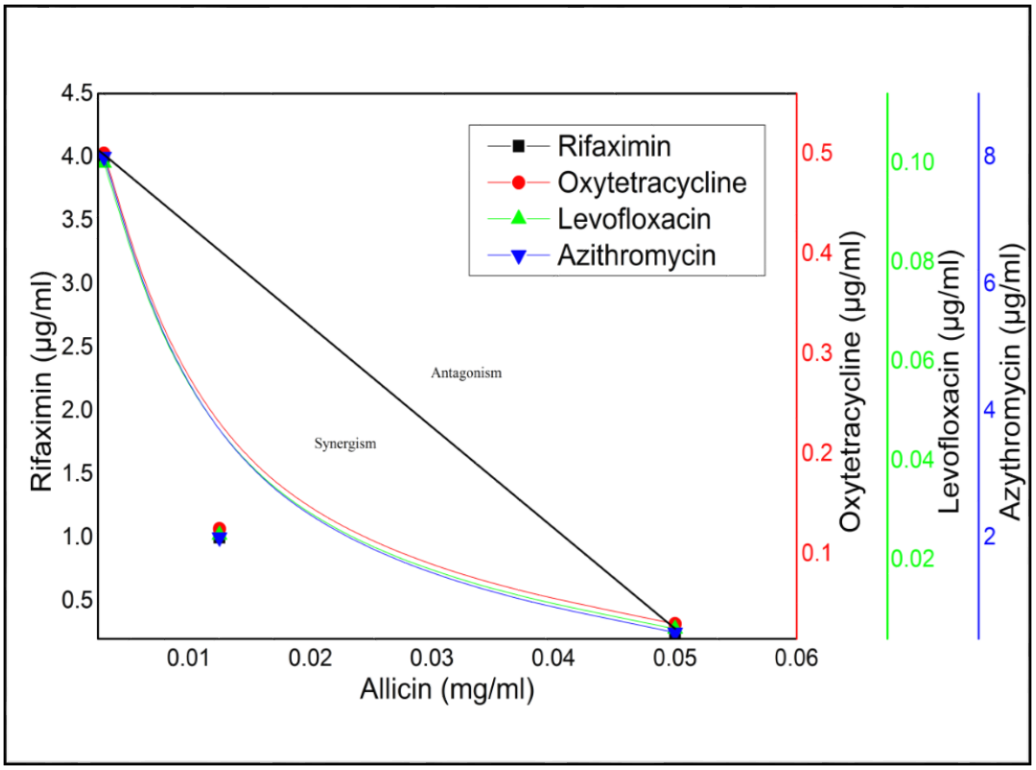


Figure 5.23: Isobologram of antibacterial agents against *Bacillus subtilis*

Figure 5.23 shows the synergistic combination of FGE containing allicin with rifaximin, levofloxacin, oxytetracycline and azithromycin. No synergy was obtained with metronidazole. The diagonal line is the additive line which differentiates the area of synergism and antagonism.

Bacillus subtilis are a gram-positive strain and usually most antibiotics are effective against it. Mechanism of inhibiting the cell growth by rifaximin, oxytetracycline, levofloxacin and azithromycin is already described for figure 5.22. For *Bacillus subtilis*, they follow the same mode of actions. They usually prevent translation, translocation, transpeptidation, transcription and replication to cease the bacterial growth. Allicin follows another mechanism; it inhibits active enzymes in metabolic pathways and stops cell growth. Though, the mode of action of allicin is not completely known yet. But it was clearly observed that these antibiotics have synergistic effect with FGE containing allicin.

5.8 Time kill curve with fresh garlic extract containing allicin in synergy with rifaximin, oxytetracycline, levofloxacin and azithromycin against *Escherichia coli and Bacillus subtilis*

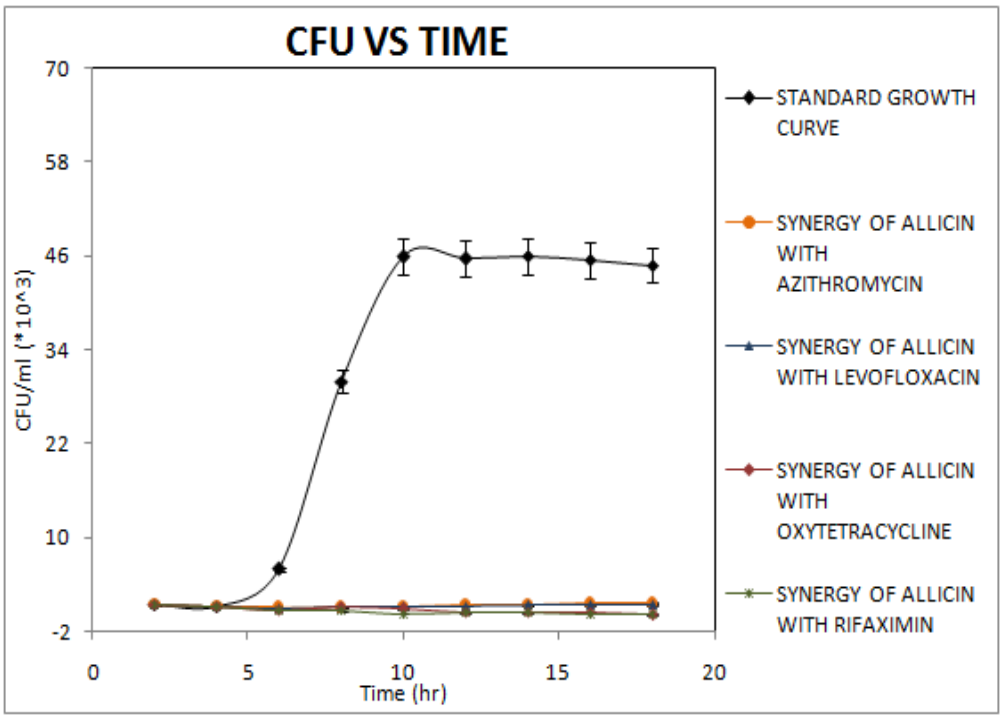


Figure 5.24: Synergy of fresh garlic extract containing allicin with antibiotics against lag phase of *Escherichia coli*

Synergistic combination of fresh garlic extract containing allicin with rifaximin, oxytetracycline, levofloxacin and azithromycin respectively was applied to the lag phase of the designated bacteria.

Rifaximin and oxytetracycline in combination with fresh garlic extract containing allicin had shown better result according to the CFU counts than levofloxacin and azithromycin. CFU count for azithromycin was the highest after applying it against the strain. Levofloxacin had shown a better result than azithromycin. In synergistic combinations, after applying the antibiotics with fresh garlic extract containing allicin no bacterial growth was observed as there was no such increase in CFU counts.

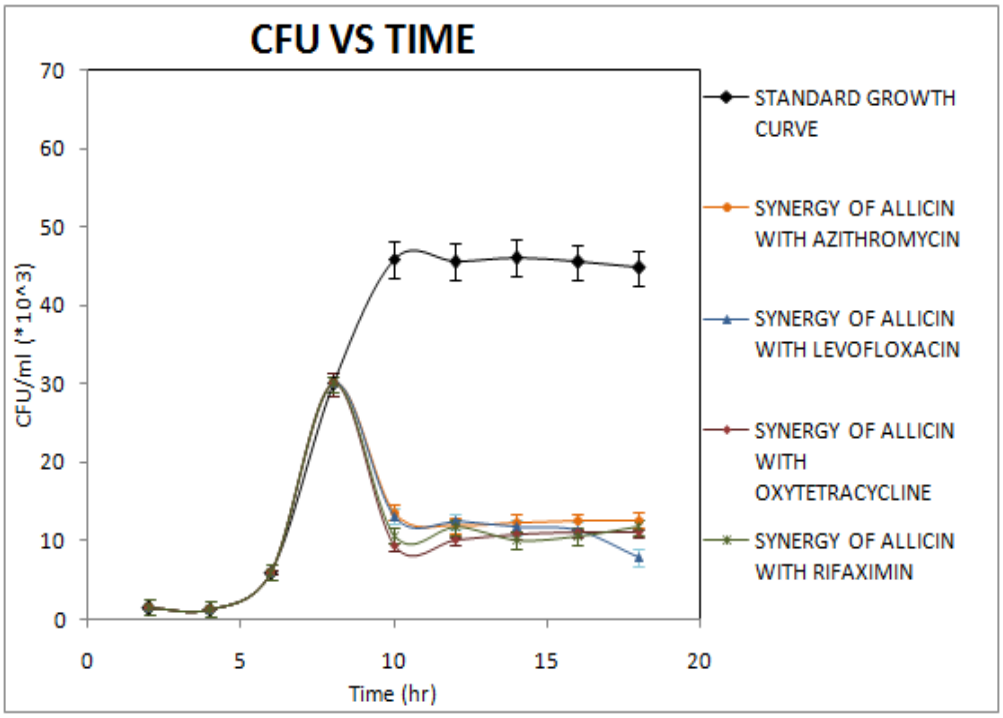


Figure 5.25: Synergy of fresh garlic extract containing allicin with antibiotics against log phase of *Escherichia coli*

Synergistic combination of fresh garlic extract containing allicin with rifaximin, oxytetracycline, levofloxacin and azithromycin respectively was applied to the mid log phase of the designated bacteria.

