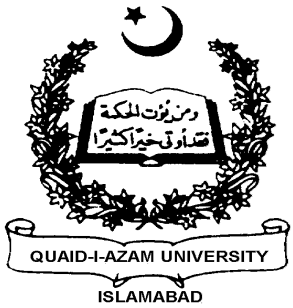
Evaluation of Pine oil in combination with Colistin against Multi-Drug Resistant *Escherichia coli* strains Isolated from Poultry



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2023

Evaluation of Pine oil in combination with Colistin against Multi-Drug Resistant *Escherichia coli* strains Isolated from Poultry

A thesis submitted in partial fulfillment of the requirements for the Degree of

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In

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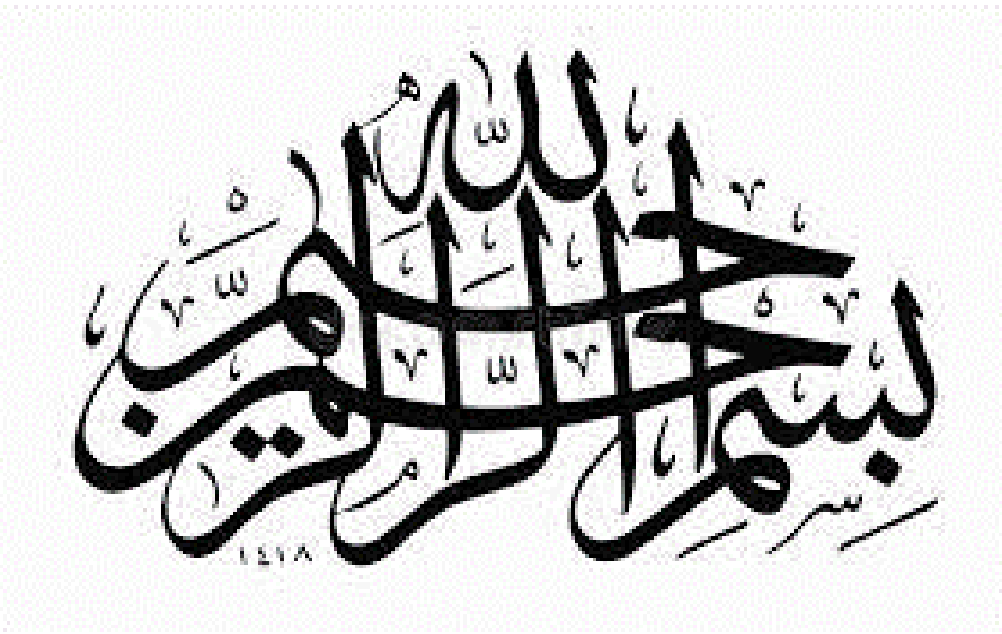
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Dedication

I would like to dedicate my work to **ALLAH Almighty** who has been my source of Strength, Grace, and Wisdom throughout my research period, and to our beloved **Holy Prophet Hazrat Muhammad (P.B.U.H).**

All the success comes with our parents' and family's support and prayers. So, I will dedicate my dissertation to:

My father Abdul Razzaq,

My mother, and my family

for their love and encouragement.

Declaration

The material and information contained in this thesis are my original work that was carried out at the Department of Microbiology, Quaid-i-Azam University, Islamabad. I have not previously presented any part of this work elsewhere for any other degree.

Mahnoor Ismail

Certificate

This thesis submitted by **Mahnoor Ismail** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

Supervisor: _____
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Chairman: _____
(Prof. Dr. Naeem Ali)

Dated: _____

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LIST OF ABBREVIATIONS

%	Percentage
^{o}C	Degree Celsius
µl	Microliter
µg	Microgram
ABR	Antibiotic Resistance
AMR	Antimicrobial Resistance
APEC	Avian Pathogenic *E. coli*
AR	Antibiotic Resistance
ATCC	American Type Culture Collection
AU	Antibiotic Usage
BDT	Broth microdilution test
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
CV	Crystal Violet
DNA	Deoxyribose Nucleic Acid
DDDA	Double Disc Diffusion Assay
DEC	Diarrheagenic *E. coli*
E. coli	Escherichia coli
EAEC	Enteroagressive *E. coli*
EO	Essential Oil
EHEC	Enterohaemorrhagic *E. coli*
ExPEC	Extra-intestinal Pathogenic *E. coli*
EIEC	Entero-invasive *E. coli*
EMB	Eosin Methylene Blue agar
FDA	Food and Drug Authority
FICI	Fractional Inhibitory Concentration Index
GNB	Gram Negative Bacteria
GPB	Gram-Positive Bacteria
GDP	Gross Domestic Product
I	Intermediate
IGs	Immunoglobulins
LPS	Lipo-Polysaccharides
MDR	Multi-Drug Resistance
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
MHA	Muller Hinton Agar
MHB	Muller Hinton Broth
NB	Nutrient Broth
OD	Optical Density
OMP	Outer Membrane Protein
PCR	Polymerase Chain Reaction
R	Resistance
RNA	Ribonucleic Acid
S	Sensitive
Spp.	Species
TSI	Triple Sugar Iron agar
WHO	World Health Organization

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In the name of Allah, the most Gracious and the Beneficent. I would like to praise Allah Almighty who gave me courage, strength, and patience to complete with work. Countless salutations upon our beloved Prophet Hazrat Muhammad (P.B.U.H) are the most exalted among human beings ever born on the surface of Earth, who forever are guidance and knowledge for humanity.

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Mahnoor Ismail

Abstract

Poultry contributes greatly to the health and economic development of the world. Antibiotic-resistant *E. coli* is the primary cause of mortality and production loss in poultry. *Escherichia coli* is a highly resistant, gram-negative, pathogenic, and rod-shaped bacteria. Infections caused by pathogenic *E. coli* are becoming harder to treat due to the rise of antibiotic-resistant strains. The rise of antibiotic resistance is a worldwide issue that affects both human and animal health. To combat this issue, new methods are needed to prevent resistance from spreading and to prolong the effectiveness of existing antibiotics such as the use of essential oils as antibiotics. This study aimed to enhance the potency of conventional antibiotics that are used against *E. coli* by combinational therapy. Colistin with Pine oil and Colistin with Oxytetracycline are two combinations that were analyzed for their effect on certain virulence factors of *E. coli*. Colistin is used as 1st line of drugs in poultry and Pine oil is used to keep the farm environment germs-free. For the evaluation of both combinations, 89 isolates of *E. coli* were processed for antimicrobial susceptibility testing; disc diffusion, MIC, and biofilm formation. The results of disc diffusion revealed 100% and 51.68% resistance in Oxytetracycline and Colistin respectively, while 100% susceptibility was observed by Pine oil. Biofilm assay presents 14.6% of isolates form biofilm and 85.3% do not develop biofilm. MIC results show 96% highest resistance against Oxytetracycline. In the case of Colistin, there is evidence of some resistance, but the resistance falls within the lower ranges of MIC. The FICIs for an antibiotic combination of Colistin and Oxytetracycline were calculated against *E. coli* based on the minimum inhibitory concentration of each agent. The Colistin and Oxytetracycline combination inhibits the growth of *E. coli* more efficiently than individual antibiotics, showing a 100% synergistic effect. Whereas, the combination of Colistin with Pine oil shows a 100% antagonistic effect against all tested MDR strains of *E. coli* that were measured by the disc diffusion method and evaluated by comparison of inhibition zones. The result of the current study concludes that the combination of Colistin with Oxytetracycline is a better substitute against *E. coli* than the combination of Colistin with Pine oil.

Introduction

Escherichia coli belongs to the Enterobacteriaceae family and is frequently found in the surroundings and the digestive systems of warm-blooded creatures. This bacterium is a noteworthy commensal organism that inhabits the lower part of the gastrointestinal tract of chickens. The environmental quality of the birds is checked through the number of commensal *E. coli* residing in their meat. *E. coli* lives in the lower digestive system of chickens, much like it does in human beings, and colonizes it during the first 24 hours of hatching or birth (Stromberg et al., 2017). Some *E. coli* strains can be harmful to poultry, causing colibacillosis. These pathogenic strains, known as Avian Pathogenic *E. coli*, have varying levels of virulence and can be influenced by factors such as stress. Around 30% of broiler flocks in the US have subclinical colibacillosis (Nguyen et al., 2022).

In 1885, Theodor Escherich discovered and described the gram-negative bacterium *Escherichia coli*, which has a rod-like shape, after isolating it from infant feces. Commensal *E. coli* strains, which live in harmony with humans, provide mutual benefits. However, in people with weakened gastrointestinal barriers or immunocompromised hosts, commensal *E. coli* can cause illness (Pakbin et al., 2021). Some of these can lead to serious human illnesses such as hemolytic uremic syndrome and hemorrhagic colitis (Momtaz et al., 2012).

Escherichia coli, a bacterial pathogen found in poultry, can harm human health because of its unique toxicity, treatment sensitivity, and evolution into multidrug-resistant (MDR) bacterial strains. Only 10%–15% of *E. coli* serotypes are harmful, and most of the *E. coli* strains, are used as indicators of fecal contamination in foods (Sarker et al., 2019). Pathogenic *E. coli* are capable of infecting humans and causing various infections, including septicemia in infants, and urinary tract infections (UTI). Researchers have identified a potential reservoir of *E. coli* with zoonotic potential in the intestines of birds, which could spread directly to humans (Furtula et al., 2010).

Organ lesions, perihepatitis, airsacculitis, and pericarditis are symptoms of enteric illnesses brought on by pathogenic *E. coli* colonization in the gastrointestinal tract of agricultural animals, particularly chickens. These conditions cause growth retardation, death, and finally severe economic losses (Daneshmand et al., 2019). ExPEC infections

are critical to human health and a significant source of financial loss for the poultry sector. ExPEC infections in people and poultry are costly in the US, costing over $4 billion annually. Similar to non-pathogenic commensal *E. coli*, ExPEC strains may colonize the gut, but they also possess virulence characteristics that enable them to spread illness to extraintestinal locations. Poultry homes, in addition to the gut, act as a reservoir for APEC (Stromberg et al., 2017). Human urinary tract infections (UTI), bacterial meningitis, and bloodstream infections are only a few of the illnesses caused by ExPECs. ExPECs are categorized into different sub-pathotypes, such as UPEC for urinary tract infections, NMEC for neonatal meningitis, and sepsis-associated *E. coli* (Zhuge et al., 2020).

The greater extent of transmission of resistant genes to human bacterial environments from poultry leads to a public health risk. Additionally, one of the most significant reservoirs for infectious bacteria is found in poultry and other foods. Normally, there are very few or no germs in the flesh of healthy animals, but contamination can occur during slaughter, transportation, and processing. Worldwide, food-borne infections have been the leading cause of disease and increased rate of mortality. The general public is becoming more aware of food-borne diseases as they have an impact on both health and the economy (Bantawa et al., 2019). The primary method of controlling bacterial infection is antibiotic therapy; however, because of the overuse of antibiotics in both human treatment and animal farms, there is now an issue with antimicrobial resistance. It has been predicted that antibiotic resistance may result in approximately 10 million mortality rates worldwide in 2050. In response, the World Health Organization has recommended the creation of a guide for the era following the use of antibiotics (Rondón-Barragán, 2020). Many MDR *E. coli* strains also can spread to other animals. They could spread to people. Transferable genetic material allows many of these AMR factors to spread to other human bacterial infections (Islam et al., 2023). The rise in chicken production was facilitated by the application of antimicrobial drugs as growth promoters in chicken feed. Nevertheless, almost 90% of the recommended amount of these antibiotics can be eliminated from the bird’s feces since they are not absorbed by the chicken's gut. This raises apprehensions regarding the existence of antibiotics and their possible consequences on both humans and the environment. This is particularly concerning since agricultural waste is frequently employed as fertilizer. (Furtula et al., 2010).

In the poultry business, outbreaks among flocks, result in large financial losses because of the condemnation of carcasses, decreased egg production, illness, and fatalities, as well as expenses for cleaning, and antibiotic therapies. Because of the expansion of the poultry business worldwide, there are currently many more chickens than people living on Earth (Sivaranjani et al., 2022).

Because of its high protein and essential nutrient content in both meat and eggs, this animal product is available at a lower price compared to other meat sources., The poultry sector makes a substantial contribution to addressing the nutritional gap in several countries (Abd El-Hack et al., 2022). Antibiotic resistance has long been a serious problem for nosocomial infections in hospital settings, and meat is a key vector for the transmission of antibiotic resistance from animals to people. Such transfer can happen in one of three ways: through the transmission of resistant food-borne pathogens, through the absorption of resistant components of the original food microflora, or the transfer of antibiotic residues to food (Pesavento et al., 2007). Poultry meat is one of the most commonly consumed meats worldwide. Its popularity is due to several factors, including the short time it takes to fatten the birds, its efficient use of space, high reproductive rates, good feed conversion, nutritious meat, and affordable prices (Kralik et al.,2017).

Commercial chicken farming started in Pakistan at the beginning of the 1960s and has been speedily growing ever since. The poultry industry is a significant part of the country's agricultural sector, contributing 1.3% to its Gross Domestic Product (GDP). The Commercial poultry business of Pakistan is an essential and indispensable sector that contributes a staggering 31% to the country's meat production, thereby stabilizing the prices of red meat. It received an incredible investment of over 700 billion rupees during the 2016-2017 fiscal year and provides a source of livelihood for nearly 1.5 million people, both directly and indirectly (HUSSAIN et al., 2015).

Chicken-based food stuff is gaining fame all over the world and chasing the first rank on the meat list because it can be prepared faster and mixed with several food products. Consumers nowadays in both developed and developing nations rely on chicken meat products as their go-to lunch option due to their current lifestyle. The small amount of saturated fat and low-calorie content of chicken flesh make it preferable to red meat. Due to chicken's nutritional profile, those with fatal or cardiac disorders can also use

chicken in their diet. Additionally, chicken meat has a low collagen content, making it digestible. Chicken meat, in comparison with other categories of meat, is cheap and easily available in markets. It is also a bucket of different vitamins as a whole: vitamin B6 and A, vitamin B3 (niacin) (L. Zainab et al., 2022). The greatest producer of livestock is Asia (42.1%), followed by America (31.4%), Europe (19.0%), and Africa (5.5%) (Manzoor et al., 2023). Over 1.5 million people were employed directly and indirectly in 2017 as a result of the predictable 1,022 million chickens in the nation, which make 17,083 million and 1,270,000 tons of eggs and meat respectively. Over the past 20 years, the commercial chicken business in Pakistan has risen at a rate of 8–10% on an average basis. In terms of the number of chickens produced, Pakistan ranked 11th in the world in 2015. Since then, both the public and private sectors have boosted their investment, going from 200 billion Pakistani rupees PKR to 700 billion PKR (4.47 billion USD) from 2015 to 2018 respectively (Aslam et al., 2020).

The reckless usage of antibiotics in intensive livestock farming contributes significantly to the development of antibiotic-resistant bacteria. Studies have shown that chickens are more likely to carry *E. coli* and are highly resistant to antibiotics than other animals used as food. Therefore, monitoring avian *E. coli* for signs of antimicrobial resistance can be a useful tool for protecting public health and guiding the appropriate use of antibiotics. By keeping an eye on *E. coli*, we can also gain insights into the prevalence of antimicrobial resistance in harmful bacteria. Numerous studies have been conducted across the globe to analyze the occurrence of multi-drug-resistant *E. coli* in poultry. As frequently as practicable, such surveillance should be localized since resistance patterns might differ within areas of the same city and, more so, in nations where the use of antibiotics is not carefully regulated (Benklaouz et al., 2020). Doxycycline, ampicillin, nalidixic acid, tetracycline, neomycin, colistin sulfate, ciprofloxacin, and sulfonamides are among the antibiotics often administered to chickens (Amin et al., 2020). Due to concerns about human health and antimicrobial resistance in the poultry industry, veterinarians have limitations on the antimicrobials they can use. (Momtaz et al., 2012).

In the last 20 years, there has been an alarming rise in the introduction of MDR strains. Pathogenic bacteria can be used to gather data on trends in antimicrobial resistance using the antimicrobial resistance patterns of indicator bacteria (Sarker et al., 2019). The use of antimicrobials in chicken feed can cause a shift in the gut flora by promoting

resistant bacterial populations like *E. coli* that could spread to the environment and food chain. This puts selection pressure on these resistant bacteria. (Furtula et al., 2010). Antimicrobial resistance is on the rise due to the frequent, and inappropriate usage of antibiotics. Antibiotic use is selected for resistance in endogenous flora of affected people or communities as well as in pathogenic bacteria. Due to the considerable selection pressure for antibiotic resistance in chickens, a relatively large number of resistant bacteria can be found in their waste flora (Momtaz et al., 2012). The overuse of antibiotics in industrial poultry farming may be one of the probable sources of resistance in chicken isolates. The habitat in which free-range hens, forage for food may include drug residues and bacteria resistant to antibiotics. According to a recent study in Bangladesh (Dhaka and Gazipur), 215 out of 260 poultry farmers use antibiotics for the avoidance of disease and growth promotion on their farms without a veterinarian's prescription (Amin et al., 2020). *E. coli* acquires its resistance genes by mutation, induction, or selection pressure. Bacterial antimicrobial-resistant (AMR) genes may spread to other bacteria both horizontally and vertically, and they can also enter the human food chain (Islam et al., 2023). There has been considerable debate about the degree of impact, but the development of plasmid-mediated resistance to (last resort) antimicrobials such as colistin in livestock production has boosted the argument that the use of antibiotics in food animal production is a major contributor to the global problem of antibiotic resistance in humans. It is believed that the use of antibiotics in livestock production plays a role in the selection, spread, and maintenance of antibiotic-resistant bacteria on farms. (Nhung et al., 2016).

The introduction of antibiotics to treat potentially fatal diseases is the biggest achievement of the modern medicinal world. However, the emergence of resistance to their antimicrobial action has gradually come after this. However, during the past two decades, there has been a sharp fall in the discovery and progress of new antibiotics. The striking rise in resistance to those antibiotics that are already on the market. The increasing frequency of illnesses brought on by Gram-negative, multidrug-resistant microbes such as Pseudomonas aeruginosa, Acinetobacter, *E. coli*, and Klebsiella pneumoniae, is of special concern globally (Li et al., 2006). Antibiotic resistance is now a top public health concern globally. Antibiotic resistance is increasing worldwide, making common infectious diseases harder to treat and sometimes even incurable. Overuse of antibiotics and easy accessibility without prescription accelerate the spread

of resistance. Overprescription by practitioners and veterinarians also contributes to the problem. We run the risk of approaching a post-antibiotic era when prevalent conditions and small injuries might be lethal if we don't take immediate action. We will have to deal with a higher death rate in the coming years since infectious illnesses will be exceedingly difficult to treat in that period (World Health Organization, 2020).

The bacterial genes that cause antimicrobial resistance can spread both horizontally and vertically, and they can also get into the human food chain. Due to selection pressure, induction, or mutation, Escherichia coli acquires the resistance genes (Sarker et al., 2019). The environment is regarded as a significant indicator of the spread of AMR due to the introduction of numerous developing environmental pollutants, such as antibiotic resistance genes (ARGs) (Zhang et al., 2020). Poultry litter is a reservoir for several antibiotic-resistant *E. coli* and a cause of antimicrobial residues (Furtula et al., 2010). Integrons have a crucial role in the bacterial dissemination of resistance. Integrons are specialized genetic elements that may bind, delete, and express genes. They are frequently found in mobile elements like plasmids, which promote gene transfer across bacteria. Recently, substantial research on antimicrobial resistance genes has been conducted utilizing molecular techniques, notably polymerase chain reaction. (Momtaz et al., 2012).