From the graph, it is visible that initially rifaximin in combination with fresh garlic extract containing allicin ceased the growth maximum in comparison with others after adding to the culture followed by oxytetracycline, levofloxacin and azithromycin respectively according to the CFU counts. But for rifaximin and oxytetracycline, after certain time slight increase in the CFU count was observed after which there was no change in CFU counts. CFU count of azithromycin almost remained the same. Fresh garlic extract containing allicin in combination with levofloxacin had shown the better result finally as there was a continuous decrease in the CFU values. It is evident from the graph that finally CFU count decreased the mostfor garlic extract containing allicin in combination with levofloxacin.

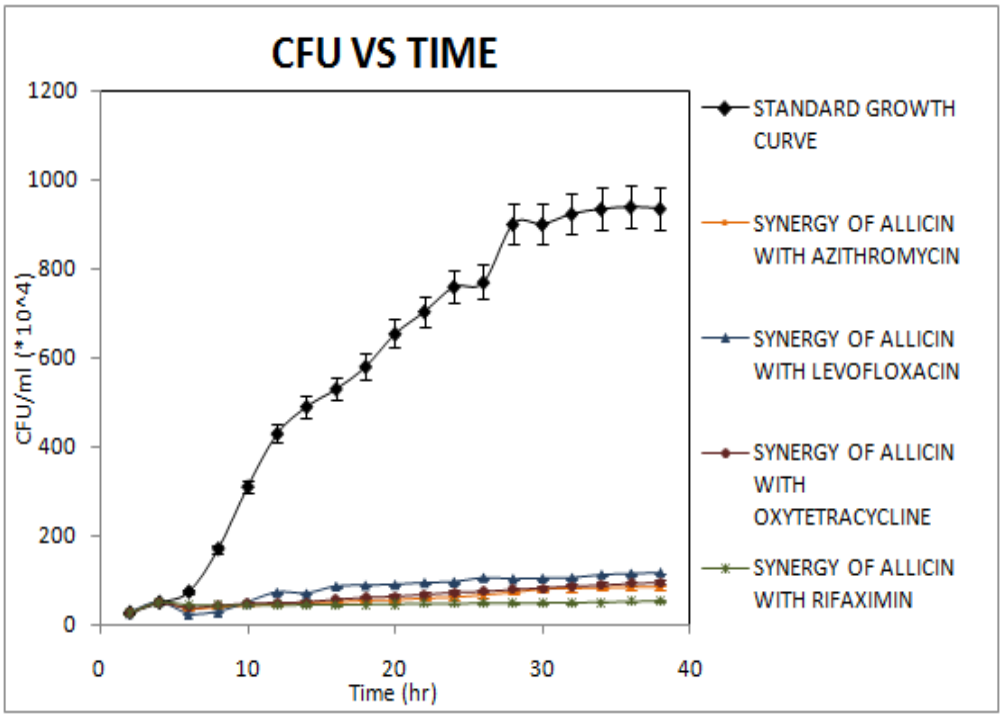


Figure 5.26: Synergy of fresh garlic extract containing allicin with antibiotics against lag phase of *Bacillus subtilis*

Synergistic combination of fresh garlic extract containing allicin with rifaximin, oxytetracycline, levofloxacin and azithromycin respectively was applied to the lag phase of the selected bacteria.

Rifaximin in combination with fresh garlic extract containing allicin had shown better result according to the CFU counts than levofloxacin, azithromycin and oxytetracycline. CFU count for levofloxacin was the highest after applying it against the strain. Levofloxacin had resulted in slightly higher CFU counts than azithromycin.

In synergistic combinations, after applying the antibiotics with fresh garlic extract containing allicin no such bacterial growth was observed as there was no such increase in CFU counts. For first 2 hours there was direct decrease of CFU counts, after certain time minimal increase in CFU/ml was observed but it stayed almost constant throughout the whole time. So, in this comparative study with standard curve the growth had ceased and had shown a better and fruitful result.

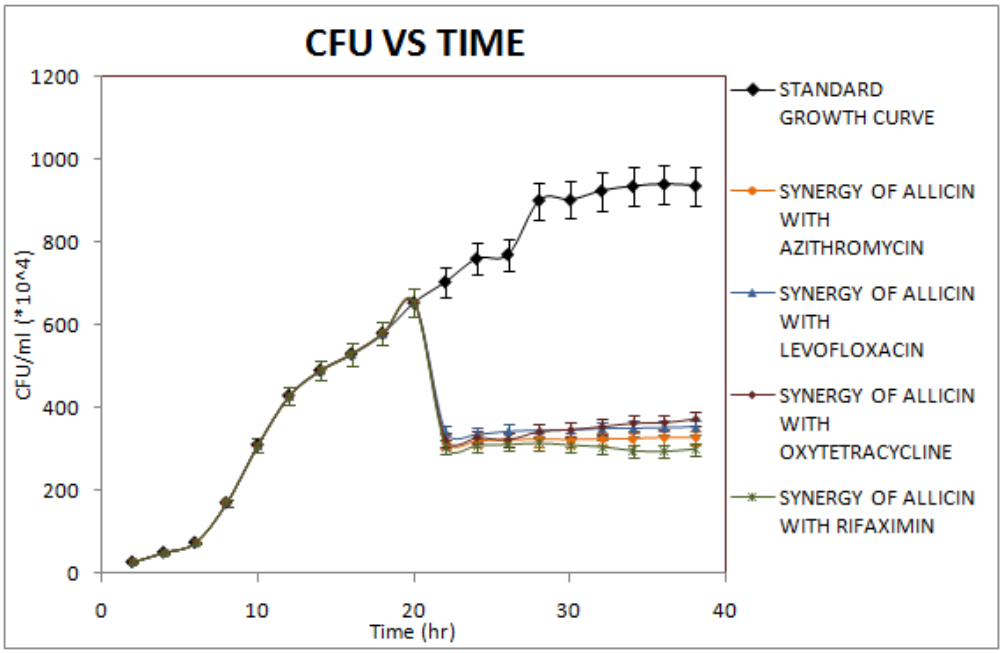


Figure 5.27: Synergy of fresh garlic extract containing allicin with antibiotics against log phase of *Bacillus subtilis*

In this synergistic study, fresh garlic extract containing allicin with rifaximin, oxytetracycline, levofloxacin and azithromycin respectively was applied to the log phase of *Bacillus subtilis*

Rifaximin in combination with fresh garlic extract containing allicin had shown better result according to the CFU counts than levofloxacin, azithromycin and oxytetracycline. Initially the CFU count for rifaximin had decreased the most and stayed constant throughout the phase.CFU count for oxytetracycline was the highest after applying it against the strain. This particular strain is resistant to oxytetracycline as found from literature survey. Thus, there was minimum increase in CFU count. Levofloxacin had resulted in slightly higher CFU counts than azithromycin.

In synergistic combinations, after applying the antibiotics with fresh garlic extract containing allicin no such bacterial growth was observed as there was no such increase in CFU counts. For first 2 hours there was direct decrease of CFU counts, it stayed almost constant throughout the whole time. This study showed that synergistic combination of allicin with antibiotics had inhibited the growth of bacteria successfully.

5.9 Growth kinetic study and time kill kinetics

Table 8: Standard growth curve

Strain Name	**Net Specific Growth Rate (μ) (hr^{-1})**	**Doubling Time (T) (hr^{-1})**
Escherichia coli	0.6	1.12
Bacillus subtilis	0.12	5.7

Table 8 shows the net specific growth rate and doubling time for standard growth of both strains in absence of any antibacterial agent.

Table 9: Time kill kinetics with fresh garlic extract containing allicin at MIC values

Strain Name	**Growth Phase**	**Death Rate Constant (K) (hr^{-1})**
Escherichia coli	Lag phase	0.07
	Mid-Log phase	0.32
	Stationary phase	0.11
Bacillus subtilis	Lag phase	0.05
	Log phase	0.47
	Stationary phase	0.16

Table 9 shows the death rate of FGE containing allicin at MIC values against selected bacterial strains at 3 different stages. For *Escherichia coli* and *Bacillus subtilis*, high killing rate was obtained at log phase (mid exponential). For lag phase, the microbial load amount was too less

and no such change was observed so it indicated a slower killing rate. *Bacillus subtilis* had a higher death rate at log phase.

At stationary phase, the killing rate was moderate because the bacterial growth was higher at this stage and cell numbers were comparatively greater than the other two phases at inoculation time.

Table 10: Growth rate and doubling time of bacteria after applying fresh garlic extract containing allicin at MIC values

Strain Name	**Growth Phase**	**Net Specific Growth Rate (μ) (hr^{-1})**	**Doubling Time (T) (hr^{-1})**
Escherichia coli	Lag phase	0.106	6.54
	Mid-log phase	0.055	12.6
	Stationary phase	0.05	13.8
Bacillus subtilis	Lag phase	0.045	15.4
	Mid-log phase	0.04	17.32
	Stationary phase	0.028	24.1

Table 10 shows the growth rate of both strains and their doubling time after applying FGE containing allicin. The growth rate was very low considering the standard growth rate of both strains. The doubling time was much higher than a normal growth curve. For stationary phase, though the microbial load was initially high, it resulted in a slower growth rate than log phase. As the amount of nutrient was limited in this phase so some bacterium could die due to limitation of nutrients. This could be the probable reason of the slower growth rate at stationary phase for both strains than log phase.

Growth of bacteria was observed after certain time of treating it with antibacterial agent, it could be due to the poor stability of allicin. As it started degrading, growth of bacteria was observed but the growth rate was very minimal.

Table 11: Time kill kinetics with fresh garlic extract containing allicin at MBC values

Strain Name	**Growth Phase**	**Death Rate Constant (K) (hr^{-1})**
Escherichia coli	Lag phase	0.085
	Mid-Log phase	0.591
	Stationary phase	0.236
Bacillus subtilis	Lag phase	0.197
	Log phase	0.595
	Stationary phase	0.226

Table 11 shows the death rate of FGE containing allicin at MBC values against selected bacterial strains at 3 different stages.

For *Escherichia coli* and *Bacillus subtilis*, the highest killing rate was obtained at log phase (mid exponential). For lag phase, the amount of microbial load was too less and no such change was observed. It indicated a slower killing rate. *Bacillus subtilits* had a higher death rate in this phase.

At mid exponential the killing rate was high for both strains. Almost similar death rate constant was obtained for both designated strains. At stationary phase, the killing rate was moderate because the bacterial growth rate was higher at this stage and cell numbers were comparatively greater than the other two phases at inoculation time. The death rate was higher for MBC value of FGE containing allicin than MIC value. From the result, it can be said that if the amount of allicin increases, it may result in higher death rate.

Table 12: Growth rate and doubling time of bacteria after applying fresh garlic extract containing allicin at MBC values

Strain Name	**Growth Phase**	**Net Specific Growth Rate (μ) (hr^{-1})**	**Doubling Time (T) (hr^{-1})**
Escherichia coli	Lag phase	0.08	8.66
	Mid-log phase	0.0384	18.05
	Stationary phase	0.02	13.8
Bacillus subtilis	Lag phase	0.046	15
	Mid-log phase	0.033	22.1
	Stationary phase	0.03	27.72

Table 12 shows the growth rate of both strains and their doubling time after applying FGE containing allicin at MBC values. The growth rate was very low considering the standard growth rate of both strains. The doubling time was much higher than a normal growth curve. For stationary phase, though the microbial load was initially high; it resulted in a slower growth rate than log phase. As the amount of nutrient was limited in this phase so some bacterium could die due to limitation of nutrients. This could be the probable reason of the slower growth rate at stationary phase for both strains than log phase. *Bacillus subtilis* had a lower growth rate than *Escherichia coli* and FGE containing allicin had a better impact on *Bacillus subtilis* according to the death rate.

Growth of bacteria was observed after certain time of treating it with antibacterial agent, it could be due to the poor stability of allicin. As it started degrading, growth of bacteria was observed but the growth rate was very minimal.

Table 13: Time kill kinetics with fresh garlic extract containing allicin in synergy with rifaximin, oxytetracycline, levofloxacin and azithromycin against *Escherichia coli*

Strain Name	Growth Phase	Antibiotics in Combination with FGE Containing Allicin	Death Rate Constant (K) (hr^{-1})
Escherichia coli	Lag phase	Rifaximin	0.1143
		Oxytetracycline	0.140
		Levofloxacin	0.136
		Azithromycin	0.083
	Mid log phase	Rifaximin	0.587
		Oxytetracycline	0.515
		Levofloxacin	0.413
		Azithromycin	0.395

Table 13 shows the synergistic effect of FGE containing allicin with different antibiotics and a better death rate was observed for each condition than only treated by FGE containing allicin. It is evident that rifaximin had shown a better death rate followed by oxytetracycline, levofloxacin and azithromycin. The death rate indicated the higher potency of rifaximin in synergistic combination with allicin. But after initial decrease in the CFU count, there was no further change observed for rifaximin, oxytetracycline and azithromycin. *Escherichia coli* are usually resistant to azithromycin but in this case it had shown a promising result in synergistic combination. There was no further growth observed. It shows that FGE containing allicin works better in synergistic combination with these antibiotics.

Table 14: Time kill kinetics with fresh garlic extract containing allicin in synergy with rifaximin, oxytetracycline, levofloxacin and azithromycin against *Bacillus subtilis*

Strain Name	Growth Phase	Antibiotics in Combination with FGE Containing Allicin	Death Rate Constant (K) (hr^{-1})
Bacillus subtilis	Lag phase	Rifaximin	0.163
		Oxytetracycline	0.123
		Levofloxacin	0.124
		Azithromycin	0.105
	Mid log phase	Rifaximin	0.378
		Oxytetracycline	0.35
		Levofloxacin	0.325
		Azithromycin	0.357

Table 14 shows the synergistic effect of FGE containing allicin with different antibiotics and a better death rate was observed for each condition than only treated by FGE containing allicin. Rifaximin had shown the best result according to the death rate for both gram-positive and gram-negative strains in combination with FGE containing allicin. Azithromycin had resulted better for *Bacillus subtillis*. Oxytetracycline and levofloxacin had similar effect for lag phase of the selective strain. In the study conducted for log phase, best result was observed for rifaximin followed by azithromycin, oxytetracycline and levofloxacin. Table 14 shows that allicin at synergistic combination had shown a positive result for each antibiotic even at four fold diluted MIC values.

5.10 Determination of E_{max} value by maximum effect model of fresh garlic extracts containing allicin for *Escherichia coli* and *Bacillus subtilis*

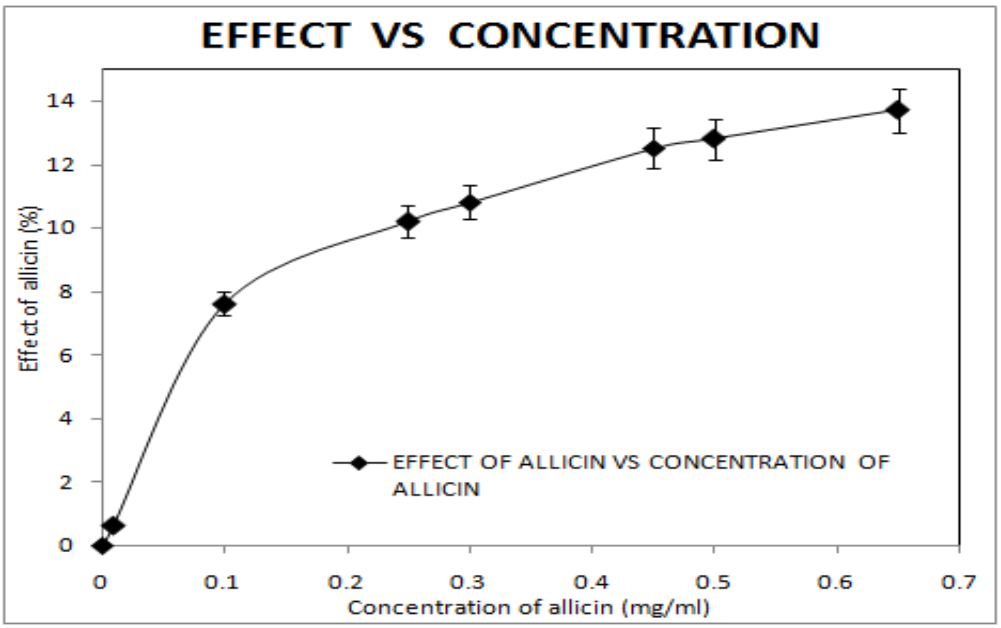


Figure 5.28: Effect of FGE containing allicin with respective concentration against *Escherichia coli*