A physiological condition known as a biofilm is one in which bacterial cells adhere to surfaces permanently and are encased in a self-secreted extracellular polymeric matrix made of proteins, polysaccharides, and nucleic acids (Kostakioti et al., 2013). Due to the difficulties in eliminating them using standard therapies, biofilm-related illnesses continue to be a significant healthcare problem. The matrix that surrounds biofilms, together with other antimicrobial tolerance mechanisms produced inside them, creates a significant barrier to the effectiveness of therapy, allowing biofilm to be immune-evading and extremely resistant to many antibiotics (Ballén et al., 2022).

Polymyxin B, a member of the family of hydrophobic, cationic, multi-component, cyclic-peptide antibiotics known as polymyxins, was first generated by the bacteria Paenibacillus polymyxa. Polymyxins are a type of antibiotic that is used as a final solution for severe diseases caused by multidrug-resistant gram-negative organisms. However, the emergence and worldwide spread of mobilized colistin resistance (MCR) determinants have endangered the effectiveness of this last-resort medication. The

widespread inclusion of polymyxin E (colistin) into routine therapeutic therapy is restricted by its possible nephrotoxicity and neurotoxicity. However, colistin is once again being considered a last resort for the cure of serious bacterial infections due to the current appearance of multidrug-resistant or exceptionally drug-resistant gram-negative bacteria (Sun et al., 2018). In animal production, polymyxin B and colistin are frequently utilized for preventative and therapeutic purposes. Infections brought on by Enterobacteriaceae in a variety of farm animals are treated with colistin in Europe. In various Asian nations, such as Japan, India, and China colistin is broadly used as a growth promoter in feed to increase its efficiency and improve in body weight of veterinary animals. The Food and Drug Administration in the USA has approved colistin usage, and it is frequently used in livestock production in Brazil as a growth booster for animals, primarily pigs and poultry. For numerous years, colistin has been utilized in the field of veterinary medicine. The mobilized colistin-resistance gene (mcr-1) was found in 2015 in Southern China, which caused colistin's use in veterinary for medication and as a feed additive to be re-examined. As a result, colistin was completely outlawed in China as a growth booster for cattle in 2017. The use of colistin in animals has aroused severe concerns from the European Medicines Agency (EMA) regarding the growing risk to people (Sun et al., 2018). Doctors and scientists have had to reassess the effectiveness of colistin, an older antibiotic called polymyxin, due to the lack of new treatments for Gram-negative infections. Colistin, also known as polymyxin E, is often used as a last resort for seriously ill patients with these infections. (Li et al., 2006).

Tetracyclines are broad-spectrum bacteriostatic antibiotics that were first identified in the 1940s. One of the earliest tetracycline antibiotics ever created was oxytetracycline. Oxytetracycline is commonly used to cure a variety of infections in chickens, including, infectious coryza, and fowl cholera. It is typically given to birds through their feed and drinking water (Pokrant et al., 2021). Its cheap cost, effectiveness, and absence of adverse effects, is the reason that it is one of the most often used antibiotics in poultry. Oxytetracycline for 2-5 days is a common antibacterial regimen used to treat digestive and respiratory ailments in birds. Today, there has been a noticeable rise in the occurrence of resistant *E. coli* strains that have been identified from poultry. Tetracycline resistance has been linked to 43 distinct tet/otr genes, according to research. These genes may prevent the effect of tetracyclines on the ribosome, pump

tetracycline out of the cell, or enzymatically destroy the drug (Nouri Gharajalar & Shahbazi, 2020).

Essential Oils usually referred to as zoo-technical additives, are one of the most effective and secure alternatives to antibiotics. Comparing essential oils to synthetic antibiotics ensures animal welfare and food safety since they are less harmful and frequently leave less residue (Chalikwar, 2020). Essential oils produced from plants have long been used as flavorings in foods and drinks, and because they include a variety of antibacterial components, they have the potential to be used as natural food preservation agents. Many tiny terpenoid and phenolic chemicals, which have also been demonstrated to have antibacterial action in pure form, are responsible for the essential oil's antibiotic activity. Gram-positive and negative bacteria are among the many microorganisms that essential oils are acknowledged to be effective against (Fekih et al., 2014). Several infections in chickens are brought on by Escherichia coli and several Salmonella serovars, which result in large economic losses. Antibacterial resistance has arisen as a result of the non-selective and widespread usage of antibacterial medicines in chicken flocks, which has become a serious public health risk. Alternatives to antibacterial agents include several therapeutic plant extracts, such as essential oils (Haji Seyedtaghiya et al., 2021). Some of the components of essential oil had bactericidal effects and others had bacteriostatic and these compounds in essential oil show their combined effect against bacteria (El-Shenawy et al., 2015).

The family Pinaceae includes the genus Pinus, whose essential oils serve multiple purposes such as adding fragrance to cosmetics, enhancing the flavor of food and drinks, providing scents to everyday products, and being utilized as intermediates in the production of perfume chemicals. Pine essential oil has been reported in many studies to have various therapeutic properties (Fekih et al., 2014).

Unfortunately, we are no longer able to just depend on the development of novel antibiotics to combat the fast-spreading bacterial resistance; we also need to study sensible methods for using more established antibiotics, like colistin. Review of recent clinical data with an emphasis on effectiveness assessment, developing resistance, potential risk factors, and combination treatment (Li et al., 2006). For this reason, this study focused on the development of novel combinations of antibiotics with other antibiotics and pine essential oils that are already in use in poultry.

In our study, we isolated the MDR strains of *E. coli* from poultry through antibiotic susceptibility testing by Kirby Bauer disc diffusion assay. Then these MDR strains for minimum inhibitory concentration against different antibiotics used in poultry production through microdilution assay. Identified isolates of *E. coli* were screened for biofilm formation via the microtiter plate method. *E. coli* isolates showing resistance against Colistin were exposed to two combinational therapies of Colistin with Oxytetracycline via checkerboard assay and the other one was Colistin with Pine Essential Oil via disc diffusion assay. *E. coli* isolates showed high resistance against Oxytetracycline, Enrofloxacin, Florfenicol, Ciprofloxacin, and Amoxicillin. But shows less resistance to antibiotics such as Colistin, Cefepime, Cefixime, and Doxycycline demonstrated through antimicrobial susceptibility testing. Pine essential oil also shows high antibacterial activity against MDR strains of *E. coli.* Whereas, the combination of Colistin and Pine essential oil showed an antagonistic effect against all isolates. The combination of Colistin with Oxytetracycline showed 75% synergistic against *E. coli* isolates through a checkerboard assay. This study reveals that 85% of *E. coli* isolates were non-biofilm formers the others were weak biofilm former and only 1% were moderate biofilm former. This study concluded that a combination of Colistin and Oxytetracycline should be a better option in the future but the combination of Colistin with pine oil needs more investigation.

AIM AND OBJECTIVES

Aim

This study was performed to evaluate the combined effect of pine essential oil with colistin against MDR strains isolated from poultry and the combined effect of oxytetracycline with colistin against resistant strains of *E. coli* isolated from cloacal samples of chicken.

Objectives

- Isolation and identification of *E. coli* strains from poultry
- To perform antimicrobial susceptibility testing for Colistin Sulphate, Cefepime, Cefixime, Amoxicillin, Doxycycline, Oxytetracycline, Enrofloxacin, Ciprofloxacin, Norfloxacin, Oxalinic Acid, Florfenicol, and Fosfomycin against *E. coli* through the Kirby Bauer test.
- To determine the minimum inhibitory concentration (MIC) for Colistin Sulphate, Oxytetracycline, Enrofloxacin, and Florfenicol against resistant strains of *E. coli* through the microtiter plate method.
- To check the ability of poultry-isolated *E. coli* to be classified as strong, moderate, weak, or non-biofilm former by the microtiter plate method.
- To determine the synergism of colistin and oxytetracycline against MDR strains of *E. coli* by checkerboard assay.
- To calculate the antibacterial activity of Pine oil against *E. coli* isolated from poultry.
- To evaluate the combined effect of colistin sulfate and Pine essential oil against MDR strains of *E. coli* by the disc diffusion method.

Literature Review

2.1 Poultry

The current primary objectives of the poultry business have been disease control, high output, product quality, and affordable production costs. To prevent disease transmission and reduce the use of antibiotics, constant efficient, and goal-oriented healthcare is required to fulfill per capita consumption and human welfare (Hafez & Attia, 2020).

There are two types of poultry production:

2.1.1 Conventional Production:

The primary source of chicken meat and eggs globally is conventional poultry farming. Conventional poultry farms are made up of big, enclosed buildings with a lot of birds inside. The mortality rate was lower and the end broiler weight was often larger in traditional methods. Antibiotics are often used on conventional chicken farms for both therapeutic and preventive purposes. Antibiotics have historically been used to broiler feed on conventional farms to promote growth and increase feed effectiveness. From farm to fork, the traditional grill manufacturing chain offers various chances for bacterial contamination (Golden et al., 2021).

2.1.2 Alternative Production:

Organic, pastured, and free-range poultry farming methods are other options. While pastured poultry operations need mobile housing that is moved to new pastures every day, organic poultry farms are characterized by farms that grow birds without the use of antibiotics and give the birds access to the outside in an open environment. Additionally, slow-growing bird breeds are frequently used in alternative poultry production techniques. Alternative poultry enterprises are dealing with higher bird death rates as a result of these practices, with necrotic enteritis being a major issue (Ricke, 2021).

2.2 Importance of the Poultry Industry

Farms that produce commercial broilers have grown in size and scope as a result of developments in genetics, nutrition, and climate-controlled housing, among other things. These developments have made it possible for the poultry industry to achieve

vertical integration and for the worldwide markets for poultry meat products to grow over the past few decades (Ricke, 2020). Due to its meat and egg products' high protein and vital nutrient content at a lower price than other animal meat sources, the poultry sector makes a substantial contribution to addressing the nutritional gap in several countries (Abd El-Hack et al., 2022). Poultry creates jobs for everyone in the globe, raises average incomes, and ends poverty. It provides the world's population with meat and eggs, which are a rich source of vitamins and proteins and are essential for the growth of good health. For rural communities, it is a quick business (Khan et al., 2022).

2.3 Food security

The chicken business continues to face serious concerns about food safety for the general public. Food-borne diseases can come into contact with chicken throughout the whole production process, from hatching to processing to retail and meal preparation (Ricke, 2020). Many infectious pathogens can cause illnesses in poultry, including bacteria, viruses, parasites, and fungi. These pathogens can be spread in farms through horizontal or vertical transmission. In cases where there are poor hatching conditions or insufficient cleanliness in the hatchery, disease transmission after hatching is primarily vertical, which can lead to omphalitis or yolk sac infection. (Hafez & Attia, 2020).

The most significant foodborne pathogen, Salmonella enterica, is frequently linked to the contamination of chicken products. Around 93 million reports of gastroenteritis and 155,000 fatalities are caused by Salmonella each year globally. Antibiotic therapy is the primary method of controlling this type of bacterial infection; however, because of the overuse of antibiotics in both human treatment and animal farms, there is now an issue with antimicrobial resistance. According to predictions, diseases triggered by antibiotic resistance are likely to cause around 10 million deaths globally in 2050, in addition The World Health Organization (WHO) advised preparing a guide for the post-antibiotic age.

Various antibiotics were shown to have various degrees of antibiotic resistance in the chicken production chain. The results of several studies show that active surveillance and the search for antibiotic substitutes are the best strategies for governmental organizations, poultry researchers, and farmers to lessen the negative effects of antibiotic usage in chickens (Rondón-Barragán, 2020).

2.4 Poultry industry globally

Over 23 billion chickens are kept across the world, or three per person. Chickens produce primarily meat, eggs, and manure for agricultural implantation and are contained and reared using a variety of farming methods. Various cultures, customs, and religions worldwide use poultry meat and eggs regularly, making them essential for food resilience and safety. The poultry subsector is highly efficient in terms of resources and protein, making it a standout in the livestock industry. This is due to its capacity to meet the increasing demand for poultry worldwide. (MOTTET & TEMPIO, 2017). Bangladesh produces tons of chicken. The Department of Livestock Services reported in 2015 that more than 115,000 farms in Bangladesh produced over 170 million broiler and layer chickens. The chicken industry is regarded as a crucial subsector for economic development and job creation (Sarker et al., 2019). In Bangladesh, contamination of chicken meat with ESBL and amp C-producing bacteria such as *E. coli* is now posing a food security risk. Uncontrolled use of antibiotics to cure or avoid diseases in food animals raises the possibility of growing bacterial strains that are resistant to treatment. Bangladesh is also showing the same situation; as in many other developing nations, there is inadequate antimicrobial drug resistance monitoring and hygienic raw food processing technology (Rahman et al., 2020). Due to the increased feeding of meat and eggs, which makes them more readily available, nutritious, and reasonably priced, the poultry industry is today regarded as one of the agriculture and veterinary fields' fastest-growing subsectors (Rafiq et al., 2022).

Did you know that the poultry sector is a significant contributor to British Columbia's economy? In the year 2006, the Fraser Valley alone produced 85% of chicken in the province. With the increasing popularity of high-density agricultural methods, the production of poultry has also expanded in recent years (Furtula et al., 2010). Because of the world's growing inhabitants, the demand for poultry is rising steadily. The world's population is at 7.6 billion, and it will shortly surpass 8 billion, driving up global demand for poultry. According to the report, poultry plays a significant part in global development. It eliminates poverty, raises people's income levels, and creates jobs for everyone around the globe. The USA leads the world in both poultry production and export. Brazil is at the top in the world, while Japan is the best country in the world for imports. 40% of the world's meat comes from poultry. The United States is the top

producer of poultry globally, followed by Brazil, China, India, Argentina, Ukraine, Russia, and Mexico. Pakistan ranks eleventh in poultry production. (Khan et al., 2021)

2.5 Poultry Industry in Pakistan

One of the biggest and most active industries in Pakistan is poultry. The poultry business of Pakistan has a significant and mounting influence on the Gross Domestic Product (GDP) around 1.5% of the country as a whole. Economically after textiles, the the poultry business is the second-largest industry in the nation and has a rapid growth trend. Within the next several years, it is expected that this industry will create 1.5 million new employments (Khan et al., 2021). According to a survey conducted in 2018, Pakistan is the world's 11th-largest producer of chickens, producing 48.83 million layers, 1.02 billion broilers, and 11.8 million breeding stock annually. By 2022, Pakistan's poultry industry will generate 1245 million kilograms of chicken meat annually and 11,250 million table eggs. In Pakistan, 40% to 45% of all meat intake is made up of chicken meat. According to the Pakistan Economic Survey, there are expected to be significant annual increases in commercial layer, breeder, and broiler stocks. Specifically, there is predicted to be a 7.0% increase in commercial layer stocks, a 5.0% increase in breeder stocks, and a 10% increase in broiler stocks. The vigorous expansion of the chicken industry in Pakistan, counting the anticipated huge rise in petitions for chicken goods, is explained by the fact that there is already a far bigger demand for chicken than local producers can currently meet. This necessitates paying extremely careful attention to the sector's production and efficiency (Khan et al., 2022). About 1.5 million people in the nation are employed by the poultry business, which is the foundation of the economy. With yearly sales of US two billion dollars, poultry is Pakistan's second-largest business after textiles (Rehan et al., 2019).

2.6 Consumption of poultry product

Result of its huge protein content and capacity to guarantee nutritional sufficiency for customers, particularly in developing states, chicken meat has been categorized as a beneficial section of an individual's regular food, with poultry farming being the main business in this respect. Over 40 proteins, including bactericidal, potent antigenic, and antihypertensive proteins, found in eggs make them one of the most important foods, having a similar biological value to breast milk. 18 distinct amino acids, including 9 that are essential for life, are also present in eggs (Khan et al., 2022). The GDP of

Pakistan is significantly influenced by the poultry industry. The capacity of Pakistan's chicken business to sustain economic productivity, animal health, and public health is threatened by E. coli and other environmental problems. Asia's chicken population is expected to grow by 129% between 2010 and 2030, partly as a result of increased demand for poultry meat in Pakistan and India, population growth, and inadequate antibiotic control measures (HUSSAIN et al., 2015)

2.7 Nutritive value of chicken meat

The perceived healthier nutritional profile of chicken meat compared to beef meat and its cheaper cost are the two key factors driving the success of poultry meat in both developed and developing nations. Additionally, the lack of religious restrictions, the relative simplicity of cuisine preparation, and the abundance of accessible processed goods have all contributed favorably to the growth of its consumption (Petracci et al., 2014). Poultry products are known for their high protein content, low fat content, and stable ratio of polyunsaturated fatty acids from n-6 to n-3. They also contain low levels of cholesterol and some functional components, making them a healthy and nutritious food choice (Petracci et al., 2013).

Choosing white chicken meat over red meat has several benefits, but its main advantage is its lower calorie and saturated fat content. Individuals who aim to reduce their fat intake and those with heart and coronary diseases should include white chicken meat in their diet. Chick meat has an excellent pedigree of protein, which makes it an ideal food for people who require quality, and comfortably digestible protein. This includes sportspersons, kids, and the youngsters. Chicken meat has the added advantage of containing low levels of collagen. This makes it easier to digest compared to other types of meat that are high in structural protein collagen. Did you know that poultry meat is a great source of essential vitamins and minerals? Compared to the meat of other animals, it contains higher levels of calcium, phosphorus, magnesium, and sodium. Additionally, chicken meat is particularly rich in niacin, or vitamin B3, and also contains higher amounts of vitamins A and B6 (Kralik et al., 2018).

2.8 Food-borne pathogens

Food-borne pathogens in people that might not be responsive to antibiotic therapy can be caused by bacteria expressing antibiotic-resistant genes present in the digestive

systems of farm animals. These bacteria can infect corpses. As a result, it's crucial to keep an eye on changes in the antimicrobial sensitivity of zoonotic and mutualistic organisms. In this regard, several veterinary monitoring programs accumulate microbes from livestock at slaughter and assess their susceptibility to antibiotics important for human medicine (de Jong et al., 2011). *E. coli*, a significant contributor to foodborne illnesses, is a typical resident of poultry, other animals, and humans' gastrointestinal tracts. Butchery contaminated with *E. coli* is caused by unhygienic slaughter procedures (Rahman et al., 2020).

2.8.1 Gut Microbiota of Poultry Chickens

The host's digestive system is home to the bacteria that make up the gut microbiota. The gut microbiota and the host's health and illness status are strongly related. The microbiota in different parts of a bird's gastrointestinal pathway, such as the colon, duodenum, gizzard, proventriculus, and ileum, is varied because of the various circumstances like pH levels, substrate availability, redox potential, and the antimicrobial action of host secretions. Lactobacilli lead in the crop and gizzard as a result of the strong selection caused by pH levels. Additionally, the development of facultative anaerobic microbe is supported by the proximal gut, and the small intestines of healthy hens contain few stringent anaerobes. Only the cecum and colon have a considerably diversified microbiome. Gram-positive Firmicutes and Gram-negative Bacteroides typically colonize the cecum of a healthy adult chicken, making up about 90% of the total microbiota. Gram-positive Actinobacteria and Gram-negative Proteobacteria typically make up the remaining phyla of the gut (Rubens et al., 2023).

2.8.2 Microbiological concerns faced by poultry

Salmonella species and campylobacters are typical pollutants in poultry and animals, along with other foodborne diseases (Doyle & Erickson, 2006). The avian gut is a mysterious organ system that harbors a variety of microbial populations, either commensals or pathogens (Calhoun & Hall, 2019). Staphylococcus aureus, Salmonella spp., *Escherichia coli*, Listeria monocytogenes, Campylobacter jejuni, and Clostridium perfringens are the primary foodborne pathogens that are also related to the poultry production environment and spread diseases to the human environment (Żbikowska et al., 2020). Prebiotic substances specifically promote the growth of helpful

gastrointestinal microbes, improving host health and preventing the spread of foodborne infections (Ricke, 2021).

2.8.3 Salmonella

Salmonella belongs to the Enterobacteriaceae family of bacteria. This species has gram-negative, rod-shaped, motile, facultatively anaerobic organisms. The Salmonella genus currently has more than 2,500 distinct serotypes. The most frequent source of foodborne diseases worldwide is still Salmonella enterica. Salmonella spp. infections are thought to cause around 155,000 deaths and 93.8 million gastroenteritis cases annually worldwide, with about 80.3 million of the cases being foodborne, according to estimates from 2010 (Golden et al., 2021). The most prevalent zoonotic diseases, including chicken, that have been identified in farm animals and are a severe threat to human health, animal welfare, and the global food economy belong to the genus Salmonella (Mali et al., 2019). Animal goods, mostly poultry products like eggs and raw chicken, are frequently the cause of contagious gastroenteritis (Castro-Vargas et al., 2020).

2.8.4 Campylobacter

Gram-negative, non-spore-producing rods with either a curved or spiral shape make up Campylobacter. This species belongs to the family Campylobacteriaceae. The Campylobacter group is known as the “thermophilic group”, This group is composed of C. jejuni, *E. coli*, and C. upsaliensis (Percival & Williams, 2014). In the early 20th century, veterinary medicine had an extensive overview of Campylobacter spp. World Health Organization reported that Campylobacter is the primary cause of 1 in 4 diarrheal infections worldwide, which results in major health and economic damages. Animal contact, contaminated or undercooked meats (typically poultry), tainted food and water supplies, and animal contact are the main causes of Campylobacter illness (Summers et al., 2023). Since Campylobacter can colonize and spread swiftly within a flock of poultry, this is one of the most frequent reasons for bacterial food-related diseases in the United States (Pendleton et al., 2015).

2.8.5 Staphylococcus aureus

Easily contaminating meat, food, and the environment is the gram-positive coccal facultative anaerobic bacteria Staphylococcus aureus. Chicken flesh may readily become contaminated by Staphylococcus aureus, and this contamination is frequently

correlated with a high level of virulence and antibiotic-resistance genes. It is a significant contributor to pneumonia, nosocomial bacteremia, postoperative wound infections, and food poisoning in humans (Momtaz et al., 2013).

2.8.6 Pseudomonas aeruginosa

Pseudomonas aeruginosa is an aerobic, oxidase-positive, gram-negative bacteria. It is common in soil and aquatic environments. It is the main cause of endocarditis, sinusitis, sinus infections, pseudomoniasis, and respiratory diseases in poultry animals including chickens and geese (Kousar et al., 2021).

2.8.7 Clostridium

A rod-shaped, anaerobic, Gram-positive bacteria called Clostridium perfringens produces spores but is not mobile. It is a typical resident of the intestinal microbiota of chickens and is widely distributed in the natural world. It is non-pathogenic at low population levels (less than 104 CFU); however, the majority of its pathogenicity is linked to toxins. One of the most serious issues for poultry is necrotic enteritis. Additionally, chicken meat contaminated with enterotoxin-positive Clostridium perfringens may be a source of human intoxication (Żbikowska et al., 2020).

2.9 *Escherichia coli*

2.9.1 Background

Warm-blooded organisms including humans commonly harbor *Escherichia coli* in their gastrointestinal tracts, and it is also present in soil and freshwater settings. The species contained pathogenic and commensal strains, which can infect a variety of hosts and cause sickness while the commensal strains help digestion and produce some essential vitamins for the body. The most common cause of human diarrhea is pathogenic *E. coli* strains. Here are a few of the factors that contribute to the extensive research on *E. coli*. Fast growth in the presence of oxygen, the flexibility of environmental adaptation, and the relative simplicity of genetic manipulation. The existence of numerous phylogenetic groups (phylogroups) that have been detected using a variety of different methodologies reflects the genomic diversity of the species, in which the genus Shigella has been suggested to be included (Abram et al., 2021). Animal products are frequently contaminated during the slaughter and preparation of the meat at the slaughterhouse.

When it comes to crops, using polluted water for irrigation and animal dung as fertilizer are two sources of contamination. Ingestion of contaminated food or water by feces from infected people or animals can result in *E. coli* pathotypes dispersal, the cause of foodborne infections. Additionally, *E. coli* exhibits the capacity to produce biofilms in a variety of environments across the whole food production chain. The ability of the bacteria to form biofilms is renowned for its mechanisms for acid resistance, which enable it to tolerate the harsh conditions present in food processing settings, such as those resulting from the use of disinfectants in the battle against food pathogens (Sateriale et al., 2022). The most frequent cause of community-acquired bacteremia, and sepsis, and the primary causes of newborn meningitis and neonatal sepsis, both of which frequently result in fatal complications, is *E. coli*. Along with playing a significant role in nosocomial pneumonia and intraabdominal infections, *E. coli* can also be found in extraintestinal illnesses such as wound infections, osteomyelitis, and cellulitis. Some disorders that, when present together, can result in serious health issues, decreased productivity, and higher healthcare expenses are among the most typical causes of UTI (Johnson & Russo, 2002). When fecal pathogens may be present, *E. coli* is utilized as an indicator bacterium to look for them (Furtula et al., 2010).

2.9.2 Diseases caused by *E. coli* in poultry

There are three pathotypes of *E. coli* commensal, intestinal, and pathogenic *E. coli*. The disease-causing strain of *E. coli* that inhabits birds and poultry is termed Avian Pathogenic *E. coli* (APEC). APEC is the subdivision of extra-intestinal pathogenic *E. coli* (ExPEC) and the reason behind colibacillosis in birds (Vounba et al., 2019). Pathogenic strains possess an arsenal of virulence genes that directly influence these bacteria's capacity to induce colibacillosis (De Carli et al., 2015). ExPECs have a special capacity to spread infections outside the host intestinal tract. ExPEC, the specialized *E. coli* isolates that are responsible for the majority of extraintestinal infections, pose a serious yet unappreciated health risk. The cause behind their evolution is still a mystery. Other than the intestinal pathogenic varieties currently making headlines, "bad" *E. coli* do exist; they are ExPEC. Effective preventative measures to hide from the pathogenicity of *E. coli*, of which there are presently few, may result from greater knowledge of the existence and significance of ExPEC as well

as their unique virulence mechanisms, reservoirs, and transmission pathways (Johnson & Russo, 2002).

2.9.3 Discovery

Escherich initially isolated and characterized this organism, now called *E. coli*, in 1885 while researching newborn diarrhea. At first, Escherich called the organism Bacterium coli commune. It belongs to the family Enterobacteriaceae. Then it was known as Bacillus coli for a short period. Theodor Escherich, a German-Austrian pediatrician and bacteriologist who discovered this organism, was honored by acknowledging his discovery by its name, *Escherichia coli*, through time ("Etymologia: Escherichia Coli," 2015). By using his anaerobic culture techniques, where they developed white, non-liquefying colonies, and Gram's staining technique, which demonstrates that these bacteria quickly took color with all dyes but lost color after being treated with alcohol and potassium iodide, Escherich isolated a variety of bacteria from infant feces. To understand how the gut flora changes after breastfeeding, he examined the meconium and feces of neonates. He gave a name to the slender, short rods present in the preparation of fecal samples for microscopy. He also demonstrated how these bacteria were grown to have the capacity for fermentation by demonstrating how the formation of acid caused the milk to coagulate over time (Escherich, 1988).

2.9.4 Taxonomic Hierarchy of *E. coli*

Table: 2.1 Presentation of *E. coli*'s taxonomic hierarchy

Domain	Bacteria
Phylum	Pseudomonadota
Class	Gammaproteobacteria
Order	Enterobacterales
Family	Enterobacteriaceae
Genus	Escherichia
Species	*E. coli*

2.9.5 Habitat of *Escherichia coli*

Escherichia coli is a bacterial type that can be institute in the intestines of warm-blooded creatures, as well as in water, sediment, and soil. These environments have varying physical characteristics and levels of nutrients. According to mathematical calculations, an *E. coli* cell is typically created in the animal's intestine and lives there for approximately half of its lifespan. Afterward, it is evacuated into the environment, where it devotes the rest of its lifecycle before eventually dying or, in rare cases, infecting a new host (Savageau, 1983). In open settings, their capacity to consume nutrients and cling to surfaces is essential to their existence. *E. coli* is a common environmental pathogen that has been used to assess the water's safety and quality by acting as a sign of fecal contamination (Chekabab et al., 2013). In an inclusive variety of pressure 1-400 atm and temperature 23-40°C, *Escherichia coli* may multiply and develop. The doubling temperature of *E. coli* is greater than 37^{o}C (Kumar & Libchaber, 2013).

2.10 Structure of *Escherichia coli*

2.10.1 Cell wall

The two concentric lipid bilayers, cytoplasmic membrane, outer membrane, and gap in between make form the intricate cell wall that shields *E. coli* cells. The defense, transportation, sensing, detoxification, motility, and energy generation of the cell wall are only a few of its physiological functions. Several chemicals help the cell wall's structural function (Nikaido, 2003). Lipopolysaccharides are molecules made up of a complex network of long-chain polysaccharides. Lipids with several fatty acid tails are found all across the outer membrane. It is based on the structure of the lipid core on the lipopolysaccharide's attachment to the membrane protein FhuA (1fcp). Each pentasaccharide repeat, which makes up the heterogeneous polysaccharide chains, measures roughly 15 Angstrom and there are 3.5 million of them per cell (Bonhivers et al., 1998).

2.10.2 Periplasm

The periplasm, an aqueous region within the cell envelope, is located between an asymmetrical outer membrane and an asymmetrical inner membrane. Numerous distinct proteins that are necessary for gram-negative bacteria to develop and proliferate

well reside in the periplasm. These proteins are known to move freely and be connected to the cell wall or the inner or outer membrane (Boags et al., 2019). These include the vitamin B12 carriers BtuF and BtuCD and the molybdenum transporters ModA and ModBC, which assemble minute molecules and transmit them to transporters in the membrane of cytoplasm. Among the defending enzymes are beta-lactamase, lysozyme inhibitor, and a monomeric copper-zinc superoxide dismutase. The chaperone proteins proline isomerase FkpA and heat shock protease DegP are also present (Nikaido, 2003).

2.10.3 Inner membrane

The inner membrane contains several different kinds of proteins. I attempted to use a range of examples, such as proteins from power generation, transportation, peptidoglycan manufacturing, sensing, and defense, to offer an overview of the processes taking place. Several transporters are among them, including the calcium pump (1su4), the two small molecule transporters already revealed, the magnesium transporters CorA (2bbj) and MgtE (2yvy), the sodium-proton antiporter NhaA (1zcd), and the zinc transporter YiiP (2qfi). A secretory channel, the SecY complex in the membrane, and SecA and SecB inside the cell can all be seen on the right side of the micrograph (1 rhz, 2 fsg, 1 ozb). Drug efflux pumps include AcrAB (1iwg) and TolC, a large complex spanning both membranes on the left (1ek9). Also included are a large number of other energy-producing enzymes, such as succinate dehydrogenase (1nek), ubiquinol oxidase (1fft), nitrate reductase (1y4z), and NADH dehydrogenase (based on electron microscopy data) (Friedrich & Böttcher, 2004).

2.10.4 Outer membrane

Both product export and nutrient uptake need transmembrane transport, and the outer membrane, which serves as the effective permeability barrier, is outfitted with sophisticated nanomachines that span the cell envelope. The OM is also in charge of maintaining cell sizes and shapes (Guo et al., 2020) (Wang, Pors, et al., 2018). The control of cellular metabolism may alter in response to modifications of the OM's structure. In addition to regulating cell shape, division, phenotypes, and stress responses, the OM of *E. coli* is essential for intracellular metabolism. The cell's shape is also determined by the envelope, which also gives the cell the ability to tolerate powerful mechanical forces like turgor pressure (Raetz & Whitfield, 2002). The OM, which is also assumed to be the reason for *E. coli*'s intrinsic resistance to antibiotics,

detergents, and colors, is supposed to block large hydrophilic molecules and hydrophobic compounds from entering (Iwadate et al., 2011). Outer membrane proteins (Omps), phospholipids, lipopolysaccharides, exopolysaccharides, flagella, and type I fimbriae are all structural features of *E. coli*. These OM proteins are found in the periplasmic and exterior leaflets of the OM, respectively, and a variety of Omps populate this membrane. The passageway of small molecule solutes into the interior of the cell is regulated by the most substantial outer membrane porin proteins in *E. coli*, OmpC, and OmpF. OmpA, another significant porin, contributes structurally to the solidity of the surface of microbial cells (Wang et al., 2021).

2.10.5 Lipopolysaccharides

Lipopolysaccharides generally consist of a distal polysaccharide or O-antigen, a non-repeating (core) oligosaccharide, and a hydrophobic domain known as lipid A or endotoxin. This also increases the rigidity of the outer membrane and the mechanical integrity of bacteria. Various operons in E. rigidity list the unimportant polysaccharide components of LPS, EPS, flagella, and fimbria. Each *E. coli* cell has 106 LPS molecules and 107 glycerophospholipids (Raetz & Whitfield, 2002) & (Brown, 2019). Lipopolysaccharide is an endotoxin that may be documented as a pathogen-associated molecule by immune cells and cause a potent immune response. Additionally, *E. coli* may generate and transfer polysaccharides to the cell membrane (Wang et al., 2021).

2.10.6 Genome

Each strain of *E. coli* may autonomously acquire new genes due to its open pangenome. A pangenome, which consists of a core genome with genes found in all strains and an accessory genome with genes found in certain strains but not others, is more accurate at identifying the genetic characteristics of a species. A huge pangenome (89,000 genes) and a tiny core genome (3,100 genes) were discovered after analysis of more than 2,000 *E. coli* genomes. This can be partly ascribed to the adaptable way of existence of *E. coli* (Carter et al., 2021). Because harmful *E. coli* strains need several distinct properties or so-called virulence factors, their genomes are frequently bigger. These are encoded by virulence-associated genes (VAGs), which are often clustered in a DNA region called a pathogenicity island (PAI) (Schmidt & Hensel, 2004).

2.10.7 Plasmid

Small, circular, double-stranded DNA fragments known as plasmids exist independently from chromosomes in a supercoiled condition. Most bacteria have chromosomal DNA as well as extrachromosomal DNA in the form of plasmids for their genetic material. Resistance (R) factors are antibiotic-resistant genes based on plasmids. R factors are present in the majority of the bacteria that are drug-resistant and have been identified in human and veterinary patients. R factors have been discovered in all known bacterial species however, they are most prevalent in the family Enterobacteriaceae (Hoffman., 2001).

2.11 Antibiotic

2.11.1 History of antibiotics

The knowledge of microbes and viral illnesses was lacking during the pre-antibiotic period. Millions of people died as a result of the ineffectiveness of the methods employed to cure and stop the spread of these deadly diseases, which frequently reached epidemic levels (Uddin et al., 2021).

The primary antimicrobial particles found were chemical compounds. Paul Ehrlich discovered arsphenamine in 1909, which was active against Treponema pallidum, the agent of syphilis, and was derived from arsenic (Durand et al., 2019). The first antibiotic, Salvarsan, was utilized in 1910. In 1928, Alexander Fleming revealed penicillin, which marked the beginning of the golden age of natural product antibiotic research, which peaked in the middle of the 1950s. In barely over a century, antibiotics have tremendously influenced contemporary medicine and extended human life expectancy by 23 years. (Hutchings et al., 2019). The drug business was motivated by the Waksman period, which prompted the discovery of all present antimicrobials between the 1940s and 1970s. During this golden era, more than 20 classes of antimicrobials were found in many microbial species and fungi (Durand et al., 2019).

2.11.2 Introduction of antibiotics

Antibiotics are defined as substances that can kill or inhibit the growth of microbes and provide a way to treat and block bacterial infections (Calhoun & Hall, 2019). The pharmacology of antibiotics involves killing the cells of bacteria by either ceasing cell division or varying a crucial cellular process or purpose. The classification of antibiotics

depends on their behavior towards bacteria; they are called bactericidal because of their killing action and called bacteriostatic if they stop the growth of bacteria. The bactericidal activity depends on either time or concentration (Calhoun & Hall, 2019).

2.11.3 Mechanism of action of antibiotics

An antibiotic can injure a germ in several ways, such as by stopping RNA synthesis, and DNA replication, denaturing essential proteins, reducing protein synthesis, denaturing cell walls, and blocking the creation of tetrahydrofolate (Finch, 2004).

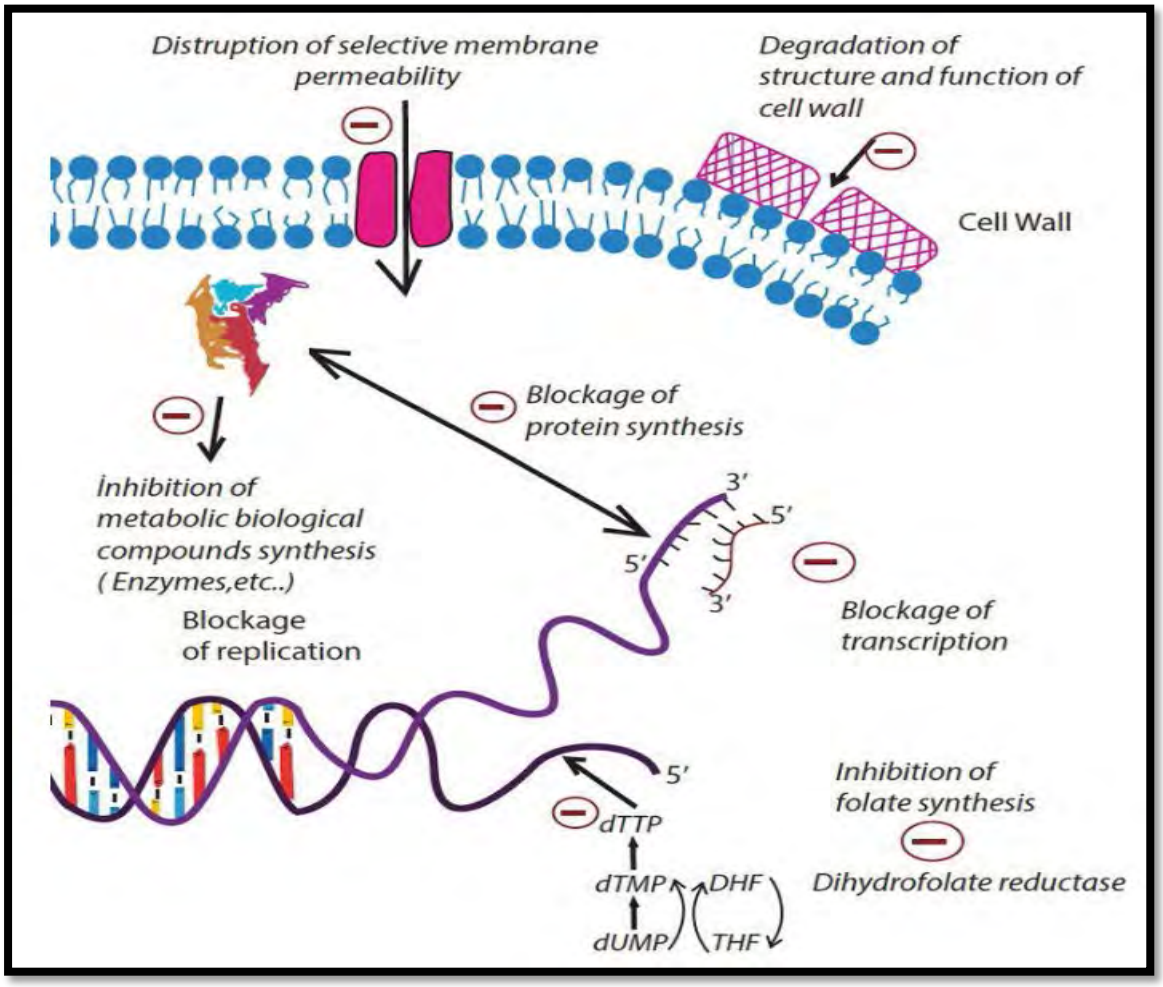

Figure 2.1: The diagram presents the Different Targets for Antimicrobials (Kırmusaoğlu, 2019)

Table 2.2: Antibiotics with targets of action and resistance mechanisms. (Davies & Davies, 2010) (C Reygaert, 2018).