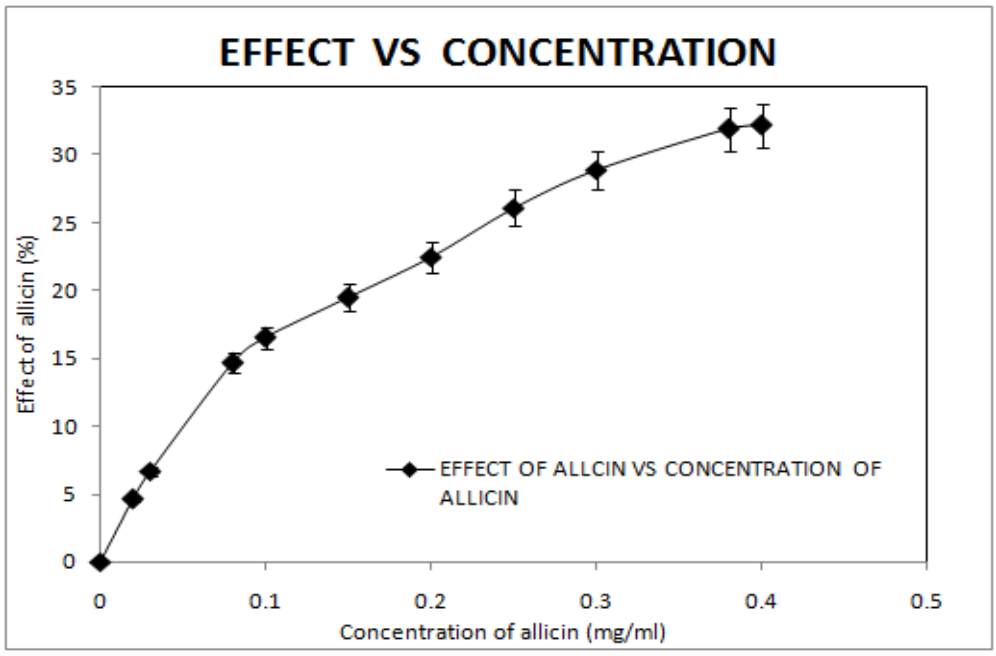


Figure 5.29: Effect of FGE containing allicin with respective concentration against *Bacillus subtilis*

Table 15: E_{max} and EC_{50} value of allicin against *Escherichia coli*

Strain Name	**E_{max} (%)**	**EC_{50}(mg/ml)**
Escherichia Coli	15.9 ≈16	0.095 ≈ 0.1

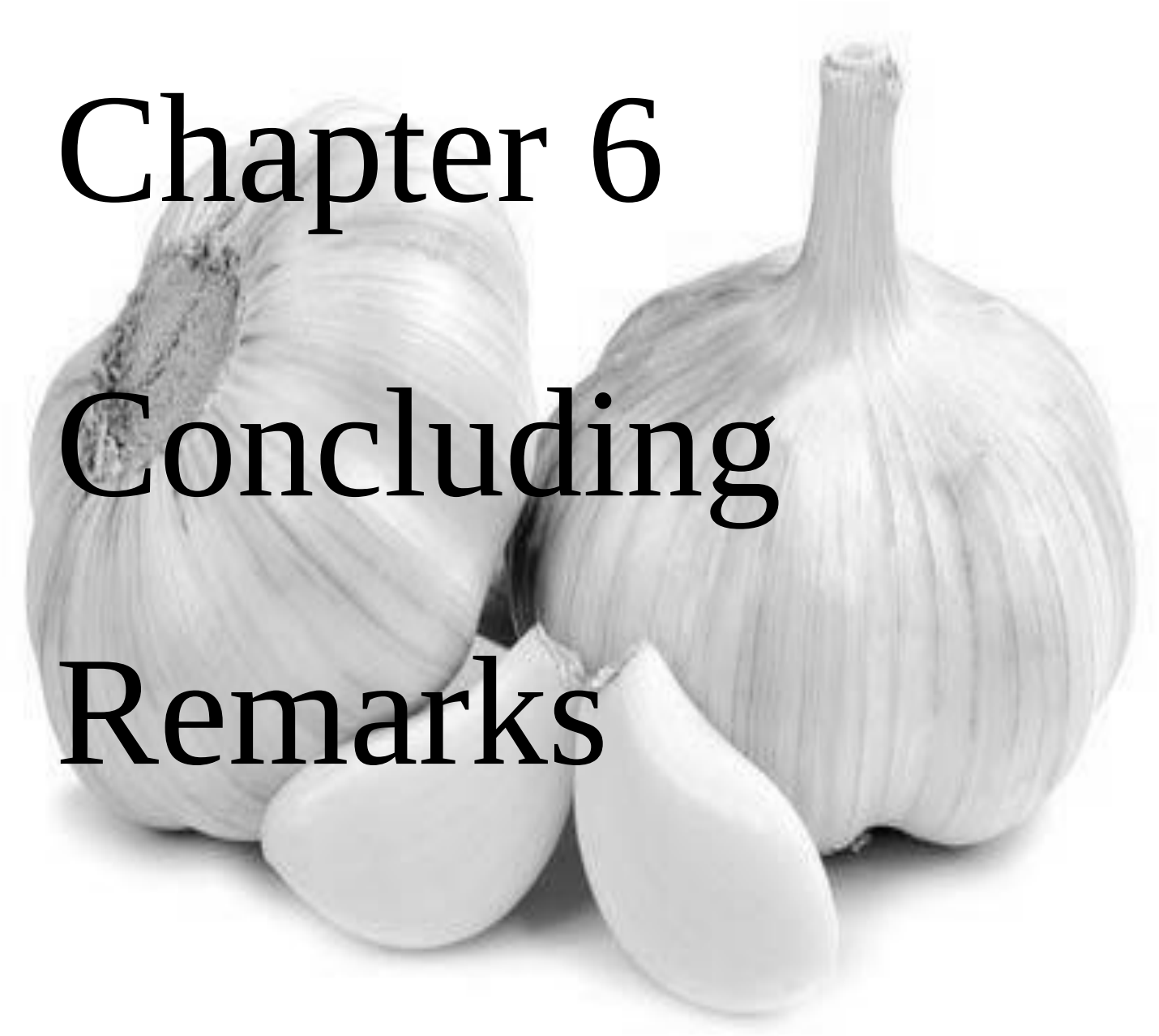
Table 16: E_{max} and EC_{50} value of allicin against *Bacillus subtilis*

Strain Name	**E_{max} (%)**	**EC_{50} (mg/ml)**
Bacillus subtilis	47.6	0.14

Maximum effect of allicin and EC_{50} were calculated using the equation 11 from 4.11.3. E_{max} value was higher for *Bacillus subtilis*, the probable value was 47.6 %. For *Escherichia coli*, E_{max} was found to be almost 16% which indicated that it can kill maximum 16% of the viable bacterial strain. The probable reason for such low value is *Escherichia coli* is a gram-negative strain and have higher resistance against antibacterial agents. EC_{50} values are 0.1 and 0.14 for *Escherichia coli* and *Bacillus subtilis* respectively. The lower EC_{50} value indicated that the drug reached the 50% response at a low concentration but after that increasing the concentration didn't trigger any further high response. The lower E_{max} value for *Escherichia coli* referred to the less efficacy of the drug against this particular strain. This could be due to its lower affinity towards the target or its poor stability. Allicin degrades quickly so after a certain point it loses the ability to inhibit the growth of bacteria and results in a reformation of bacterial growth. For *Bacillus subtilis*, as it is a gram positive bacteria so the maximum effect of allicin was higher and indicated greater potency of allicin against gram-positive bacteria.

Chapter 6

Concluding Remarks



6.1 Conclusion

The outbreak of severe resistant infectious diseases and side effects of used antibiotics have impelled researchers to find an alternative of commercial antibiotics. Misuse of antibiotics has created resistant microbial species. Nutraceutical compounds can play a major role in such scenario to overcome the threat to human lives.

Allicin is found in fresh garlic extract and is solely responsible for antibacterial activity of garlic. This study was conducted to check the potency of FGE containing allicin against *Bacillus subtilis* (gram-positive strain) and *Escherichia coli* (gram-negative strain). Fresh garlic extract was prepared and allicin concentration was found to be 1.6 mg/ml. Quality parameters of the extracts were checked and total protein, phenol and flavonoid content were determined. It was observed that the extract had antioxidant property by DPPH radical scavenging assay. It had 386mg/L total protein, 580µg/ml total phenol and 86µg/ml total flavonoid content. The garlic extract could have some amount of precursor allin and enzyme allinase along with allicin.