Classes	Antibiotics	Target of action	Resistance mechanism
Cephalosporins	Cefixime, Cefepime	Peptidoglycan biosynthesis	Target alteration, efflux pump, hydrolysis
Penicillin	Amoxicillin	Inhibition of Cell wall synthesis	Target alteration, efflux pump, hydrolysis
Tetracycline	Doxycycline, Oxytetracycline	Translation, inhibition of protein synthesis	Target alteration, efflux pump, mono-oxygenation
Fluoroquinolones	Enrofloxacin, Ciprofloxacin, Norfloxacin, Oxalinic acid	Translation inhibits the synthesis of nucleic acid	Target alteration, efflux pump, acetylation
Phenicol	Florfenicol	Translation	Target alteration, efflux pump, acetylation
Phosphonic Acid	Fosfomycin		
Polymyxin class	Colistin Sulphate	Cell membrane	Target alteration, efflux pump

2.11.4 Beta-Lactam Antibiotics

Penicillin and Cephalosporins are two subclasses of the Beta-lactam class of antibiotics. A significant event in human history was the discovery of β-lactam antibiotics enhanced the treatment of microbial infections and changed the course of medical research. It also helped save countless lives. Being the largest section of the worldwide antibacterial

medicine market, they are among the medications that are most frequently given (Lima et al., 2020).

2.11.5 Colistin

Colistin Sulphate belongs to the polymyxin class of antibiotics. It was exposed in 1949 in Japan and formed by Bacillus polymyxa (Andrade et al., 2020). The last-resort antibiotic colistin works by making the membranes of gram-negative bacteria permeable. It is widely used in veterinary and agricultural medicine (Sarker et al., 2019). However, because of its toxicity and the evolution of resistant organisms, its therapeutic utility has been restricted (Xie et al., 2022). Due to a process resembling that of a detergent, it is bactericidal against Gram-negative bacteria, particularly Pseudomonas aeruginosa (Dosler et al., 2016). The academic and public health groups are paying close attention to and becoming concerned about MCR-1. Unexpected variety is present in a rising number of novel mcr-like variations, suggesting that various evolutionary phases may be subject to unseen selection forces. To fight transferable resistance to polymyxin, the last line of defense against pathogens with carbapenem resistance, there is an urgent need for collective effort. As a result, we must acknowledge that MCR-like colistin resistance may present worrying stress on clinical rehabilitation and public well-being (Sun et al., 2018).

To destroy Gram-negative germs, polymyxin disrupts membrane permeability through hydrophobic and polar connections. Among these connections, an electrostatic contact exists between the negatively charged lipid and the positively charged polymyxin residues. A lipopolysaccharide (LPS) moiety is attached to the bacterial membrane's outer leaflet (Sun et al., 2018).

2.11.6 Oxytetracycline

In Japan, oxytetracycline (OTC), a bacteriostatic antibiotic with a broad spectrum of activity, has been licensed as a medication for the control of livestock disorders. It is frequently used to treat acute coliform mastitis as a first-line medication. Due to the minimal lipopolysaccharide release that accompanied the removal of *E. coli* (Yasunori Shinozuka et al., 2019).

A broad-spectrum antibiotic called tetracycline stops bacteria from synthesizing proteins by blocking aminoacyl-tRNA from attaching to the ribosome.

One or more of the 36 identified tet genes provide resistance to the antibiotic. These genes encode for a resistance mechanism: an efflux pump, a technique of ribosomal safety, or direct enzymatic deactivation of the drug. While ribosome defense mechanisms are more dominant in Gram-positive bacteria, efflux mechanisms seem to be more dominant in Gram-negative bacteria. Tetracycline resistance among bacteria has expanded quickly as a result of the placement of tet genes on transposable genetic elements such as plasmids, transposons, and integrons (Avijit Kumar Das et al., 2020).

2.12 Antibiotic usage in Pakistan

Antimicrobials play a vital role in maintaining the health of both humans and animals. To treat *E. coli* infections in both community and hospital settings, a range of antibiotic drugs are used. Many of the antimicrobials used for animals are also used for people. However, it is important to note that some antimicrobials used in human medicine have not been authorized for use in animal studies (Benklaouz et al., 2020).

2.12.1 Antibiotics used in poultry

According to the Report of the 22nd meeting of the World Health Organization Expert Committee, some of the currently available antimicrobials (fluoroquinolones, aminoglycosides, and cephalosporins) are listed as critically important veterinary antimicrobials and are also regarded as highly important medicines for humans (Benklaouz et al., 2020). In poultry, numerous antibiotics, together with Amoxicillin, Neomycin, Lincomycin, Spectinomycin, and Oxytetracycline, are authorized for usage in hens such as broilers that are utilized as meat. Chlortetracycline is approved for the therapy of birds that lay eggs such as layers (Liu et al., 2020).

2.12.2 Antibiotic usage in poultry farms in Pakistan

Antibiotics can be provided to poultry for several beneficial purposes, including therapeutic, preventative, and growth-promoting effects. However, pathogenic bacteria, deadly chemical residues in meat, and the potentially dangerous progress of resistance in human flora undermine the beneficial effects of antibiotics. Immunoglobulins (IGs) found in yolks of eggs were exploited on a wide gauge for research as well as medical objectives, such as the prohibition of gastrointestinal illnesses. A natural substitute is essential for consumer health as an effect of chemical deposits from routinely used

drugs in chicken farms. Chemical leftovers have the potential to hurt consumers of meat (Hussein et al., 2020).

Table 2.3: Antibiotics used in commercial poultry farms in Pakistan (Ume Habiba et al., 2022).

Classes of Antibiotics	**Antibiotics**	**% of farms using Antibiotics**
Aminopenicillins (Beta Lactams)	Amoxicillin trihydrate	7.5
Tetracyclines	Chlortetracycline	2.5
	Oxytetracycline	2.5
	Doxycycline	22.5
Polymyxins	Colistin	60.0
Macrolides	Tylosin	25.0
	Erythromycin	5.0
Fluoroquinolones	Ciprofloxacin	2.5
	Enrofloxacin	35.0
Penicillin	Penicillin	5.0
Polypeptides	Bacitracin	10.0
Trimethoprim	Trimethoprim	2.5
Sulfonamides	Sulfa methoxy pyridazine	2.5
	Sulfamethazine	2.5
Aminoglycosides	Neomycin	10.0
	Streptomycin	5.0
	Gentamycin	5.0

Nitrofurans Derivatives	Furaltadone	2.5
Polymyxins/ Aminopenicillins	Amoxicillin trihydrate + colistin sulfate	60.0

2.13 Antibiotic Resistance

The capacity of microbes to survive the effects of antimicrobial drugs is called antibiotic resistance, and it progresses when an antibiotic drops its capability to efficiently prevent the growth of bacteria (Pulingam et al., 2022). Due to its capacity to evolve its antimicrobial resistance phenotypes in environments other than the host set up a wide range of species and transform from a commensal, antimicrobial-susceptible microbe into a resistant, pathogenic organism. *E. coli* is a significant ESBL-producing species. Human intake of meat and animal byproducts, as well as direct contact with food animals, can spread ESBL-producing *E. coli* to humans. ESBL-producing *E. coli*, ESBL resistance genes, and mobile genetic elements that promote horizontal gene transfer have all been linked to environmental reservoirs such as streams or soil polluted with domestic animal feces. Companion animals like cats and dogs may harbor ESBL-producing *E. coli* and facilitate transmission to people via related pathways. Companion animals may occasionally serve as hosts for ESBL-producing *E. coli* between humans and food animals (Mitman et al., 2022). A serious bacterial infection epidemic caused by resistant microbes can worsen with time such as the greater chance of getting an infection or serious disease for a patient going through surgery, chronic infections brought on by improper antibiotic use, increasing expenses and mortality rates in humans as well as in animals. Generally, immunocompromised elderly people, children, and even younger patients who need medical assistance regularly are the greatest victims of antibiotic resistance. The rate of antibiotic resistance is also higher in hospitals or medical centers (Pulingam et al., 2022). Politicians, medical professionals, and scientists are concerned about the life-threatening issue of AMR since it makes antimicrobial medication treatments to be unsuccessful. Antimicrobial resistance (AMR) is increasingly posing a danger to global health in developing or under-developed countries like Bangladesh and Pakistan (Sarker et al., 2019). The environment is regarded as a significant indicator of the spread of AMR due to the introduction of numerous developing environmental pollutants, such as antibiotic

resistance genes (ARGs) (Zhang et al., 2020). Around 700,000 people worldwide pass away from AMR infections each year, and if no effective mitigation measures are put in place, that figure is expected to rise to 10 million by 2050, surpassing the current death toll from cancer (Guo et al., 2019).

Antimicrobial resistance has plateaued in several high-income countries since the publication of the inaugural State of the World's Antibiotics Report in 2015, but it has continued to increase in many low- and middle-income nations. Antibiotic usage in people, as together in terrestrial and aquatic animals reared for human food, is a significant contributor to resistance. Between 2000 and 2015, human antibiotic usage scaled globally by 65 percent, and animal antibiotic consumption is predicted to grow by 11.5 percent between 2017 and 2030. Between 2015 and 2030, antibiotic usage is projected to increase globally by 200 percent if nothing is done to modify these trends (CDDEP, 2021).

2.13.1 Antibiotic resistance in Pakistan

AMR has been steadily expanding over the past few decades, and it already causes about 7 million mortalities annually, with estimates that this number will increase to 10 million by the year 2050, with 90% of these deaths occurring in LMICs in Africa and Asia (Ume Habiba et al., 2022).

2.13.2 Antibiotic Resistance in Poultry:

Antibiotic resistance is the behavior of a bacteria to protect itself from the action of any antibiotic used against it. Antibiotic resistance is a natural process due to the evolution of bacteria but the overuse and misuse of antibiotics against these bacteria in humans and poultry farms to treat the infection and as growth promoters leads to speed up this process. The infections caused by resistant bacteria are harder to deal with rather than the bacteria sensitive to the drug (World Health Organization, 2023). Bacteria can acquire the genetic material encoding resistance from other bacteria or develop it de novo through gene mutation. Bacteria with the resistance gene are forced to live and proliferate due to the selection pressure caused by the extensive use of antibiotics (Machowska & Stålsby Lundborg, 2018). In Pakistan these days, the chicken farming industry is flourishing. Antibiotics that are often utilized by bacteria are losing their effectiveness due to selection pressure. To keep the control sheds at a consistent

temperature, exhaust blowers are employed. The germs and antibiotic residues from the chicken farms are dispersed into the environment by these fans. Chicken litter contamination of the soil may cause bacteria that live there to develop resistance (Kousar et al., 2021). Poultry litter is a reservoir for several antibiotic-resistant *E. coli* and a source of antimicrobial residues (Furtula et al., 2010).

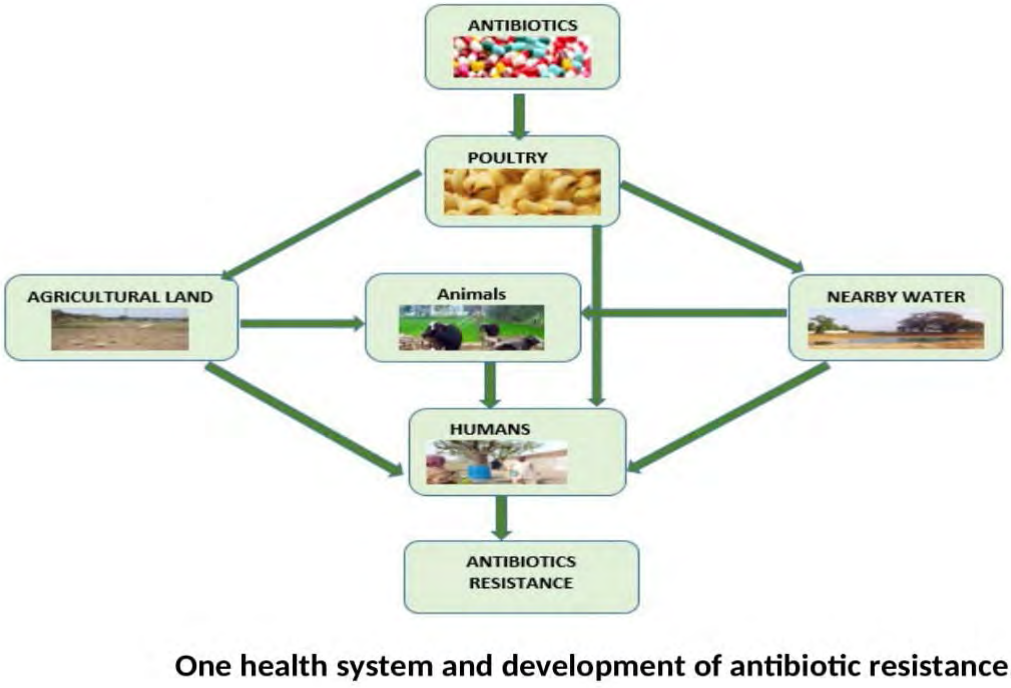

Figure 2.2: Antibiotic Resistance is one health concept. (Ume Habiba et al., 2022)

2.13.3 Resistance caused by antibiotics in poultry farms

To overcome the resistance in poultry, antibiotics should be eliminated from the poultry sector. Therefore, some alternatives could be helpful to eliminate the antibiotics from poultry. Antibiotic growth promoters (AGPs) have been substituted by a diversity of feed additives, such as biologically synthesized nanoparticles, probiotics, prebiotics, herbal extract, essential oils, organic acids, enzymes, and essential amino acids, among others (Abd El-Hack et al., 2022). The practice of antibiotics as growth promoters in livestock animals was excluded in the European Union in 2006 due to the rising threat of antibiotic-resistant bacteria, although it is still practiced in other areas of the world. Following the prohibition, multiple countries noted a detrimental effect on animal welfare, the resurgence of previous infectious illnesses in poultry, and an intensification

in the use of antibiotics for therapeutic purposes in poultry farms. The main bacterial causes of human illnesses nowadays are thought to be foodborne bacterial infections. It makes sense that so much scientific work has gone into creating and applying new technologies to attack pathogens in an era of rising multidrug-resistant bacteria and an acute shortage of new, potent antibiotics (Żbikowska et al., 2020).

2.13.4 Resistance in microbiota in poultry animals

Through selection pressure, the use of antibiotics for therapeutic and, in particular, non-therapeutic purposes on poultry farms encourages the development of antibiotic resistance in microorganisms that were earlier sensitive to antibiotics. The Food and Drug Administration (FDA) first acknowledged the threat that antibiotic resistance poses to human health when antibiotics are administered to animals at dosages below therapeutic levels more than 40 years ago. The FDA has enacted new regulations on the use of therapeutically relevant antibiotics in animal food production. Antibiotics that are used to treat human illnesses are considered clinically relevant. Clinically relevant antibiotics are no longer permitted in poultry and other food animals as growth promoters or for feed efficiency as a result of these new regulations. Despite the significant regulatory improvement, antibiotics deemed (not medically relevant) are still permitted in poultry feed (Sanchez et al., 2020).

2.13.5 Antibiotic resistance gene in poultry

The Antibiotic resistant genes (ARGs) with higher abundances, particularly tetracycline resistance gene (tetM), aminoglycoside resistance gene (strB), and sul2, were probably carried by bacterial species that were already widely distributed in the environment or were not the product of antibiotic-induced selection. The streptomycin resistance genes strA and strB are frequently related to the plasmid-borne sul2 gene, which has been discovered in plasmids from several incompatibility groups. These tet genes are recognized to exist in a broad variety of bacterial species, some of which being Clostridium, Bacteroides, and Corynebacterium, members of the main flora of the typical chicken gastrointestinal system and chicken litter. The ribosome protection type tet genes make up around 30% of the total ARG abundance in the chicken cecum, according to metagenomic research, and numerous tet genes are frequently linked to mobile elements in a variety of species. All chickens shed samples were found to have large amounts of the ermB gene, which confers macrolide resistance. Like the tet genes,

ermB was found in a variety of bacterial species and was linked to conjugative transposons, which may help to explain the gene's widespread distribution in the environment (Liu et al., 2020).

2.14 Antibiotic Resistance as One Health Concept

Antibiotic resistance has long been a serious problem for nosocomial infections in hospital settings, and meat is a key vector for the transmission of antibiotic resistance from animals to people. Such transfer can happen in one of three ways: through the transmission of resistant food-borne pathogens, through the absorption of resistant components of the original food microflora, or the transfer of antibiotic residues to food (Pesavento et al., 2007). In addition to their therapeutic uses, antibiotics also have prophylactic and growth-promoting applications. Another way that resistant pathogens are introduced to the soil from animal farms is through the manure-based disposal of animal waste. The practices of concentrating on animal feeding have made this issue worse. As a consequence, resistant bacteria and antimicrobial residues build up in the soil and spread among bacteria that live there (Kousar et al., 2021). Due to its involvement in the advancement of antibiotic resistance and as a vector of infectious illnesses, poultry poses a threat to human health. Furthermore, poultry consumes a lot of natural resources and has a big influence on the environment. The industry uses a lot of land, water, and nutrients to produce feed materials, even though this process is typically regarded as efficient in turning natural resources into edible goods. It also pollutes air and water and contributes to climate change, mostly through the manufacture of feed (MOTTET & TEMPIO, 2017). Infections caused by *E. coli* are a medical concern as this species is developing resistance to current antimicrobials. This is particularly concerning with the rise of isolates that produce extended-spectrum Beta-lactamases (ESBLs). Multidrug-resistant *E. coli* infections are associated with increased morbidity and mortality rates in poultry as well as in humans making them increasingly significant (Benklaouz et al., 2020). To lessen the prevalence of ESBL-producing organisms in poultry and their subsequent spread into the environment, it is crucial to regulate the prophylactic use of antibiotics, particularly those that are crucial for human health. To drive the intervention methods intended to control AMR, systematic AMR surveillance should be carried out while considering a One Health perspective (Amin et al., 2020).

2.15 Mechanism of antibiotic resistance

There are some mechanisms of resistance (C Reygaert, 2018).

- Restricting drug consumption
- Changes to a therapeutic target
- Drug inactivation (Beta-lactamases)
- Drug's active efflux
- Biofilm formation

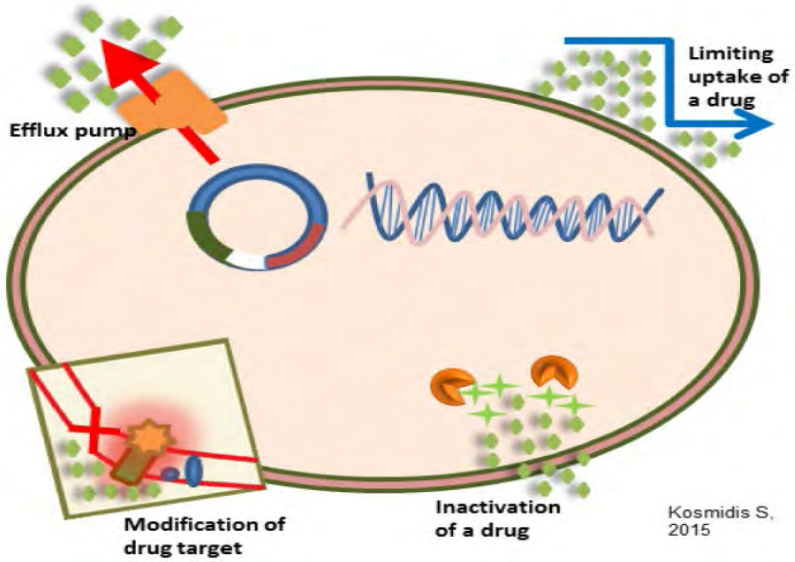

Figure 2.3: Presents the general mechanism of resistance to drugs (C Reygaert, 2018)

2.15.1 Restricting drug consumption

Certain types of chemicals are blocked by the gram-negative bacteria's LPS layer's structure and activities. Because of this, certain bacteria have an inbuilt resistance to specific classes of powerful antibiotic agents (C Reygaert, 2018).

2.15.2 Changes to a therapeutic target

Several bacterial cell components may be targets for antimicrobial agents, and an equal number of targets may be altered by the bacterium to confer drug resistance. Point mutations in certain genes can lead to very quick and simple resistance mechanisms, with little to no effect on the fitness of the microorganism. For example, the type IIA topoisomerases needed for uncoiling supercoiled DNA at replication forks and

decatenation of daughter strands during bacterial DNA replication are the target of synthetic fluoroquinolone antibiotics like ciprofloxacin. Single changes in target genes, like gyrA, result in significant levels of resistance (D. Wright, 2011).

2.15.3 Drug inactivation (Beta-lactamases)

Bacteria can inactivate medications in one of two ways: by degrading the drug or adding a chemical group to the drug. The broad family of drug-hydrolyzing enzymes is known as Beta-lactamases.

2.15.4 Drug's Active Efflux

Genes for efflux pumps are chromosomally encoded in bacteria. Some are permanently expressed, while others are induced or overexpressed (high-level resistance is often caused by a mutation that alters the transport channel) in response to certain environmental cues or in the presence of an appropriate substrate (C Reygaert, 2018). The MexXY multidrug efflux system is a key factor in Pseudomonas aeruginosa's aminoglycoside resistance, according to growing research. A timely overview of the MexXY pump from P. aeruginosa and other aminoglycoside efflux pumps from a variety of bacteria. Typically, transcriptional regulators that either activate or inhibit the transcription of the multidrug efflux genes regulate the expression of the bacterial multidrug efflux system (Lin et al., 2015).

2.15.5 Antibiotic resistance in biofilm

E. coli is protected by biofilms from the immune system and antibiotic therapy. Compared to planktonic bacteria, they are up to 1000 times more resistant to drugs. The following processes are the primary causes of antibiotic tolerance: Reduced growth rates and stress responses, efflux pumps, persister cells, limited antimicrobial penetration, and horizontal gene transfer (Ballén et al., 2022). Biofilm-forming microbial cells differ physiologically from planktonic cells of the same organism. Large sets of genes are differently regulated and a cell changes its phenotypic behavior as it transitions to the biofilm mode of growth. Finally, because bacteria in biofilms adapt collectively rather than as individual cells to changing environments, they become more resilient to antibiotics and the host's immunological defense mechanisms (Dosler et al., 2016).

2.15.6 Beta-lactamases

By hydrolyzing the beta-lactam ring, beta-lactamases are bacterial enzymes that give resistance to beta-lactam antibiotics like penicillin and cephalosporin. Extended-spectrum beta-lactamases (ESBLs) and AmpC beta-lactamases are two novel varieties of beta-lactamase enzymes that have appeared in recent years. TEM, SHV, OXA, CMY, and CTX-M beta-lactamases are the most prevalent beta-lactamases in Gram-negative bacteria. The majority of ESBLs and AmpCs are found on plasmids or integrons, which are mobile genetic elements. Through horizontal gene transfer processes including conjugation, transformation, and transduction, these mobile genetic elements spread to the cells of other bacteria. Food animals and retail meat assist as reservoirs for *E. coli* that produce ESBL and AmpC (Rahman et al., 2020). Inactivation by enzymes is arguably the peak of bacterial antibiotic resistance (D. Wright, 2011).

Quorum sensing is a special form of inter-cell communication that controls the expression of genes involved in virulence and antibiotic resistance. As a result, the primary quorum-sensing mediators, such as acylated homoserine lactone, have shown to be appealing targets for antimicrobial treatment (Lin et al., 2015).

2.15.7 Intrinsic Resistance

Intrinsic resistance is also known as natural resistance. When a bacterial genome contains genes that have the potential to produce a resistant phenotype, such as proto-resistance or quasi-resistance, this is referred to as intrinsic resistance. A variety of taxa, species, strains, and other entities display various antibiotic response characteristics (Davies & Davies, 2010). A feature that uniformly exists within the species of bacteria, unaffected by prior antibiotic exposure, and isolated to horizontal gene transfer is referred to as intrinsic resistance. Reduced outer membrane permeability, particularly caused by lipopolysaccharide, and naturally occurring efflux pumps are the two most common bacterial developments implicated in intrinsic resistance. An additional distinctive mechanism of induced resistance is the multidrug-efflux pump (C Reygaert, 2018).

2.15.8 Acquired Resistance

Acquired resistance types develop in response to stresses on a bacterial population due to selection. Antibiotic resistance genes are frequently present in a population before

exposure to a certain medicine. Even though antimicrobial medications seldom result in genetic changes, their existence puts pressure on the selection of mutants. A single organism has to modify its DNA to change its genetic composition to display resistance. Antibiotic targets are typically modified by chromosomal alterations that lead to antimicrobial resistance, such as by altering bacterial cell walls. A single organism must quickly propagate antibiotic resistance to the rest of the population once it has acquired it. There are only three ways for donor microorganisms to pass genetic information to a recipient: transposon, plasmid, or chromosomal. The genetic material exchange is through three procedures; Transduction, Transformation, and Conjugation (Hoffman., 2001).

2.15.9 Mechanism of antibiotic resistance in *E. coli*

Due to selection pressure, induction, or mutation, Escherichia coli acquires the resistance genes (Sarker et al., 2019). Gene mutation or the acquisition of external resistance determinants are two ways to acquire antibiotic resistance. Several potential processes might account for the bacteria's resistance to β-lactam antibiotics. Gram-negative resistance is primarily caused by four distinct factors: the susceptibility of the target enzymes “penicillin-binding proteins”, the effectiveness of the active efflux system, the periplasmic Beta-lactamases' quantity and characteristics, and the outer membrane's permeability (Lima et al., 2020).

2.16 Prevalence of antibiotic resistance

Antibiotic resistance is caused by misuse and overuse of antibiotics to treat infections or diseases with less or no knowledge. Antibiotics belonging to the same class cause cross-resistance, which further results in the development of high resistance rates. A key cause of antibiotic resistance is the irrational use of antibiotics. Lack of public knowledge and awareness, easy access to antibiotics without a written prescription, unused antibiotics, insufficient medical training, pharmaceutical publicity, a lack of patient-doctor connection, and a lack of quick and adequate diagnostic testing are important issues facing healthcare professionals (Machowska & Stålsby Lundborg, 2018). On a chicken farm, antibiotics often kill the isolates that are susceptible to them, leaving only the isolates with specific features of antibiotic resistance. These bacteria then use plasmids or horizontal gene transfer to spread their characteristics and genes to other bacteria. It has been demonstrated that chicken farms are home to resistant bugs

and that the amount of resistance increases with agricultural activity (Kousar et al., 2021). The usage of antibiotics in farming is thought to be one of the sources of drug-resistant *E. coli* in people (Benklaouz et al., 2020). In chicken farming, veterinary medications are commonly used to avoid and deal with microbial illnesses, as well as improve feed efficiency. However, concerns have been raised regarding the impact of their use on public and environmental health. Poultry litter, which comprises antibiotic residues and resistant bacteria, is often used as fertilizer. This raises concerns about the quantity and potential environmental consequences of these medications and bacteria. Poultry litter is a reservoir for several antibiotic-resistant *E. coli* and a source of antimicrobial residues (Furtula et al., 2010). Antibiotic-resistant bacteria, such as *E. coli*, can readily spread from animals to humans through polluted water, foodstuff, waste, or other ecological factors (Hossain et al., 2020). Lateral gene transfer, notably integrons, may allow the transmission of resistance in bacterial populations from one habitat to another. Integrons, which have been referred to as "a genetic construction kit for bacteria," are bacterial genetic components that permit the shifting of smaller mobile elements known as gene cassettes. Genetic mobile elements, such as plasmids, which may also carry virulence factors, are associated with the spread of resistance (Kheiri & Akhtari, 2017).

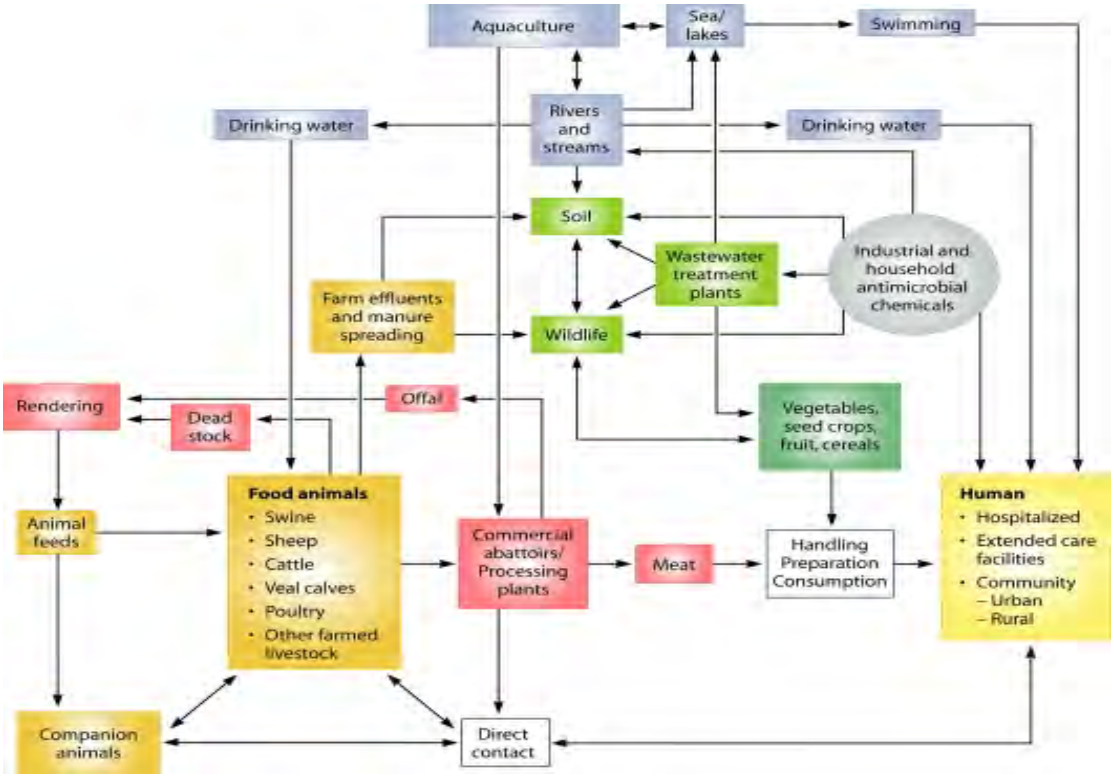

Figure 2.4: Irrational use of antibiotics and antibiotic resistance (Isaacson et al., 2006) (Linton, 1977)

2.17 Prevention and control of antibiotic resistance

- There are the following instructions provided by WHO to prevent antibiotic resistance:
- Obey the instructions of your healthcare worker while taking antibiotics.
- Use antibiotics only as instructed by a qualified doctor.
- It is important to prevent infections by washing your hands regularly, preparing food correctly, avoiding sick people, and making sure your vaccinations are up-to-date.
- Never make use of or distribute extra antibiotics (World Health Organization, 2020).

2.18 Biosecurity measures

The presence of ESBL/AmpC-generating *E. coli* in broiler meat poses a danger to human health because human infection has been substantially proven and may make illness treatment more challenging (Becker et al., 2021). To prevent diseases from poultry and their prevalence in the food chain by the transmission of pathogens to

humans and the environment, we had to develop and follow some biosecurity measures such as; Access to the staff should be controlled, with few entrances (Ridley et al., 2011). There should be secure entrances to the chicken coop. Another potential source of virus introduction is among farm laborers. Jackboots, overalls, double foot dips, hand sanitizers, and changing rooms are a few amenities that might be helpful. Insects and rodents are key players in the disease's horizontal spread. At this point, a successful intervention would lessen the spread (Newell et al., 2011). The used litter needs to come from reputable sources and should be thoroughly cleaned before usage. The used litter has to be collected and dumped right away. To stop the virus from spreading, water sanitation is also essential (Messens et al., 2009). The usage of chlorinated water can also be an effective preventative approach, as can the addition of organic acids like lactic acid to drinking water helps to avoid deadly pathogens of poultry (Umaraw et al., 2015). Water and feed must be regularly sampled and tested. Before reusing it, farm equipment has to be cleaned and sanitized. The entire chicken house has to be cleaned and sanitized in between flocks (Abraham et al., 2023).

2.19 Biofilm

To cling to biological or abiotic surfaces, microbes form well-organized communities called biofilms that are encased in an extracellular polymeric substance. Biofilm communities display unique qualities not seen in free-living cells, such as defense against environmental abrasions like temperature and pH (Stewart, 2002). Biofilm-forming microbes can colonize a variety of medical devices, including prosthetic heart valves, intravenous and urine catheters, contact lenses, and endotracheal tubes. This leads to higher mortality and morbidity rates and the development of chronic illnesses from infections that are already present (Ballén et al., 2022). A biofilm's cell population is diverse; it includes both types of cells that can grow slower and faster as well, some of them are resistant due to the expression of inactivating enzymes and efflux pumps, and others that are glaringly devoid of these systems. Therefore, the interaction of the whole cell population determines the overall resistance, and treatment must target a multicellular community (Lambert., 2002).

2.19.1 Biofilm formation in *E. coli*

The well-studied bacteria *E. coli* is crucial to the composition of the human microbiome. Some strains, however, have the potential to develop into pathogens and

infect people by forming biofilms not just in the gastrointestinal tract but also in other areas of the body. In *E. coli*, the production of biofilms is a complicated process that comprises three stages: reversible and irreversible attachment, maturation, and dispersion.

2.19.2 Strategies to prevent biofilm formation in *E. coli*

There are the following strategies used to prevent biofilm formation

- Anti-adhesion substances
- Phage therapy
- Quorum sensing inhibition
- Antimicrobial Peptides

2.20 Combination therapy

We had no time to just depend on the development of novel antibiotics to combat the fast-spreading bacterial resistance; we also need to study sensible methods for using more established antibiotics, like colistin. Review of recent clinical data with an emphasis on effectiveness assessment, developing resistance, potential risk factors, and combination treatment (Li et al., 2006). Combinations of antibiotics are frequently employed in medicine to broaden the antibacterial susceptibility range and provide synergistic effects. Antibiotic resistance can be slowed down while using medication combination techniques that are successful against MDR bacteria (Zhong et al., 2020). The effectiveness of currently available antibiotics in the suppression of MDR strains of bacteria, such as *E. coli*, may be improved by the innovative and opportunistic method of combining antibacterial drugs (Hossain et al., 2020). Although it has been observed that antimicrobial medicines can be combined with important essential oils to increase their antibacterial efficiency, such as thymol, carvacrol, citral, and cinnamaldehyde (Ma et al., 2022). Therapeutic studies have also demonstrated that antibacterial combination treatment can produce positive therapeutic results and reduce death rates in patients.

2.21 Synergistic effect

Combining antibacterial agents can result in a stronger effect than using them individually, known as the synergistic effect. Synergy is characterized as a decrease in the viability of the organism as a result of the combination when compared to the most

effective antibiotic tested alone. Some antibiotic combinations have greater therapeutic benefits in vitro than the sum of their separate effects. Our defense system is harmed by an antimicrobial agent's hazardous side effects, which can occasionally kill bacteria and remove toxic substances. However, by reducing the dosage of a single antimicrobial drug through synergistic combination treatment, these negative effects can be significantly mitigated (Xu et al., 2018). Synergy is quantified in vitro using the Fractional Inhibitory Concentration Index (FICI), the gold standard for assessing whether two drugs interact synergistically or antagonistically. Using the checkerboard test, FICI quantifies the growth of microorganisms in the presence of different concentrations of two antibiotics in 96 different combinations. The amount is calculated by dividing the minimum inhibitory concentrations (MIC) of each antibiotic by the total of each antibiotic's MICs. A FICI of 0.5 shows synergy. FICI values between 0.5 to 4.0 imply "no interaction," whereas values over 4.0 suggest antagonistic behavior (Hu et al., 2018) (Jiang et al., 2021).

2.22 Essential Oil (EOs)

Essential oils (EOs) have been employed as food preservatives since ancient times, but there has recently been an upward trend in scientific interest in their usage in the food industry and food products. Essential oils are fragrant oily liquids that may be extracted from plant material such as roots, leaves, stems, flowers, and others using a variety of techniques. They may be a superior alternative to some manufactured chemical-based modifications. They are a group of bioactive compounds with a variety of unique biological characteristics, including antioxidant, antibacterial, antifungal, and antibiofilm qualities, which make them useful in several industries, including cosmetics, medicine, pharmaceuticals, and the food sector. Various aromatic plants, including herbs and spices, can be used to produce EOs (Sateriale et al., 2022).