In batch process, under favorable condition, *Bacillus subtilis* and *Escherichia coli* had achieved a higher growth rate without any inhibitory compound in the media. A comparative study was carried out and changed in CFU values were obtained after applying FGE containing allicin at 3 different phases of bacterial growth. Firstly, a direct drop in CFU counts was observed but after certain time there was a slow increase in CFU counts per ml for both MIC and MBC values at different stages. From growth and time kill kinetics, it was determined that highest death rate was achieved at the log phase, followed by stationary and lag phase. But the proliferation of bacterial growth was high for both stationary and log phase. It indicated that FGE containing allicin was an inhibitory agent and could cease the growth for certain time. But due to its poor stability, it degraded quickly and resulted in formation of bacterial growth. FGE containing allicin at MBC value had shown a better result at exponential phase according to the time kill study. From the investigation, it was also noticed that FGE containing allicin was more effective on *Bacillus subtilis* than *Escherichia coli*.

Synergistic combinations of FGE containing allicin with rifaximin, oxytetracycline, levofloxacin and azithromycin were applied to both designated strains at log and lag phase. The best result was obtained for synergistic combination of rifaximin with allicin. *Escherichia coli* is usually

resistant to azithromycin but this investigation illustrated that synergistic combination of garlic extract containing allicin with azithromycin had successfully inhibited the growth of bacteria. It shows that antibiotics can work better in synergy.FGE containing allicin had also shown better antibacterial activity against both strains in synergistic combinations. It worked even at a lower concentration than MIC of allicin. So, it was found that FGE containing allicin had a synergistic effect when it was combined with antibiotics and resulted in better killing rate of bacteria. E_{max} value of FGE containing allicin indicated a higher effect against *Bacillus subtilis.*

The present study had shown that FGE containing allicin is a potent antibacterial agent and was more effective against gram-positive bacteria. Study with synergistic combinations had indicated that it resulted better with antibiotics. The synergistic study suggested that the use of FGE containing allicin along with antibiotics may overcome the resistance developed by bacteria. Finally it can be concluded that fresh garlic extract containing allicin is a potential antibacterial agent and can inhibit proliferation of bacteria in synergy with other antibiotics. Considering the potentiality of allicin, it can be stated that in future it has possibilities to be used as a therapeutic antimicrobial drug.

6.2 Future Scope

The positive result from this experimental study suggested that FGE containing allicin is effective as an antibacterial agent. However, some further research should be done to use it commercially.

- Study to increase the stability of allicin.
- Study to form a pure extract of allicin from garlic.
- Study to optimize the pH and ionic conditions for higher stability of allicin.
- Toxicological study on the garlic extracts using gas chromatography & mass spectroscopy & others.
- Study to find out specific mode of action of allicin against bacterial strain.
- Application of allicin on mammalian cell line to find a better practical scenario of affectivity of allicin.

Appendix

Table A: Composition of starch agar

Ingredients	**Amount (g L^{-1})**
Soluble starch	10
Peptone	5
Agar	15

Table B: Instrument credentials

INSTRUMENT NAME	**MANUFACTURED BY**
AUTOCLAVE	CONCEPTS INTERNATIONAL
INCUBATOR-SHAKER	CONCEPTS INTERNATIONAL
VORTEX	REMI
MIXER-GRINDER	PHILIPS INDIA PVT. LTD.
MICROSCOPE	OPTIKA [ITALY] XDS-2
PH METER	LABMAN
RO WATER SYSTEM	SARTORIUS STEDIM BIOTECH
LAMINAR FLOW	N.R SCIENTIFIC
ANALYTICAL BALANCE	SARTORIUS BT 124 S
CENTRIFUGE	SUPERSPIN R-V/Fm
UV –VIS SPECTROPHOTOMETER	VARIAN 50 BIO
HOT AIR OVEN	CONCEPTS INTERNATIONAL
REFRIGERATOR	SAMSUNG
MAGNETIC STIRRER	REMI
MICROPIPETTE	TARSON, SARTORIUS
DEEP FRIDGER	BLUE STAR

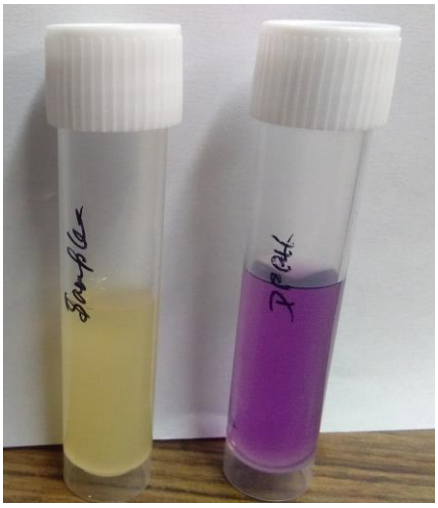


Figure A: Color change observed in DPPH assay

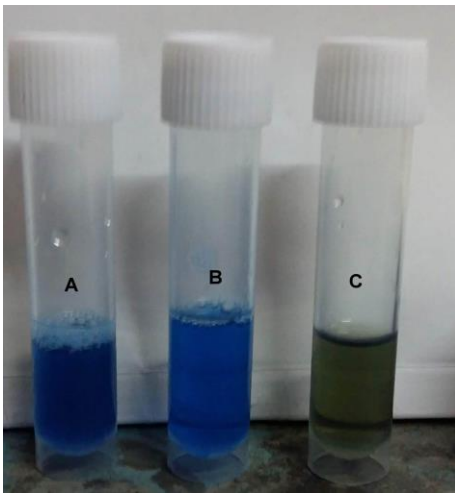


Figure B: Bradford assay
A and B: Samples at different dilution C: Blank

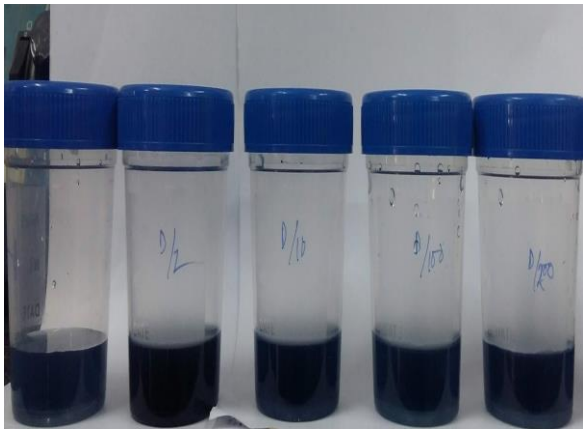


Figure C: Total phenol content assay

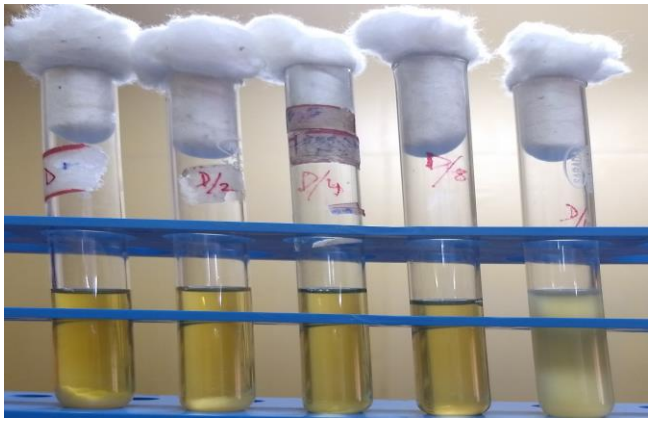


Figure D: Determination of MIC value of FGE containing allicin against *E.coli*

Figure E: Determination of MIC value of rifaximin and oxytetracycline against *E.coli* and *B.subtilis*

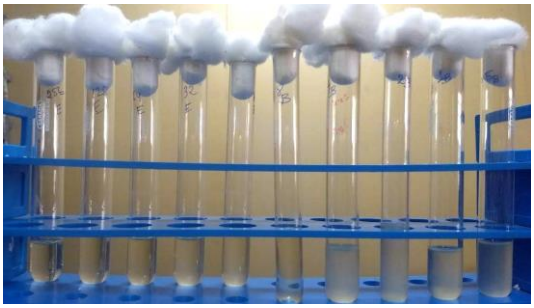


Figure F: Determination of MIC value of azithromycin against *E.coli* and *B.subtilis*

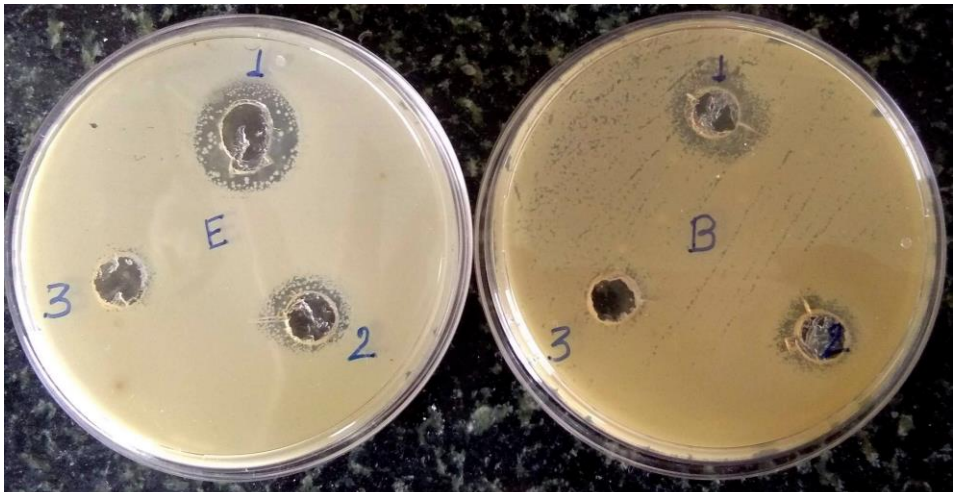


Figure G: Agar well diffusion method to determine MBC value of FGE containing allicin