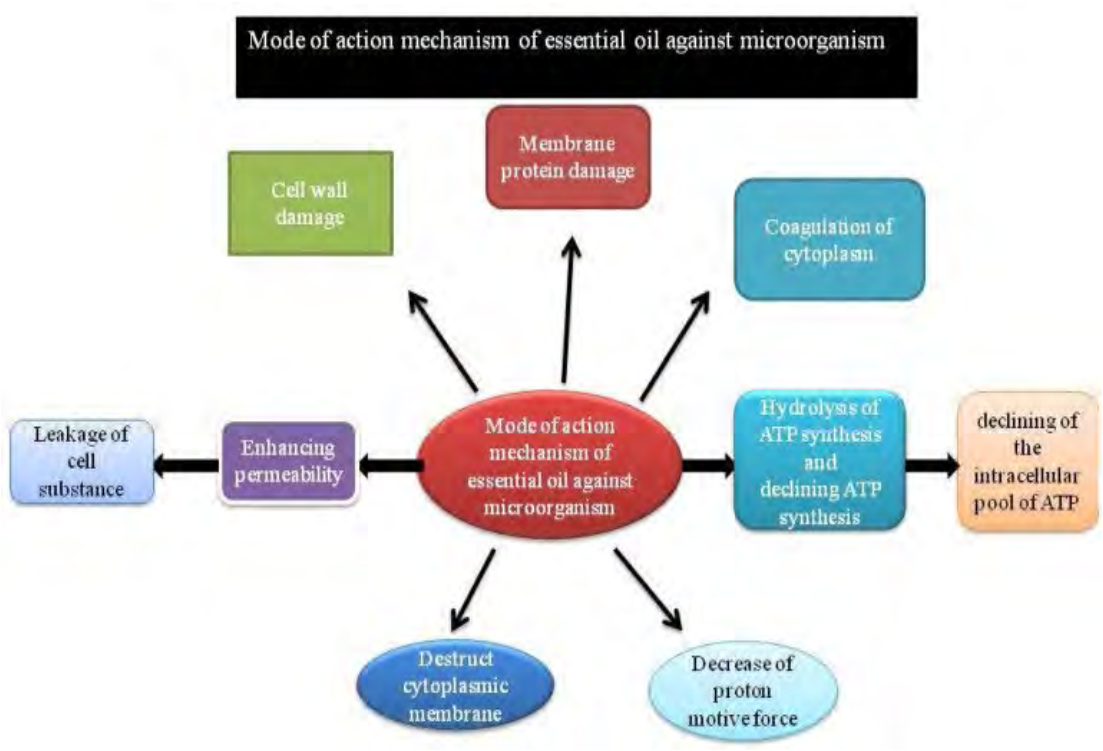

Figure 2.5: Presenting the mode of action of essential oil (Alam et al., 2022)

2.22.1 Essential oils for gram-negative bacteria

Antibiotics are losing their effectiveness against microorganisms with resistance. Essential oils (EOs) have been employed as alternative medicines, particularly as antibacterial agents to treat different pathogens spread with microorganisms. They are the essential naturally occurring bioactive compounds collected from plants, commonly referred to as volatile oils (Zakaria Nabti et al., 2020). Essential oils may have a remarkable effect on the developing issue of antimicrobial resistance because of their inherent antibacterial resistance. Therefore, it is essential to develop new, powerful antibacterial techniques that can be combined with established ones. The emergence of multi-resistant bacteria, notably foodborne ones, has been facilitated by the inappropriate and excessive use of conventional antimicrobials, with serious consequences for human health. Therefore, it is very beneficial to research the antibacterial properties of an individual or combined essential oils. According to several indications, essential oils might serve as potent weapons against microorganisms that have developed resistance, even when combined with or used as adjuvants with conventional antibiotics. Essential oils may damage a microorganism's cellular structure and have several targets for inhibiting it, making it more susceptible to other

antimicrobial chemicals (Sateriale et al., 2022). Since many years ago, synthetic preservatives have been extensively used to preserve food quality, increase shelf life, and guarantee food safety. However, their continued use has caused a buildup of chemical residue in the food chain, the emergence of bacterial resistance, and adverse impacts on human health. There is another way by which essential oils can treat the microbes: by the vapor method. The antibacterial activity of essential oils in the vapor phase has several uses in the fields of air disinfection, food preservation, agricultural product preservation, and fungus prevention. Studies have indicated that many processes, such as cell wall breakdown, cell membrane damage, membrane protein structural alterations, cytoplasm condensation, and altered nuclear activity, are responsible for the essential oil's antibacterial action during the vapor phase. However, the active center of the antibacterial component, the target site, and the mechanism of action on bacteria have not been described concerning essential oils (Wu et al., 2019). In poultry feeding essentials oil’s mechanisms of action. Spices, which are added to meals as flavoring agents, are also thought to have several advantageous physiological benefits. The usage of EOs in the production of chicken is increasing as a result of the prohibition on the practice of antibiotics as growth promoters. The bioactive substances in birds have not yet been fully described in terms of how they work, though. We identified various pathways of the EOs effects based on the most recent avian research, which we then categorized into four groups: sensory, metabolic, antioxidant, and antibacterial (Brenes & Roura, 2010).

2.22.2 Essential oil's activity against *E. coli*

The use of essential oils could be a different approach to combat bacteria with antibiotic resistance (Zakaria Nabti et al., 2020).

2.23 Pine oil

There are many sources for obtaining different essential oils, such as Eucalyptus, Sage, Thymus plants, Ginger plants, Mint leaves, and many more. Pine is one of them and belongs to the family Pinaceae, as it is the most important source of essential oil worldwide. It had the 10 most important components out of the 50 components it was made of. Pinene, camphene, sabin, 3-carene, myrcene, terpinolene, α-terpineol, limonene, caryophyllene, bornyl acetate, p-cement, felandren, γ-terpene, germacrene D, and spathulenol are the primary constituents of pine essential oil. These components

play an important role in the improvement of white blood cells. White blood cells then take charge of eliminating microorganisms from the body. Significant antioxidant activity was shown by pine needle essential oil, particularly against hydroxyl and superoxide radicals. The main constituents of pine essential oil, known as terpenoids, have demonstrated antibacterial, antiallergic, antifungal, antiviral, antispasmodic, and anti-inflammatory characteristics supportive in the anticipation and treatment of several disorders, including cancer (Gheorghita et al., 2022).

2.23.1 Antibacterial activity of pine oil

Some antibacterial and antifungal activities can be found in pine needle essential oil. These pine-derived essential oils stop bacterial infections from growing. Pine essential oil components have been known to prevent microbial development in the past. In particular, it has been shown that α-pinene and β-pinene have antibacterial action against methicillin-resistant Staphylococcus aureus (MRSA) strains. The fact that essential oils are probably not carcinogenic and, despite their capacity to kill cells, do not result in alterations in live cells is another characteristic that supports the use of essential oils as an antibacterial agent. For both people and animals, this antimicrobial action may be used to combat a variety of common infections (Sakul, 2016).

2.23.2 Mechanism of action of pine oil

Although essential oils and the various elements that make them up have been researched for a long time, it is still unclear how they work against microbes. Most of the time, one or two of the oils' constituents play a crucial role and inhibit or prevent bacterial development. The primary modes of action are modifications to the membranes and structures of microbial cells, disruptions of ion channels or transport systems, extremely quick interactions with OH radicals (from both internal and external sources), and attacks on receptors and enzymes (Pesciaroli et al., 2020).

MATERIAL AND METHODS

3.1 Course Duration

For a duration of one year, this study was conducted in the Applied Environmental and Geomicrobiology Laboratory, Department of Microbiology, Quaid-e-Azam University, Islamabad, Pakistan.

3.2 Sample collection

A total of 120 samples were collected by cloacal swabs from two different sheds of a poultry farm. Out of 120 samples, 30 samples were collected from randomly the 1st-week chicken of shed 1, and 30 were collected on the 5^{th} week of chicken growth. This phenomenon is the same for shed 2. The cloacal swabs were transferred to the lab in cold and sterile containers for further processing.

3.3 Sample source

Samples were collected from the cloaca of chicken. Sterile swabs were used to collect the samples from the cloaca by rubbing them around the cloaca of the chicken After that these swabs were in sterile covers with having transport medium. Transported immediately to the lab for further study.

3.4 Study Criteria

Gram-negative *E. coli* was isolated from the collected sample on which this study was based and all other bacterial isolates were excluded.

3.5 Morphological Identification of *E. coli*

E. coli can be identified by the following methods (Md. Golam Hakkani et al., 2016).

3.5.1 MacConkey Agar

The very first step to identifying the required microbe is their appearance, size, and shape on the media plates. To identify and isolate the required microbe I used MacConkey agar (selective and differential) media. *E. coli* grows flat, round, dry pink colonies on MacConkey agar due to lactose fermentation and darker corners indicate digestion of bile salts present in agar. *E. coli* had small to moderate colony size.

3.5.1.1 Principle

MacConkey agar is a selective as well as a differential medium for gram-negative bacteria as it obstructs the growth of gram-positive bacteria due to the selective constituents including bile salts and crystal violet while lactose acts as a differential component. After 24 hours of incubation lactose fermenting, pink colonies again streak on the MacConkey agar plates for purification and better identification. The round, pink, lactose fermenting colonies were picked.

3.5.1.2 Procedure

Freshly prepared MacConkey agar plates were directly streaked by the cloacal swabs on the day of arrival and then incubated aerobically under 37ºC for 24 hours.

3.5.1.3 Result Evaluation

After 18 to 24 hours of incubation, petri plates streaked with sample swabs were observed in the biosafety cabinets to ensure the purified colonies of *E. coli*.

3.5.2 Eosin methylene blue agar (EMB):

E. coli may be quickly and accurately distinguished from other gram-negative pathogens by using eosin-methylene blue agar. EMB is a selective and differential medium, that inhibits the growth of gram-positive organisms. EMB differentiates different organisms like *E. coli* that ferment lactose and those that do not, like Salmonella, and Shigella using a color indication.

3.5.2.1 Principle:

Eosin Y and Methylene blue up to some extent inhibit the growth of gram-positive microbes. Lactose fermenting gram-negative bacteria release acid in the media which drops the pH of the medium resulting in the formation of dark purple colonies, *E. coli* produces metallic green sheen colonies on EMB (Reza, 2009).

3.5.2.2 Procedure

A sterile loop used to pick up an isolated colony grown on a fresh culture (24hr or 48hr old) plate of MacConkey agar or preserved stock was streaked on a labeled EMB agar plate. After streaking plates were covered by cling film and put in the incubator at 37ºC for 24 hours. Plates were observed after the completion of 24 hours.

3.5.2.3 Results Evaluation

After 18 to 24 incubation hours, the EMB-streaked plates were observed for metallic green sheen colonies of *E. coli* in the biosafety cabinet.

3.5.3 Gram Staining:

3.5.3.1 Principle:

The basis of Gram staining lies in the ability of the bacterial cell wall to retain the crystal violet dye following solvent treatment. It is important to note that gram-positive bacteria possess a higher concentration of peptidoglycan, while gram-negative bacteria, such as E. coli, contain a greater number of lipids.

All bacteria initially absorb the crystal violet dye. However, the lipid coating of gram-negative bacteria is dissolved by the solvent, resulting in the loss of the main stain. This dissolution causes the violet-iodine complex to be unable to diffuse due to closed pores. The solvent ruthlessly dehydrates the cell walls of gram-positive bacteria, which in turn leaves them marked (Tripathi & Sapra., 2022).

3.5.3.2 Procedure

3.5.3.3 Equipment and reagents

Glass Slides, Distilled Water, Bunsen Burner, Toothpicks, Spirit, Immersion oil, Compound microscope, Fresh culture plate, Crystal Violet, Safranin, Gram's iodine, Ethanol, Dropper, slides rack, blotting paper, fresh culture plate (24 to 42hours).

3.5.3.4 Slides Preparation

Take a clean slide rinse with methylated spirit and tap dry with tissue then label the slides properly with isolate number and marked area for smear formation. The label slide then passes through the Bunsen burner to avoid contamination by heating.

3.5.3.5 Smear Formation

For the smear formation place a drop of distilled water with a dropper on the marked area for smear on the slide. Took a colony from the fresh culture plate (prepared after incubation of 24 hours) with a sterilized toothpick then spread it over the slide in a circular motion for the proper emulsion of distilled water with an isolated colony. Leave the slide to air dry after 2 mins pass it through the flame two to three times for heat

fixation. Picking an inoculum that is too heavy will reduce visibility because it will block light from passing through while performing microscopy. Additionally, smear fixation for a longer time may damage cell morphology.

3.5.3.6 Staining of Slides:

The smear was treated with an initial stain that is crystal violet. Place three to four drops of crystal violet on the smear to stain, and leave the stain for 60 seconds After passing 60 seconds slide was rinsed with distilled water. It is important to remove the stain without damaging the fixed culture. This must be done carefully to ensure the culture remains intact. The next step is 'dye fixation' which is done by adding iodine solution over the smear for 30 seconds then rinse with running water in the tilted position of the slide. Excess water was removed by shaking the slide. Dye fixation is followed by solvent treatment for which decolorizer is used and we used ethanol as a decolorizer. Avoid using excess decolorizer or using it for longer periods which may produce false results. The final step is done by using counterstain which is safranin exposing the slide to safranin for 40 to 50 seconds and rinse the slide with tap water. Shake the slide to remove excess water and leave for air dry.

3.5.3.7 Microscopy for results interpretation

Results interpretation is done by microscopic examination. The microscope was adjusted to a 40X objective to examine the smear distribution over the slide this is the initial observation then the 100X objective was set by using oil immersion to evaluate the proper cell shape and color.

3.6 Biochemical Identification:

Biochemical tests were performed for the confirmation of *Escherichia Coli*. In this work, five biochemical tests were performed. There were five tests performed to confirm *E. coli* for my study, Catalase test, Citrate test, Indole test, Methyl red, and Voges Proskauer (Md. Golam Hakkani et al., 2016).

3.6.1 Catalase Test

3.6.1.1 Principle

A catalase test is performed to detect the presence of the enzyme "catalase" which is the reason behind the split of hydrogen peroxide into two molecules, water and oxygen.

$$H_2O_2 \longrightarrow 2H_2O + O_2$$

The oxygen released in the reaction produces a bubble which indicates the catalase is positive.

3.6.1.2 Equipment and reagents

Autoclaved glass slides, pipette, tips, hydrogen peroxide, Toothpicks/loop, stopwatch, spirit lamp, autoclave,

3.6.1.3 Procedure

The catalase test was performed by using sterile glass slides. Pick a colony from freshly prepared culture plates (24 to 48 hours) with the help of toothpicks. Place the colony on the marked glass slide in the center then add a few drops of hydrogen peroxide on the colony with the help of a dropper, then observe for rapid bubble formation. An organism giving a negative result for the catalase test was also performed as a control. Bubble formation indicates a catalase-positive test on the other hand no bubble formation is an indication of a catalase-negative test.

3.6.1.4 Result Evaluation

The positive catalase test is indicated by bubble formation occurring within 2 to 5 seconds. No bubble formation occurs by the addition of hydrogen peroxide within 20 seconds indicating catalase negative.

3.6.2 Citrate Test

3.6.2.1 Principle

Citrate is used to identify an organism's capacity to utilize citrate as the only source of carbon for its metabolism, producing alkalinity, and the ability of an organism to consume citrate as a source of energy is tested using citrate agar. Inorganic ammonium salts serve as the only supply of nitrogen in the citrate agar. Citrate-permease, an enzyme that can convert citrate into pyruvate, is produced by bacteria that can survive in this medium. The organism's metabolic cycle can then incorporate pyruvate to produce energy. Growth is a sign that citrate, a Krebs cycle intermediate metabolite, is being used. As a result of the citrate test, oxaloacetic acid, and acetic acid are produced when citrate is hydrolyzed by the citrate permease enzyme. The ammonium salts are

converted to ammonia by the bacteria as they break down citrate, which raises alkalinity. The medium's bromothymol blue indicator changes from green to blue due to the pH increase (Aryal, 2022).

3.6.2.2 Equipment and reagents

Simmons Citrate agar, test tubes, tips, pipette, inoculating needle, freshly prepared culture, aluminum foil, test tube rack, autoclave, incubator, distilled water, weighing balance, and laminar flow hood.

3.6.2.3 Procedure

To perform the citrate test Simmons Citrate agar was prepared by following the instructions of the manufacturer and autoclaving the media to sterilize the media then pouring the media into test tubes and placing the test tubes in a tilted position for slant formation. Sterile the inoculating needle red hot and cool. Pick a colony from the freshly prepared culture plates and inoculate the slant. Cover the test tubes with aluminum foil, and put them in an incubator at 37ºC for 24 hours. One test tube was left uninoculated for negative control and one was inoculated with Klebsiella pneumoniae as a positive control.

3.6.2.4 Result Evaluation

After 24 hours of incubation, the test tubes were examined for the variation in color of the slants. A change of green color to blue color is an indication of a positive citrate test but no change in color indicates citrate negative.

3.6.3 Indole test

3.6.3.1 Principle

In an indole test, the capacity to produce the tryptophanase enzyme is examined to distinguish the coliform from other Enterobacteriaceae members. Tryptophan is hydrolyzed by this enzyme into indole, pyruvic acid, and ammonia. Indole production occurs as a result of adding KOVAC's reagent and is shown as a red-colored ring (Aryal, 2019).

3.6.3.2 Equipment and reagents

SIM media, sterile test tubes, marker, weighing balance, inoculating needle, KOVAC's reagent, pipette, tip box, fresh culture plates, aluminum foil, and incubator.

3.6.3.3 Procedure

Sim media was used to perform the indole test and prepared as per manufacturer's information 1 1ml of autoclaved media in the sterile marked test tubes and then exposed to UV for two minutes and then inoculated with the organism to be tested with the help of sterile inoculating needle and one test tube uninoculated as a negative control. Cover these test tubes with foil and place them in an incubator for 24 hours at 37^{o}C. The growth is shown as the turbidity of the media after proper incubation, add 3 to 4 drops of KOVAC's reagent. Roll tubes between your palms to mix the reagent in the medium evenly and leave it for a while.

3.6.3.4 Result Evaluation

Observing the test tubes for the red ring formation on the surface later the addition of KOVAC's reagent specifies positive results. while no ring formation shows negative results. *E. coli* gives a positive indole test.

3.6.4 Triple Sugar Iron (TSI) Test

3.6.4.1 Principle

TSI test was performed to observe the ability of an organism to produce hydrogen sulfide (H_2S) gas after using lactose, sucrose, and glucose. The availability of ferrous sulfate and sodium thiosulphate satisfies the need for sulfur, while phenol red is an indicator of changes in the media's environment caused by the formation of acid or alkali. When holes appear or the material is torn into multiple pieces, gas production can be seen. The medium turns black as a result of H2S generation by the bacterium.

3.6.4.2 Equipment and reagents

TSI agar, test tubes, inoculating needle, burner, weighing balance, tips, pipette, marker, aluminum foil, laminar flow hood, spirit, test tube rack, distilled water.

3.6.4.3 Procedure

Triple Sugar Iron (TSI) agar was prepared as per the instructions provided by the manufacturer. This autoclaved media was then poured into the sterile, marked test tubes with the help of a pipette and settled in a tilted position for the slant formation. By using an inoculating needle, the media in the test tube was directly stabbed in the butt and gently streaked in a zig-zag manner with the organisms to be tested and covered with aluminum foil. Place these test tubes in the incubator at 37^{o}C for 24 hours.

3.6.4.4 Result Evaluation

Observe the color change, gas production, and H2S production. Acid with gas production without H2S generation is produced by *E. coli*. The yellow color of the butt and slant indicates that *E. coli* uses all sugar anaerobically and produces gas as it ferments. However, the Pseudomonas species gives the opposite results than *E. coli*, as it does not use any sugar and gives alkaline/alkaline results.

3.6.5 Methyl Red-Vogues Proskauer

3.6.5.1 Principle

MR-VP tests are performed using the same MR-VP broth developed by Clark and Lubs. After incubation, the inoculated media was divided into two different test tubes to perform both tests at the same time on the same media.

The Methyl Red test is essential in determining whether fermentation produces acid. It is important to note that some bacteria can convert glucose into a stable acid, including lactic acid, acetic acid, or formic acid. This process involves converting glucose into pyruvic acid, which is then processed through different pathways to create a stable acid. The type of acid generated is specific to the bacterial species and is dependent on the available enzymatic pathways. A shift in the color of methyl red from yellow to red indicates that the acid produced during this process lowers the pH. (Sagar Aryal, 2018).

If you want to know whether an organism produces acetyl methyl carbinol through glucose fermentation, you can perform the Voges-Proskauer (VP) test. If the organism does produce this compound, it will react with alpha-naphthol, strong alkalis such as 40% KOH, and ambient oxygen to form diacetyl. Although alpha-naphthol, also known as Barritt's reagent, was discovered by Barritt to intensify the color of the reaction, it

was not originally included in the technique and must be added beforehand. The peptones present in the broth containing diacetyl and guanidine molecules will then condense to form a pinkish-red polymer. (Aryal, 2018).

3.6.5.2 Equipment and reagents

MR-VP broth, autoclave, test tube, pipette, tips, inoculating needle, methyl red indicator, incubator, laminar flow hood, spirit, spirit lamp, Barritt's reagent A (alpha-naphthol), Barritt's reagent B (40% KOH), fresh culture plates.

3.6.5.2 Procedure

Prepared the MR-VP broth in distilled water as per the instructions provided by the manufacturer. and autoclaved the broth along with other stuff being used in this test. Pour the autoclaved broth into the sterile, marked test tubes with the help of blue tips using a pipette. Then inoculate the broth with organisms to be tested by using an inoculating needle. Place these test tubes for 24 hours in the incubator at 37^{o}C. After completion of the incubation time. Divide the aliquots into two test tubes for both tests per test tube 1ml broth was added.