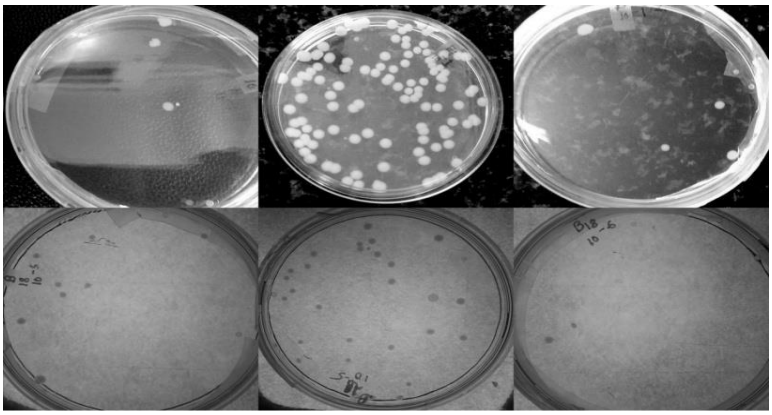


Figure H: CFU counting

Figure I: Incubator shaker

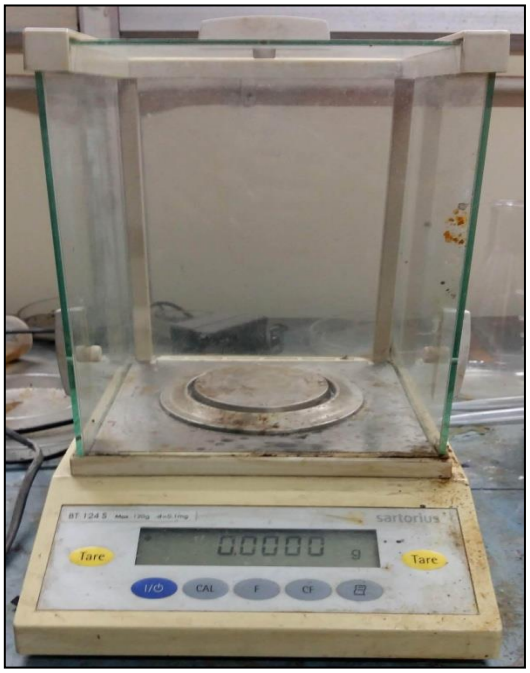


Figure J: Analytical balance

Figure K: Hot air oven

Figure L: Autoclave

Figure M: Cold centrifuge

Figure N: UV-VIS Spectrophotometer

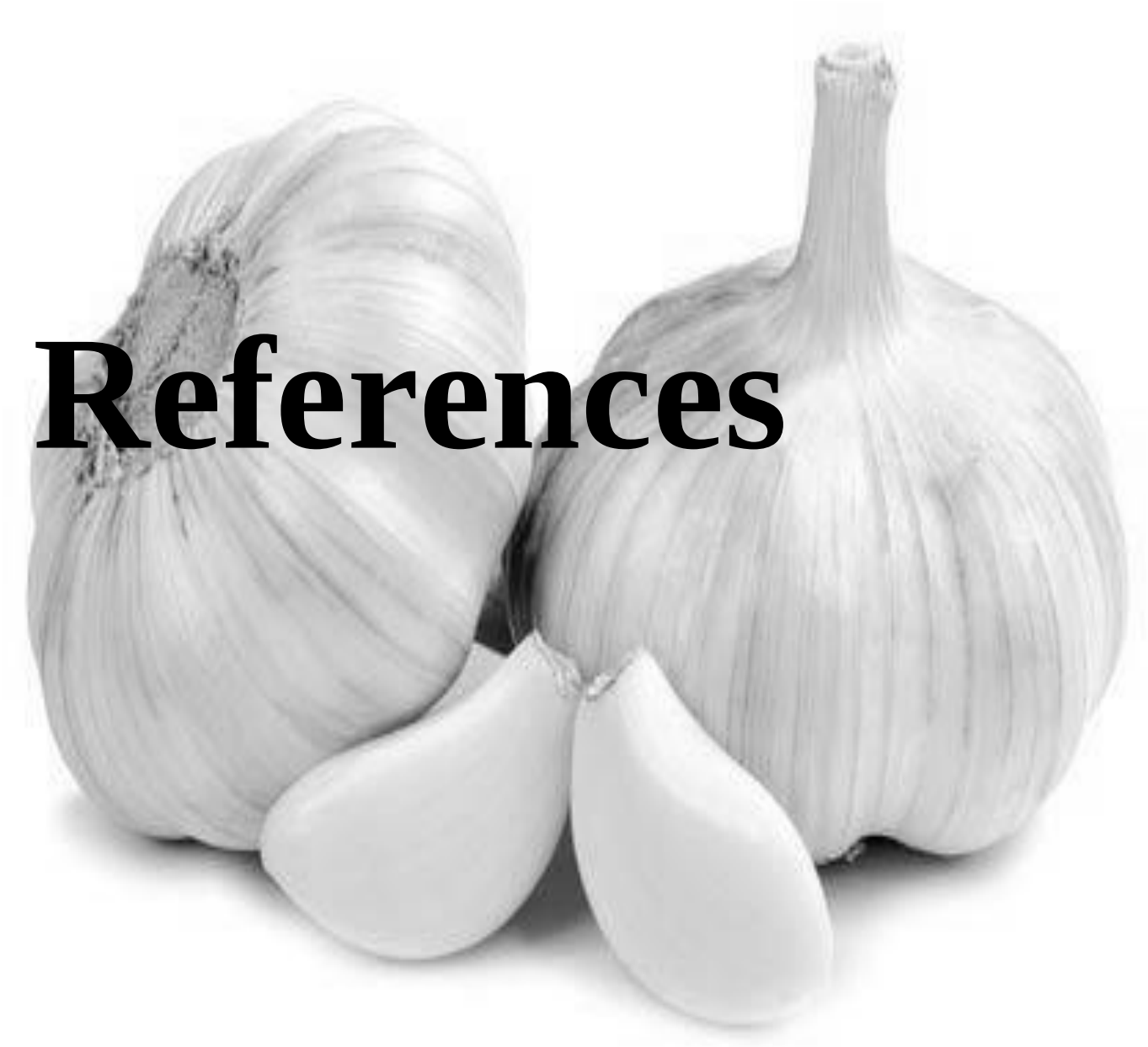
Figure O: Mixer grinder

Figure P: pH meter

Figure Q: Laminar Flow

Figure R: Microscope

References



References

1. Finlay, B.B. and Cossart, P., 1997. Exploitation of mammalian host cell functions by bacterial pathogens. *Science*, *276*(5313), pp.718-725.
2. Neu, H.C., 1992. The crisis in antibiotic resistance. *Science*, *257*(5073), pp.1064-1073.
3. Rios, J.L. and Recio, M.C., 2005. Medicinal plants and antimicrobial activity. *Journal of ethnopharmacology*, *100*(1-2), pp.80-84.
4. Abrami, L., Reig, N. and van der Goot, F.G., 2005. Anthrax toxin: the long and winding road that leads to the kill. *Trends in microbiology*, *13*(2), pp.72-78.
5. Salyers, A.A., Whitt, D.D. and Whitt, D.D., 1994. *Bacterial pathogenesis: a molecular approach* (Vol. 3). Washington, DC: ASM press.
6. Falkow, S., 1991. Bacterial entry into eukaryotic cells. *Cell*, *65*(7), pp.1099-1102.
7. Cossart, P. and Sansonetti, P.J., 2004. Bacterial invasion: the paradigms of enteroinvasive pathogens. *Science*, *304*(5668), pp.242-248.
8. Kotb, M., 1995. Bacterial pyrogenic exotoxins as superantigens. *Clinical microbiology reviews*, *8*(3), pp.411-426.
9. Aktories, K. and Barbieri, J.T., 2005. Bacterial cytotoxins: targeting eukaryotic switches. *Nature Reviews Microbiology*, *3*(5), p.397.
10. Morrison, D.C. and Ryan, J.L., 1987. Endotoxins and disease mechanisms. *Annual review of medicine*, *38*(1), pp.417-432.
11. Morrison, D.C. and Ryan, J.L., 1987. Endotoxins and disease mechanisms. *Annual review of medicine*, *38*(1), pp.417-432.
12. Dempsey, P.W., Allison, M.E., Akkaraju, S., Goodnow, C.C. and Fearon, D.T., 1996. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science*, *271*(5247), pp.348-350.
13. Medzhitov, R. and Janeway Jr, C.A., 1997. Innate immunity: impact on the adaptive immune response. *Current opinion in immunology*, *9*(1), pp.4-9.
14. Doolan, D.L., Dobaño, C. and Baird, J.K., 2009. Acquired immunity to malaria. *Clinical microbiology reviews*, *22*(1), pp.13-36.
15. Giguère, S. and Prescott, J.F., 2000. Equine immunity to bacteria. *Veterinary Clinics of North America: Equine Practice*, *16*(1), pp.29-47.

16. Droge, W., 2002. Free radicals in the physiological control of cell function. *Physiological reviews*, *82*(1), pp.47-95.
17. Gandon, S., 2004. Evolution of multihost parasites. *Evolution*, *58*(3), pp.455-469.
18. Rosenfeld, W.D. and ZoBell, C.E., 1947. Antibiotic production by marine microorganisms. *Journal of bacteriology*, *54*(3), p.393.
19. Kohanski, M.A., Dwyer, D.J. and Collins, J.J., 2010. How antibiotics kill bacteria: from targets to networks. *Nature Reviews Microbiology*, *8*(6), p.423.
20. Ray, K., Bala, M., Kumari, S. and Narain, J.P., 2005. Antimicrobial resistance of Neisseria gonorrhoeae in selected World Health Organization Southeast Asia Region countries: an overview. *Sexually transmitted diseases*, *32*(3), pp.178-184.
21. D'costa, V.M., McGrann, K.M., Hughes, D.W. and Wright, G.D., 2006. Sampling the antibiotic resistome. *Science*, *311*(5759), pp.374-377.
22. Furuya, E.Y. and Lowy, F.D., 2006. Antimicrobial-resistant bacteria in the community setting. *Nature Reviews Microbiology*, *4*(1), p.36.
23. Kuhn, M., Letunic, I., Jensen, L.J. and Bork, P., 2015. The SIDER database of drugs and side effects. *Nucleic acids research*, *44*(D1), pp.D1075-D1079.
24. Dudeja, P. and Gupta, R.K., 2017. Nutraceuticals. In *Food Safety in the 21st Century* (pp. 491-496). Academic Press.
25. Dillard, C.J. and German, J.B., 2000. Phytochemicals: nutraceuticals and human health. *Journal of the Science of Food and Agriculture*, *80*(12), pp.1744-1756.
26. Das, L., Bhaumik, E., Raychaudhuri, U. and Chakraborty, R., 2012. Role of nutraceuticals in human health. *Journal of food science and technology*, *49*(2), pp.173-183.
27. Pandey, M., Verma, R.K. and Saraf, S.A., 2010. Nutraceuticals: new era of medicine and health. *Asian J Pharm Clin Res*, *3*(1), pp.11-15.
28. Cowan, M.M., 1999. Plant products as antimicrobial agents. *Clinical microbiology reviews*, *12*(4), pp.564-582.
29. Zuchi, C., Ambrosio, G., Lüscher, T.F. and Landmesser, U., 2010. Nutraceuticals in cardiovascular prevention: lessons from studies on endothelial function. *Cardiovascular therapeutics*, *28*(4), pp.187-201.
30. Oroian, M. and Escriche, I., 2015. Antioxidants: Characterization, natural sources, extraction and analysis. *Food Research International*, *74*, pp.10-36.

31. Ilić, D.P., Nikolić, V.D., Nikolić, L.B., Stanković, M.Z., Stanojević, L.P. and Cakić, M.D., 2011. Allicin and related compounds: Biosynthesis, synthesis and pharmacological activity. *Facta universitatis-series: Physics, Chemistry and Technology*, *9*(1), pp.9-20.
32. Cavallito, C.J. and Bailey, J.H., 1944. Allicin, the antibacterial principle of Allium sativum. I. Isolation, physical properties and antibacterial action. *Journal of the American Chemical Society*, *66*(11), pp.1950-1951.
33. Cavallito, C.J. and Bailey, J.H., 1944. Preliminary note on the inactivation of antibiotics. *Science*, *100*(2600), pp.390-390.
34. Cavallito, C.J., 1946. Relationship of thiol structures to reaction with antibiotics. *Journal of Biological Chemistry*, *164*(1), pp.29-34.
35. Borlinghaus, J., Albrecht, F., Gruhlke, M., Nwachukwu, I. and Slusarenko, A., 2014. Allicin: chemistry and biological properties. *Molecules*, *19*(8), pp.12591-12618.
36. Li, G., Ma, X., Deng, L., Zhao, X., Wei, Y., Gao, Z., Jia, J., Xu, J. and Sun, C., 2015. Fresh garlic extract enhances the antimicrobial activities of antibiotics on resistant strains in vitro. *Jundishapur journal of microbiology*, *8*(5).
37. Hannan, A., Rauf, K., Ullah, M.I., Naeem, T., Raja, M., Qamar, M.U., Tahir, R. and Saba, M., 2012. Inhibitory effect of aqueous garlic (Allium sativum) extract against clinical isolates of Salmonella typhi. *African Journal of Microbiology Research*, *6*(21), pp.4475-4480.
38. Wang, H., Li, X., Liu, X., Shen, D., Qiu, Y., Zhang, X. and Song, J., 2015. Influence of pH, concentration and light on stability of allicin in garlic (Allium sativum L.) aqueous extract as measured by UPLC. *Journal of the Science of Food and Agriculture*, *95*(9), pp.1838-1844.
39. Bocchini, P., Andalo, C., Pozzi, R., Galletti, G.C. and Antonelli, A., 2001. Determination of diallyl thiosulfinate (allicin) in garlic (Allium sativum L.) by high-performance liquid chromatography with a post-column photochemical reactor. *Analytica Chimica Acta*, *441*(1), pp.37-43.
40. Rybak, M.E., Calvey, E.M. and Harnly, J.M., 2004. Quantitative determination of allicin in garlic: supercritical fluid extraction and standard addition of alliin. *Journal of Agricultural and Food chemistry*, *52*(4), pp.682-687.
41. Olech, Z. and Zaborska, W., 2012. A spectrophotometric assay for total garlic thiosulfinates content. Kinetic aspects of reaction with chromogenic thiols. *Polish journal of food and nutrition sciences*, *62*(1), pp.23-29.

42. Farías-Campomanes, A.M., Horita, C.N., Pollonio, M.A. and Meireles, M.A.A., 2014. Allicin-rich extract obtained from garlic by pressurized liquid extraction: quantitative determination of allicin in garlic samples. *Food and Public Health*, *4*(6), pp.272-278.
43. Yu, T.H., Wu, C.M. and Chen, S.Y., 1989. Effects of pH adjustment and heat treatment on the stability and the formation of volatile compounds of garlic. *Journal of agricultural and food chemistry*, *37*(3), pp.730-734.
44. YU, T.H. and WU, C.M., 1989. Stability of allicin in garlic juice. *Journal of Food Science*, *54*(4), pp.977-981.
45. Shen, C., Xiao, H. and Parkin, K.L., 2002. In vitro stability and chemical reactivity of thiosulfinates. *Journal of agricultural and food chemistry*, *50*(9), pp.2644-2651.
46. Lawson, L.D., Wang, Z.J. and Papadimitriou, D., 2001. Allicin release under simulated gastrointestinal conditions from garlic powder tablets employed in clinical trials on serum cholesterol. *Planta Medica*, *67*(01), pp.13-18.
47. Fujisawa, H., Suma, K., Origuchi, K., Kumagai, H., Seki, T. and Ariga, T., 2008. Biological and chemical stability of garlic-derived allicin. *Journal of agricultural and food chemistry*, *56*(11), pp.4229-4235.
48. Lee, S.Y., Kim, D.S. and Kyung, K.H., 2014. Factors influencing the stability of garlic thiosulfinates. *Food Science and Biotechnology*, *23*(5), pp.1593-1600.
49. Wang, H., Li, X., Liu, X., Shen, D., Qiu, Y., Zhang, X. and Song, J., 2015. Influence of pH, concentration and light on stability of allicin in garlic (Allium sativum L.) aqueous extract as measured by UPLC. *Journal of the Science of Food and Agriculture*, *95*(9), pp.1838-1844.
50. Feldberg, R.S., Chang, S.C., Kotik, A.N., Nadler, M., Neuwirth, Z., Sundstrom, D.C. and Thompson, N.H., 1988. In vitro mechanism of inhibition of bacterial cell growth by allicin. *Antimicrobial Agents and Chemotherapy*, *32*(12), pp.1763-1768.
51. Rees, L.P., Minney, S.F., Plummer, N.T., Slater, J.H. and Skyrme, D.A., 1993. A quantitative assessment of the antimicrobial activity of garlic (Allium sativum). *World Journal of Microbiology and Biotechnology*, *9*(3), pp.303-307.
52. Elsom, G.K., 2000. An antibacterial assay of aqueous extract of garlic against anaerobic/microaerophilic and aerobic bacteria. *Microbial ecology in health and disease*, *12*(2), pp.81-84.

53. Cañizares, P., Gracia, I., Gómez, L.A., de Argila, C.M., de Rafael, L. and García, A., 2002. Optimization of Allium sativum solvent extraction for the inhibition of in vitro growth of Helicobacter pylori. *Biotechnology progress*, *18*(6), pp.1227-1232.
54. Curtis, H., Noll, U., Störmann, J. and Slusarenko, A.J., 2004. Broad-spectrum activity of the volatile phytoanticipin allicin in extracts of garlic (Allium sativum L.) against plant pathogenic bacteria, fungi and Oomycetes. *Physiological and Molecular Plant Pathology*, *65*(2), pp.79-89.
55. Aronstein, K.A. and Hayes, G.W., 2004. Antimicrobial activity of allicin against honey bee pathogens. *Journal of apicultural research*, *43*(2), pp.57-59.
56. Cutler, R.R. and Wilson, P., 2004. Antibacterial activity of a new, stable, aqueous extract of allicin against methicillin-resistant Staphylococcus aureus. *British journal of biomedical science*, *61*(2), pp.71-74.
57. Cai, Y., Wang, R., Pei, F. and Liang, B.B., 2007. Antibacterial activity of allicin alone and in combination with β-lactams against Staphylococcus spp. and Pseudomonas aeruginosa. *The Journal of antibiotics*, *60*(5), p.335.
58. Booyens, J. and Thantsha, M.S., 2013. Antibacterial effect of hydrosoluble extracts of garlic (Allium sativum) against Bifidobacterium spp. and Lactobacillus acidophilus. *African Journal of Microbiology Research*, *7*(8), pp.669-677.
59. Li, G., Ma, X., Deng, L., Zhao, X., Wei, Y., Gao, Z., Jia, J., Xu, J. and Sun, C., 2015. Fresh garlic extract enhances the antimicrobial activities of antibiotics on resistant strains in vitro. *Jundishapur journal of microbiology*, *8*(5).
60. Reiter, J., Levina, N., van der Linden, M., Gruhlke, M., Martin, C. and Slusarenko, A., 2017. Diallylthiosulfinate (allicin), a volatile antimicrobial from garlic (Allium sativum), kills human lung pathogenic bacteria, including MDR strains, as a vapor. *Molecules*, *22*(10), p.1711.
61. Dwivedi, V.P., Bhattacharya, D., Singh, M., Bhaskar, A., Kumar, S., Sobia, P., Van Kaer, L. and Das, G., 2018. Allicin enhances antimicrobial activity of macrophages during Mycobacterium tuberculosis infection. *Journal of ethnopharmacology*.
62. Miron, T., Shin, I., Feigenblat, G., Weiner, L., Mirelman, D., Wilchek, M. and Rabinkov, A., 2002. A spectrophotometric assay for allicin, alliin, and alliinase (alliin lyase) with a

chromogenic thiol: reaction of 4-mercaptopyridine with thiosulfinates. *Analytical biochemistry*, *307*(1), pp.76-83.

63. Shimamura, T., Sumikura, Y., Yamazaki, T., Tada, A., Kashiwagi, T., Ishikawa, H., Matsui, T., Sugimoto, N., Akiyama, H. and Ukeda, H., 2014. Applicability of the DPPH assay for evaluating the antioxidant capacity of food additives–inter-laboratory evaluation study–. *Analytical Sciences*, *30*(7), pp.717-721.
64. Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, *72*(1-2), pp.248-254.
65. Singleton, V.L. and Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American journal of Enology and Viticulture*, *16*(3), pp.144-158.
66. Woisky, R.G. and Salatino, A., 1998. Analysis of propolis: some parameters and procedures for chemical quality control. *Journal of apicultural research*, *37*(2), pp.99-105.
67. Chang, C.C., Yang, M.H., Wen, H.M. and Chern, J.C., 2002. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of food and drug analysis*, *10*(3).
68. Luo, C., Walk, S.T., Gordon, D.M., Feldgarden, M., Tiedje, J.M. and Konstantinidis, K.T., 2011. Genome sequencing of environmental Escherichia coli expands understanding of the ecology and speciation of the model bacterial species. *Proceedings of the National Academy of Sciences*, *108*(17), pp.7200-7205.
69. Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G.O., Azevedo, V., Bertero, M.G., Bessieres, P., Bolotin, A., Borchert, S. and Borriss, R., 1997. The complete genome sequence of the gram-positive bacterium Bacillus subtilis. *Nature*, *390*(6657), p.249.
70. Gram, C., 1884. The differential staining of Schizomycetes in tissue sections and in dried preparations. *Fortschitte der Medicin*, *2*, pp.185-189.
71. Taylor, W.I. and Achanzar, D., 1972. Catalase test as an aid to the identification of Enterobacteriaceae. *Appl. Environ. Microbial.* *24*(1), pp.58-61.
72. Hemraj, V., Diksha, S. and Avneet, G., 2013. A review on commonly used biochemical test for bacteria. *Innovare J Life Sci*, *1*(1), pp.1-7.

73. Hemaiswarya, S., Kruthiventi, A.K. and Doble, M., 2008. Synergism between natural products and antibiotics against infectious diseases. *Phytomedicine*, *15*(8), pp.639-652.
74. Daina, A., Michielin, O. and Zoete, V., 2017. SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Scientific reports*, *7*, p.42717.
75. Orhan, G., Bayram, A., Zer, Y. and Balci, I., 2005. Synergy tests by E test and checkerboard methods of antimicrobial combinations against Brucella melitensis. *Journal of clinical microbiology*, *43*(1), pp.140-143.
76. McKay, G.A., Beaulieu, S., Arhin, F.F., Belley, A., Sarmiento, I., Parr Jr, T. and Moeck, G., 2009. Time–kill kinetics of oritavancin and comparator agents against Staphylococcus aureus, Enterococcus faecalis and Enterococcus faecium. *Journal of antimicrobial chemotherapy*, *63*(6), pp.1191-1199.
77. Schuler, M.L. and Kargi, F. (2001) Bioprocess Engineering: Basic Concepts (2nd edition). New Jersey: Prentice Hall PTR.
78. Wakelkamp, M., Alván, G. and Paintaud, G., 1998. The time of maximum effect for model selection in pharmacokinetic–pharmacodynamic analysis applied to frusemide. *British journal of clinical pharmacology*, *45*(1), pp.63-70.
79. Aksoy, L., Kolay, E., Ağılönü, Y., Aslan, Z. and Kargıoğlu, M., 2013. Free radical scavenging activity, total phenolic content, total antioxidant status, and total oxidant status of endemic Thermopsis turcica. *Saudi journal of biological sciences*, *20*(3), pp.235-239.
80. Guo, X.Y., Wang, J., Wang, N.L., Kitanaka, S. and Yao, X.S., 2007. 9, 10-Dihydrophenanthrene derivatives from Pholidota yunnanensis and scavenging activity on DPPH free radical. *Journal of Asian natural products research*, *9*(2), pp.165-174.
81. Lipinski, C.A., Lombardo, F., Dominy, B.W. and Feeney, P.J., 1997. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced drug delivery reviews*, *23*(1-3), pp.3-25.