For the Methyl Red test, the test tube was marked with the test name along with the organism. The aliquot was then exposed to 2 to 3 drops of methyl red indicator with the help of a pipette. Observe the test tube for a change in color from yellow to red.

For Voges-Proskauer the remaining aliquot will be treated with the addition of 5 to 6 drops of 5% alpha-naphthol and mix well then add 3 to 4 drops of 40% potassium hydroxide (KOH) and mix well by shaking the test tube between your palms. Then observe the test tube for the color change from yellow to red-pink.

3.6.5.3 Result Evaluation

MR test results: If the medium color turns to red it indicates a positive MR test (it shows the production of one or more organic acids during fermentation) and yellow color indicates negative results. And red-orange color indicates weak positive results.

VP test results: If the medium shows a change in color to pink-red indicates a positive VP test. The lack of pink-red color indicates VP negative. Rust color indicates the false VP test.

3.7 Preservation of *E. coli*

After confirmation of *E. coli* bacteria, isolated from the sample. These confirmed *E. coli* strains were preserved by following the method and preserved at -20^{0}C or -40^{0}C.

3.7.1 Equipment and reagents

Tryptic soy broth or nutrient broth, Eppendorf, Eppendorf stand, pipette, tips, autoclave, distilled water, 40% glycerol, shaking incubator, spirit, laminar flow hood, spirit lamp, toothpicks, weighing balance, paraffin film.

3.7.2 Procedure

Before performing this procedure all the required materials were autoclaved to avoid contamination. Initially, the 500µl autoclaved nutrient broth was poured into the sterile Eppendorf with the help of a pipette and blue tips. By using, toothpicks a colony from the freshly cultured plates was added to the labeled Eppendorf. These Eppendorf-containing inoculated nutrient broths were exposed to incubation at 37^{0}C for 24 hours in the shaking incubator. After 24 hours of incubation, 500µl of freshly prepared 40% glycerol was added to the turbid broth Eppendorf one by one in the sterile environment of the laminar flow hood. Cover the Eppendorf with paraffin film tightly and vortexed then preserve them at -20^{0}C or 40^{0}C.

3.8 Antibiotic Susceptibility Testing Assay of the antibiotics

Antibiotic susceptibility testing (AST) can be performed by different methods such as disc diffusion, well diffusion, broth macro-dilution, and broth microdilution. These tests were performed to identify which antibiotic inhibits or kills the bacteria we are tested.

After complete isolation and identification of confirmed *E. coli* strains. These isolates were exposed to 12 different antibiotics used in human and poultry sectors to test their antibiotic susceptibility pattern as resistant, sensitive, and intermediate, by using disc diffusion test by Kirby-Bauer method.

3.8.1 Antibiotics Used

For this study, eleven antibiotics were used from seven different classes of antibiotics with different modes of action to evaluate the antibiotic susceptibility of *E. coli*.

Table 3.1: Antibiotics Used for Antibiotic Susceptibility Testing Assay

Class	Antibiotic	Symbol	Concentration (µg)	Mode of action
Polymyxin class	Colistin Sulphate	CS	10µg	Disrupting membrane integrity
Cephalosporin Beta-lactams	Cefepime	FEP	30µg	Cell wall inhibitor
	Cefixime	CFM	5µg	
Penicillin (Beta-Lactams)	Amoxicillin	AML	25µg	
Tetracycline	Doxycycline	DXT	30µg	Inhibit 30S ribosomal subunit
	Oxytetracycline	OX	30µg	
Fluoroquinolone	Enrofloxacin	ENR	5µg	Blocking DNA replication
	Ciprofloxacin	CIP	5µg	
	Norfloxacin	NOR	10µg	
	Oxalinic Acid	OA	2µg	
Phenicol	Florfenicol	FFC	30µg	Inhibit microbial protein synthesis
Phosphonic Acid	Fosfomycin	FOS	200µg	Inhibit bacterial cell wall biosynthesis

3.8.2 Disc Diffusion test by Kirby-Bauer method

The Kirby-Bauer disc diffusion susceptibility test is an essential method in determining the resistance or susceptibility of pathogenic aerobic and facultative anaerobic bacteria to different antimicrobial drugs. This information is crucial in selecting the most effective treatment for patients. The bacteria are grown on Mueller-Hinton agar with various antimicrobial agent discs to evaluate their reaction to the drugs. The capability of an antibiotic to inhibit the growth of an organism could be measured by the absence of growth around the drug in the form of inhibition zones (Hudzicki, 2009), (Guo et al., 2019).

3.8.3 McFarland Standard

McFarland is a turbidity standard. It has significant application for the preparation of inoculum while performing antibiotic susceptibility tests. This study was done according to the 0.5 McFarland standard. For E. Coli 0.5 McFarland is equal to 1.5x 108 CFU/ml.

3.8.4 0.5 McFarland preparation

0.05 mL of 1.175% barium chloride dihydrate (BaCl2•2H2O) and 9.95 mL of 1% sulfuric acid (H2SO4) are combined to create a 0.5 McFarland standard. There are currently McFarland standards made from latex particle suspensions, which increase the stability and shelf life of the suspensions. The standard can be visually compared to a suspension of microorganisms in nutritional broth or sterile saline.

3.8.4.1 Equipment and reagents

Muller Hinton Agar, Muller Hinton broth/Nutrient broth, petri plates, Conical flask, distilled water, Eppendorf, toothpicks, cotton swabs, forceps, pipette, blue tips, antibiotic discs, autoclave, incubator, Biosafety cabinet, freshly prepared culture plates.

3.8.4.2 Procedure

3.8.4.3 Preparation of inoculum

Inoculum was prepared by adding 1ml autoclaved nutrient broth in the sterile and properly labeled Eppendorf. By using sterilized toothpicks 2 to 3 colonies of E. Coli from the freshly prepared culture plates were suspended in the nutrient broth and mixed well. Carefully close these Eppendorf and place them in an incubator at 37^0C for 15 to 20 minutes. After 20 minutes of incubation, the turbidity of the suspension was compared and modified according to the 0.5 McFarland standard.

3.8.4.4 Muller Hinton agar plate preparation

Agar was prepared and autoclaved in the conical flask according to the plates required for the test. Other material was also autoclaved with MHA. The biosafety cabinet was sterilized with the spirit then media was poured into the autoclaved plates carefully in the cabinet. Allow these plates to dry and ensure their sterility by exposing them to UV

for 2 minutes. Then mark these plates carefully for swabbing and antibiotics disc placement.

3.8.4.5 Inoculation of MHA plates

After a comparison of bacterial suspension with 0.5 McFarland standard, it is approved to be used for lawn formation. A sterile swab was dipped in the bacterial suspension of specific E. coli strains labeled on both plates and Eppendorf. The excessive inoculum was removed by tapping the swabs against the walls of the Eppendorf. This swab was then ready to streak the plate from all directions by slowly rotating and changing the position of the plate at the angle of 60^0. In this way, a perfect lawn was formed. The swab was discarded after passing it through the flame.

3.8.4.6 Discs Placement on MHA Plates

By using forceps, place the antibiotic-impregnated discs on MHA in the following way. Initially, the forceps were cleaned by using a sterile alcohol pad or passing through the flame to red hot. Carefully remove the disc from the cartridge by using forceps. Then place this disc on the labeled area on the surface of the inoculated MHA plate. Sterile the forceps again and place another antibiotic at a distance more or equal to 24mm from the other discs. Do not place the antibiotic disc at a distance of less than 24mm. One plate allowed 12 antibiotic discs to be placed at a specific distance. Do not pick up a disc once seeded on the MHA surface even if it was placed in the wrong position because some antibiotics diffuse in a short period. Once the disc is placed on the surface gently press it with the forceps to ensure its attachment. Wrap the plates with cling film and place them 24 hours in an incubator at 37^0C for better results. The same procedure was repeated for all isolates of E. coli.

3.8.4.7 Result Evaluation

After 24 hours of incubation. The zones of inhibition measured with the help of a measuring scale and compared the zone diameters with the CLSI guidelines standard 2018 to 2021 to determine the isolates as sensitive, intermediate, and resistant.

Antibiotic susceptibility by zone diameter according to CLSI 2018-2021

Table 3.2: Shows zone diameter breakpoints of antibiotics used

	Zone of Inhibition (mm)		
Antibiotic used	**Resistant**	**Intermediate**	**Sensitive**
Colistin Sulphate	≤10mm	---	≥11mm
Cefepime	≤18mm	---	≥25mm
Cefixime	≤15mm	16-18^mm	≥19mm
Amoxicillin	≤13mm	14-17mm	>18mm
Doxycycline	≤10mm	11─13mm	≥14mm
Oxytetracycline	≤11mm	---	≥15mm
Enrofloxacin	≤16mm	17-20mm	≥21mm
Ciprofloxacin	≤21mm	22-25^mm	≥26mm
Norfloxacin	≤12mm	13-16mm	≥17mm
Florfenicol	≤14mm	15-18mm	≥19mm
Fosfomycin	≤12mm	13-15mm	≥16mm

3.9 Antibiotic Susceptibility Testing Assay for Essential Oil

3.9.1 Paper Disc Diffusion Method

The paper disc diffusion method is also the Kirby-Bauer disc diffusion method to find the susceptibility pattern of Pine essential oil against gram-negative E. coli by using Whatman filter paper discs on MHA media plates

3.9.1.1 Equipment and reagents

Muller Hinton Agar, Muller Hinton broth/Nutrient broth, petri plates, Conical flask, distilled water, Eppendorf, toothpicks, cotton swabs, forceps, pipette, blue tips, Whatman filter paper discs, pine essential oil, autoclave, incubator, Biosafety cabinet, freshly prepared culture plates.

3.9.1.2 Procedure

3.9.1.3 Preparation of inoculum

Inoculum was prepared by adding 1ml autoclaved nutrient broth in the sterile and properly labeled Eppendorf. By using sterilized toothpicks 2 to 3 colonies of E. Coli from the freshly prepared culture plates were suspended in the nutrient broth and mixed well. Carefully close these Eppendorf and place them in an incubator at 37^0C for 15 to 20 minutes. After 20 minutes of incubation, the turbidity of the suspension was compared and adjusted according to the 0.5 McFarland standard.

3.9.1.4 Muller Hinton agar plate preparation

Agar was prepared and autoclaved in the conical flask according to the plates required for the test. Other material was also autoclaved with MHA. The biosafety cabinet was sterilized with the spirit then media was poured into the autoclaved plates carefully in the cabinet. Allow these plates to dry and ensure their sterility by exposing them to UV for 2 minutes. Then mark these plates carefully for swabbing and antibiotics disc placement.

3.9.1.5 Inoculation of MHA plates

After a comparison of bacterial suspension with 0.5 McFarland standard, it is approved to be used for lawn formation. A sterile swab was dipped in the bacterial suspension of specific E. coli strains labeled on both plates and Eppendorf. The excessive inoculum was removed by tapping the swabs against the walls of the Eppendorf. This swab was then ready to streak the plate from all directions by slowly rotating and changing the position of the plate at the angle of 60^0. In this way, a perfect lawn was formed. Swab was discarded after passing it through the flame.

3.9.1.6 Discs preparation and Placement on MHA plates

Six sterile Whatman filter paper discs with 6mm diameter were previously impregnated with pure Pine essential oil. 10µl of pine essential oil was poured on each paper disc and placed onto Muller-Hinton agar by using forceps in the following way. Initially, the forceps were cleaned by using a sterile alcohol pad or passing through the flame to red hot. Carefully pick the disc impregnated with pine essential oil by using forceps. Then place this disc on the labeled area on the surface of inoculated MHA plate. The blank

disc was placed as a negative control. Sterilize the forceps again and place another disc of pine oil at a distance more or equal to 24mm from the disc placed before. Do not place the paper disc at a distance of less than 24mm. Do not pick up a disc once seeded on the MHA surface even if it was placed in the wrong position due the diffusion. Once the disc is placed on the surface gently press it with the forceps to ensure its attachment. This procedure was repeated three times and the result was the mean value of them. Wrap the plates with cling film and place them in an incubator for 24 hours at 37^0C for better results. The same procedure was repeated for all isolates of E. coli.

3.9.1.7 Result Evaluation

After 24 hours of incubation. The inhibition zones were measured including the disc diameter in (mm) with the help of a measuring scale, calculating the mean value for each strain, and the result was characterized in four categories weak, medium, strong, and extremely active as described by (S. Keeratirathawat et al., 2013), (Vasireddy et al., 2018) and (Dzaferovic, 2017)

Table 3.3: Presents the Antibacterial Activity of Pine oil

Antibacterial Activity	**Weak**	**Moderate**	**Strong**	**Extreme Active**
Zone Diameter	<8mm	8-14mm	14-19mm	>19mm

3.10 Biofilm formation (96-well microtiter plate assay)

3.10.1 Principle

The bacteria had a character to form the biofilm and to characterize them as weak, moderate, and strong biofilm former, microtiter plate method is the most common and easiest way. Moreover, the microtiter plate contains 96 wells on a single plate due to which we can easily perform biofilm assay for many isolates at one time. Bacteria are grown in the microtiter plate for a specific period in the incubator for biofilm formation. When the biofilm is formed at the base planktonic cells are removed by washing. After staining of biofilm, stained biofilm is solubilized by using different solvents. Then OD is taken for interpretation of biofilm and to visualize the extent of biofilm formation.

3.10.2 Equipment and reagents

Microtiter plate, Eppendorf tubes, pipette, blue tips, phosphate-buffered saline (PBS) media, autoclave, spirit, spirit lamp, cling film, ELISA plate reader, 1% crystal violet solution, methanol, toothpicks, distilled water, incubator.

3.10.3 Procedure

3.10.4 Bacterial suspension formation

Previously labeled Eppendorf tubes with isolates no. were dispensed with 500ul MHB media in them. This procedure is done in the biosafety cabinet. Freshly streaked MacConkey agar plates of 24hrs to 48hrs old were used to pick up 2 to 3 isolated colonies of the tested strain with the help of sterilized toothpicks. Shake the Eppendorf and place it in an incubator for 15 minutes at 37ºC. the turbidity of the suspension was compared and adjusted according to the 0.5 McFarland standard.

3.10.5 Biofilm formation

A microtiter plate labeled for each isolate tested for biofilm formation in triplicate along with media control.100ul of each diluted culture was poured into the wells of a microtiter plate containing 100ul of MHB media in it. The plate was covered with its lid and transferred to the incubator for 24hrs at 37ºC.

3.10.6 Fixation of Biofilm

After discarding the planktonic cells from the plate, wells were washed with PBS. 100ul of methanol was added to fix the biofilm in each well and to ensure the attachment of bacteria after washing of plate. It was allowed to stay for 20 minutes in each well of the microtiter plate and then methanol was removed from them. The plate was allowed to dry for 6 minutes.

3.10.7 Staining of Biofilm

Biofilm was stained with 1% crystal violet dye by adding it to each well and allowing it to dry for 20 minutes. Control wells were also stained with CV. Then wells were washed thrice with autoclave PBS, 200ul in each well. Media control shows sterility whereas CV control shows cut-off values for optical density of biofilm. Plates were dried after washing of stain.

3.10.8 Quantification of biofilm

Quantification of biofilm was done by measuring optical density. The optical density of biofilm was measured by an ELISA plate reader at 540nm wavelength.

3.10.9 Result Evaluation

Strains were classified as strong, moderate, weak, and non-biofilm former by comparing the optical density values of isolates with cut-off values. (Noreen et al., 2022).

Table 3.4: Evaluation of Biofilm based on OD

Biofilm formation	**Optical density**
Strong	4ODc < ODi
Moderate	2ODc < ODi ≤ 4ODc
Weak	ODc < ODi ≤ 2ODc
Non-biofilm former	ODi ≤ ODc

3.11 Minimum Inhibitory Concentration (MIC)

MIC is the quantitative method used to report antibiotic susceptibility. It determines which antibiotic is most effective against the organism tested. MIC demonstrates the lowest concentration of drug used to inhibit the growth of the tested bacteria. In this study, the minimum inhibitory concentration of planktonic cells was evaluated. The short form of a minimum inhibitory concentration of planktonic cells is MIC-p. “p” indicates the planktonic cells. MIC-p was evaluated by broth microdilution method by using 96 wells microtiter plate.

3.11.1 Equipment and Reagents

Nutrient broth, antibiotic in powdered form, test tubes, falcon, Eppendorf, blue tips, yellow tips, pipette, test tube and Eppendorf stand, distilled water, autoclave, incubator, biosafety cabinet, spirit, lamp, toothpicks.

3.11.2 Procedure (Broth microdilution method)

3.11.3 Antibiotic Stock Preparation

Stock for the antibiotics used in this study was made according to the MIC protocol. For the preparation of stock, the solvent for the specific drug used in this study such as colistin sulfate, oxytetracycline, enrofloxacin, and florfenicol was selected according to the CLSI instructions. 10ml total stock solution was prepared for which 10ml autoclaved distilled water was poured into the sterile falcon. The weight of the drug was calculated through this formula W= C x V/P, where

- W is the weight of the drug
- P is the potency of the drug
- V is the volume of stock solution
- C is the total concentration of the solution

The solvent used for the above-mentioned drugs was distilled water for the preparation of their specific stock. The stock was prepared for all drugs with the same method.

3.11.4 Working solution and 2 folds serial dilution preparation

The working solution was prepared by adding 4.5ml of nutrient broth along with 500ul of previously prepared stock solution of the drug in the autoclaved and properly labeled test tube. A total of 5 ml of the working solution was formed. For two-fold serial dilution of antibiotics, 1 ml of sterile nutrient broth was suspended in the test tubes labeled from 256ug/ml to 0.25ug/ml. The first test tube labeled with 512ug/ml was suspended 2ml of working solution from the working solution test tube with the help of a pipette. Then pick 1ml from 512ug/ml and transfer it to the next 256ug/ml and mix them by pipette up and down. Then pick from 256ug/ml solution and transfer to the next, this procedure should repeat till the last dilution of 0.25ug/ml. In this way, a two-fold serial dilution of required antibiotics was done. 1 ml from the last test tube was discarded. The microtiter plate was then labeled with antibiotics used against 7 different E. coli strains. Antibiotic dilution was labeled horizontally and bacterial dilution was labeled vertically for isolate no. The wells of the microtiter plate were filled with 100ul of bacterial dilution and 100ul of drug dilution along rows and columns respectively. The same procedure was repeated for all isolates.

3.11.5 Bacterial Suspension and Dilution

The bacterial suspension was prepared by 1ml of NB in an Eppendorf and 2-3 colonies of test bacteria from fresh cultured plates, mixed well and placed those labeled Eppendorf in the incubator for 20 minutes at 37ºC. After incubation 20ul was picked from each Eppendorf to dilute the bacterial suspension. 20ul bacterial suspension was added to 1980ul of NB with the help of a pipette and mixed well. Out of this bacterial dilution 100ul was picked and added to each well of the row mentioned for the specific isolate already having 100ul of drug dilution from 512ug/ml to 0.25ug/ml. Media control, antibiotic control, and E. coli ATCC as control were also plated with the test organisms. This 96-well microtiter plate was covered with its cap and properly labeled with the test organisms, sample, date, and time. Place this microtiter plate in the incubator for 24 hours at 37ºC. This procedure was followed for all antibiotics used against MDR strains to find out their minimum inhibitory concentration such as Colistin, Oxytetracycline, Enrofloxacin, and Florfenicol. After 24hrs of incubation plates and test tubes were checked for turbidity. MIC is defined as the lowest concentration of a drug that inhibits the growth of the bacteria so, the last clear well with a particular concentration was marked as MIC.

Table 3.5: MIC Breakpoints of Antibiotics (CLSI 2021)

Antibiotic used	**Sensitive**	**Resistant**
Colistin	≤2µg/ml	≥8µg/ml
Oxytetracycline	≤4µg/ml	>16µg/ml
Enrofloxacin		≥2µg/ml
Florfenicol	≤4µg/ml	≥8µg/ml

3.12 Broth microdilution test (BDT) for MIC and MBC of Pine essential Oil

3.12.1 Minimum Inhibitory Concentration

MIC of pine oil was determined, where there was no visible growth of bacteria in the well of a microtiter plate. This method was performed as described in (El-Shenawy et al., 2015 & Vasireddy et al., 2018).

3.12.1.1 Equipment and reagents

Nutrient broth, Pine Oil, Tween 80, test tubes, falcon, Eppendorf, blue tips, yellow tips, pipette, test tube and Eppendorf stand, distilled water, autoclave, incubator, biosafety cabinet, spirit, lamp, toothpicks, microtiter plate, MacConkey plates.

3.12.1.2 Stock Solution and Dilutions of Essential Oil

Stock solution for essential oil by prepared by the addition of 0.5%(25µl) of Tween80 in 4.9ml of broth. Tween80 was added to emulsify the essential oil. Then 1 ml of essential oil was added to the 1 ml of broth holding Tween80 to produce a final concentration of 1% (v/v). 1 ml of sterile nutrient broth was suspended in each test tube labeled from 1% to 0.0625%. The serial dilution was made by picking 1ml from 1% concentrated essential oil dilution to the next test tube containing 1ml of nutrient broth and continuing to the last test tube with 0.0625% concentration. The contents of each tube were vortexed and mixed at high speed for 20 to 30 seconds to confirm that the EO was evenly dispersed throughout the broth. In this way, the dilution of the required essential oil was done for further performance.

3.12.1.3 Bacterial Suspension and Dilution

The bacterial suspension was the same as described above in the MIC assay for antibiotics. Then 100µl of each dilution was poured with the help of a pipette in each well of the microtiter plate horizontally and 100µl of bacterial suspension of all isolates against each dilution was suspended vertically in a microtiter plate. Then microtiter plate was covered with a lid and placed in the incubator for 24 hours at 37ºC.

3.12.1.4 Results Evaluation

The result of MIC of essential oil was determined by observation of the well with the lowest concentration where there was no visible growth. That concentration was considered as MIC value

Table 3.6: Activity of Essential Oil according to (El-Shenawy et al., 2015)

Range of MIC	**Antibacterial Activity**
≥1% - 1%	Weak activity
0.125% - 0.5%	Moderate Activity
≤0.0625% - 0.125%	Strong activity

3.12.2 Minimum Bactericidal Concentration

The minimum concentration of essential oil where there was no growth observed in the drug-free medium.

3.12.2.1 Procedure

To determine the MBC value a sterile cotton swab was dipped in the well showing no visible growth or the clear well and from the control well then streaked on the MacConkey agar plates. Incubate those media plates for 24 hours at 37^{o}C and note down the results.

3.12.2.2 Results Evaluation

That minimum concentration was considered as MBC where there was no growth observed after 24 hours of incubation.

3.13 Synergistic Antibiotic Assay (Pine oil with Colistin)

A synergistic antibiotic assay was performed to evaluate the combinational effect of Antibiotics with essential oil. So, in this study, Colistin was combined with Pine essential oil against colistin-resistant strains. This methodology was performed as mentioned in (Saquib et al., 2021)

3.13.1 Equipment and Reagents

Muller Hinton Agar, Muller Hinton broth/Nutrient broth, petri plates, Conical flask, distilled water, Eppendorf, toothpicks, cotton swabs, forceps, pipette, blue tips, Colistin

antibiotic discs, pine essential oil, autoclave, incubator, Biosafety cabinet, freshly prepared culture plates.

3.13.2 Procedure

The bacterial suspension was prepared by suspending one bacterial colony from a fresh media plate and incubating for 20 minutes then matching it with 0.5 McFarland standard. A swab dipped in this bacterial suspension was streaked on the prepared MHA media plates from all directions by rotating at a 360^0 angle. For the evaluation of synergistic effect selected antibiotic drug such as colistin was impregnated with 10µl of Pine essential oil with the help of pipette drop by drop. Then pick this Colistin disc having Pine essential oil with the help of sterilized forceps and carefully put it on the inoculated MHA media plates with tested strains. Gently press the discs with forceps to ensure the attachment. Cover the plates and put them in an incubator at 37ºC for 24 hours. Perform this test in triplicate for each strain tested. Repeat this procedure for all other strains in the same way.

3.13.3 Result Evaluation

After 24 hours of incubation, the plates were examined for the zone of inhibition and measured these zones with the help measuring scale in (mm). Calculate the mean value of the results and compare them with the standard values to characterize them as Synergistic, Additive, and Antagonistic. (Saquib et al., 2021)

Table 3.7: Combinational effect of Colistin with Pine Oil

Combinational Effect	**Combinational Activity**
Synergism	CZ>ZOP+ZOC
Additive	CZ=ZOP+ZOC
Antagonistic	CZ<ZOP+ZOC

ZOP=zone of pine oil, ZOC=zone of colistin, CZ=combinational zone

3.14 Antimicrobial Combination Therapy of Drugs by Checkerboard (CHBD) Assay

A checkerboard assay was used to determine the combinational effect of different antibiotics in a microtiter plate. This method is useful for presenting the combined effect of drugs (either two or three) against the effect of an antibiotic alone. Combinational

therapies are beneficial for their effectiveness due to synergism and the risks linked with antibiotics are reduced (Nutman et al., 2020). The results for the combination of drugs with different concentrations were performed by checkerboard assay was evaluated by FIC index. To evaluate the effect of the combination of two drugs by FIC index value, MIC values are required for the given combination. The equation used to find out the effect of the combination of drugs is given as **FICI = FICA + FICB**. FICI= (MICA in combination/MICA alone) + (MICB in combination/MICB alone) (Wang et al., 2018).

3.14.1 Various Interactions of Drugs

3.14.1.1 Synergism

When the effect of inhibition of two drugs in combination is greater than the individual effect of the drug then the interaction of two drugs is considered as synergistic. e.g., if FICI is ≤ 0.5 then the effect of the two combined drugs used in the checkerboard is interpreted as synergistic.

3.14.1.2 Additive

When the combinational effect of two drugs is equal to the sum of the effect of two drugs used individually and that does not give an enhanced inhibitory effect then the result of their combination is considered as inhibitory. However, the combination is interpreted as an additive if the FICI value is in the range of >0.5 - ≤ 1.

3.14.1.3 Indifference

The interaction of a combination of two drugs is considered as indifference if the inhibitory effect of two drugs in combination is equal to the inhibitory effect of any of two drugs used alone. If the FICI value is in the range of > 1 <4 then the combination is considered as indifference.

3.14.1.4 Antagonism

When the combination of drugs gives an effect that is either less than the combination of two individual drugs or even less than the effect of a single drug then this type of effect of combination is considered as antagonistic. In checkerboard assay, it is interpreted as if the FICI value is greater and equal to 4. (Kumaraswamy et al., 2018)

Table 3.8: Drug Interactions and their Respective FICI ranges

Types of interaction	**FICI ranges**
Synergy	≤ 0.5
Antagonism	≥ 4
Additive	>0.5 - ≤ 1
Indifference	<1 < 4

3.14.2 Procedure

96 well-containing microtiter plate was properly labeled for antibiotic dilutions of each drug alone and in combination. Moreover, controls were also labeled. Then the bacterial suspension was formed in 1ml of autoclaved MHB in an Eppendorf by adding two to three colonies of freshly cultured media plates. Incubate them for 15 to 20 minutes and turbidity was matched with 0.5 McFarland standard as done for MIC microdilution assay. Then antibiotic dilutions(2-fold) of both drugs Colistin and Oxytetracycline were separately prepared in autoclave test tubes ranging from 512ug/ml to 8ug/ml. 75ul of each drug were added as drug A was added vertically in the columns in descending order from 512ug/ml to 8ug/ml and drug B was added horizontally with different concentrations from left to right in decreasing order particularly well according to the checkerboard assay. Then 50ul of the bacteria suspension was added to each well of the microtiter plate that was labeled. Media control and antibiotics control were also run to check the sterility of media and drugs. The plate was covered with a lid and incubated overnight at optimal conditions required for bacterial growth. The next day the clear wells with a minimum concentration of drugs either singly used or in combination were noted.

3.14.3 Result Evaluation

Then these values were used to calculate the FICI values to characterize the interaction

Combination of drug A and drug B

	1	2	3	4	5	6	7	8	9	10	11	12
A	Media Control	512A 4B	512A 8B	512A 16B	512A 32B	512A 64B	512A 128B	512A 256B	512A 512B	Drug A Control	Drug B Control	Growth Control
B		256A 4B	256A 8B	256A 16B	256A 32B	256A 64B	256A 128B	256A 256B	256A 512B			
C		128A 4B	128A 8B	128A 16B	128A 32B	128A 64B	128A 128B	128A 256B	128A 512B			
D		64A 4B	64A 8B	64A 16B	64A 32B	64A 64B	64A 128B	64A 256B	64A 512B			
E		32A 4B	32A 8B	32A 16B	32A 32B	32A 64B	32A 128B	32A 256B	32A 512B			
F		16A 4B	16A 8B	16A 16B	16A 32B	16A 64B	16A 128B	16A 256B	16A 512B			
G		8A 4B	8A 8B	8A 16B	8A 32B	8A 64B	8A 128B	8A 256B	8A 512B			
H		4A 4B	4A 8B	4A 16B	4A 32B	4A 64B	4A 128B	4A 256B	4A 512B			

Drug A dilution

Drug B dilution

Figure 3.1: Table Represents the microtiter plate for the combinational effect of two antibiotics through Checkerboard Assay. (A) Colistin, (B) Oxytetracycline

RESULTS

4.1 Sample Collection

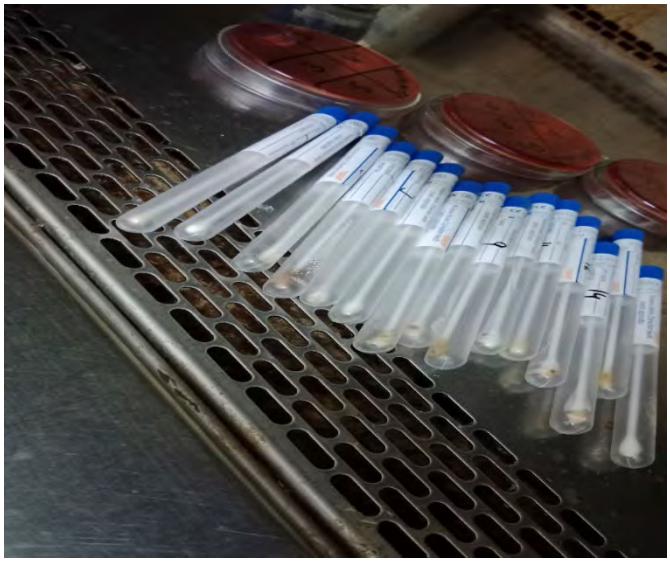

Figure 4.1: Collection of samples

4.2 Isolation and identification of *E. coli*

4.2.1 Morphological Characterization of *E. coli*

E. coli was morphologically identified by following methods

- Colony morphology on MacConkey agar
- Culturing EMB media
- Gram staining

4.2.1.1 Colony Morphology

Each isolate of *E. coli* was identified by its shape, color, size, and texture of isolates by culturing them on MacConkey Agar and EMB agar for visualization. On MacConkey agar E. coli shows pink colonies. Gram-negative E. coli grows flat, dry, round, non-mucoid pink colonies due to lactose fermentation with darker pink areas at their periphery as a result of bile salts present in the MacConkey agar.

Figure 4.2: Round Pink colonies of *E. coli* on MacConkey Agar

4.2.1.2 Pigmentation

Due to the dyes, and amide bonding in an acidic environment, lactose-fermenting coliforms like *E. coli* develop purple-black colonies with a metallic green sheen color.

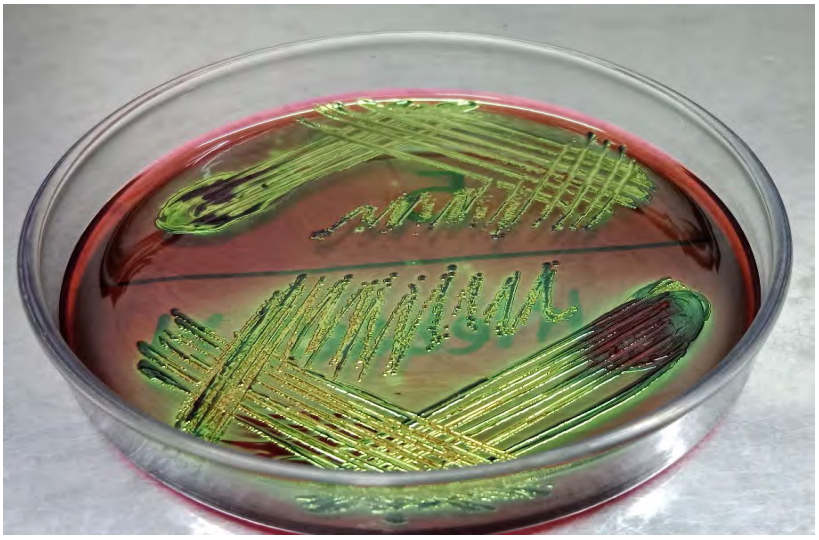

Figure 4.3: *E. coli* shows metallic green sheen colonies on EMB agar

4.2.1.3 Gram Staining

Gram-positive and Gram-negative Bacteria are distinguished by gram staining test, in this way Gram-negative rods of *E. coli* were confirmed by gram staining test. By using the compound microscope, the shape and color of bacteria were observed. Gram-

positive bacteria give a purple appearance whereas gram-negative bacteria manifest pink color. In this case, short, gram-negative rods of pink color were observed confirming gram-negative bacteria.

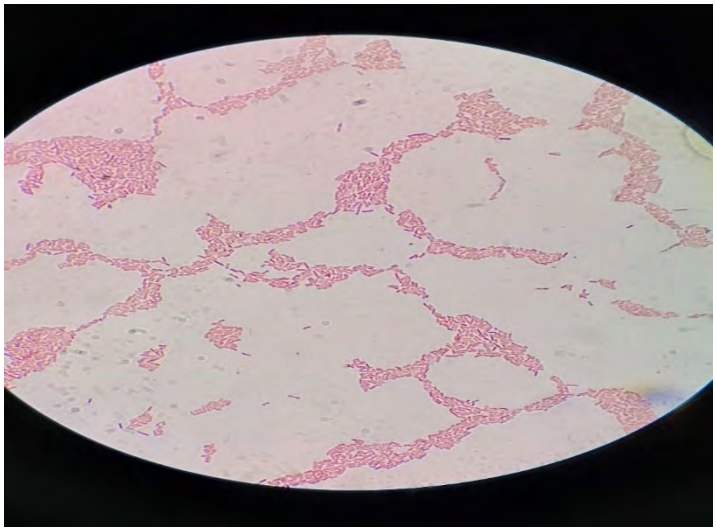

Figure 4.4: Gram staining of *E. coli* shows pink rods

Table 4.1: Morphological Identification of *E. coli*

Morphological Test	**Results**
MacConkey media	Round pink colonies
EMB media	Metallic Green Sheen colonies
Gram Staining	Gram Negative Pink rods

4.2.2 Biochemical Identification of *E. coli*

After morphological identification, *E. coli* was confirmed by biochemical tests. Following biochemical tests were performed in the lab.

4.2.2.1 Biochemical tests for *E. coli* were performed in the lab.

To identify *E. coli,* five separate biochemical tests were performed, which are:

- Indole Test
- Methyl Red Vogues Proskauer
- Catalase Test
- Triple Sugar Iron Test

- Citrate Test

The following characteristics were possessed by *E. coli* based on these biochemical tests.

Table 4.2: Results of Biochemical Identification of *E. coli*

Biochemical Test	**Results**
Indole Test	Positive (red ring formation)
Methyl Red /Vogues Proskauer Test	MR positive / VP negative
Catalase Test	Positive (immediate bubble formation)
Triple Sugar Iron Test	Positive (yellow butt, yellow slant, gas formation, H_2S negative
Citrate Test	Negative (no color change to blue)

4.2.2.2 Indole Test

An indole test was performed to find the existence of an enzyme “tryptophanase” that breaks down the amino acid tryptophan. As a result of the breakdown of amino acid indole is produced. Thus, when 2 to 3 drops of Kovac’s reagent were added it reacted with indole, and a red ring was formed. In my study result was positive showing the presence of the enzyme tryptophanase and confirming the bacterium to be *E. coli*.

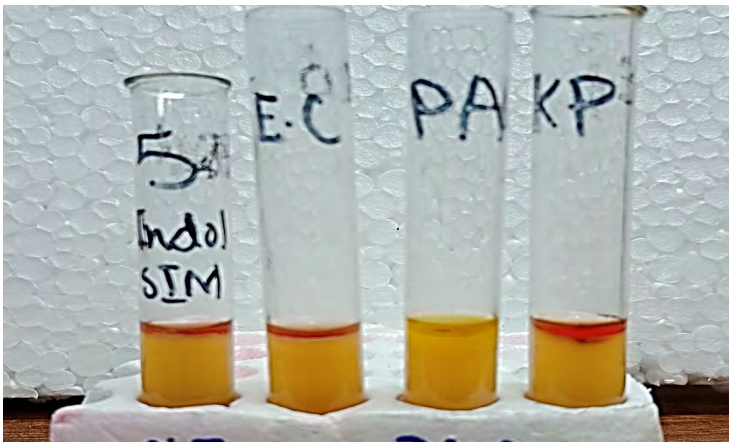

Figure 4.5: Represents *E. coli* results for the Indole test

4.2.2.3 Methyl Red- Vogues Proskauer Test

When methyl red was added, *E. coli* inoculated MR-VP broth maintained its red color. The MR test came back with a favorable result. MR-VP broth contaminated with Enterobacter cloacae turns yellow when methyl red is added. This is a negative MR result.

The media changes to a copper color when the VP reagents are introduced to MR-VP broth that has been infected with *E. coli*. The VP test came back with a negative result. The media changes color when the VP reagents are added to MR-VP broth that has been infected with Enterobacter cloacae. This is a positive VP result.

4.2.2.3.1 Results

MR: For *E. coli* the tube turns red, the test is positive for mixed acid fermentation

VP: For *E. coli* the tube didn't turn pink or red, the test is negative for acetoin.

A

B

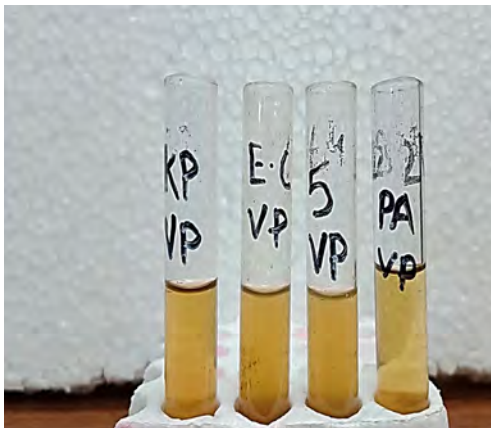

Figure 4.6: E. coli results for the MR test on the right side and VP test on the left side

4.2.2.4 Catalase Test

Catalase test for *E. coli* gives positive results by bubble formation immediately after the addition of hydrogen peroxide. Otherwise, it is considered negative even if the bubbles are produced after twenty seconds of the addition of H2O2. All organisms of *E. coli* were catalase-positive.

Figure 4.7: Represents *E. coli* results for the Catalase test

4.2.2.5 Triple Sugar Iron (TSI) Test

TSI is performed to know the pattern of carbohydrate fermentation and hydrogen sulfide production in organisms. In the case of E. coli, the slant and butt turn yellow.

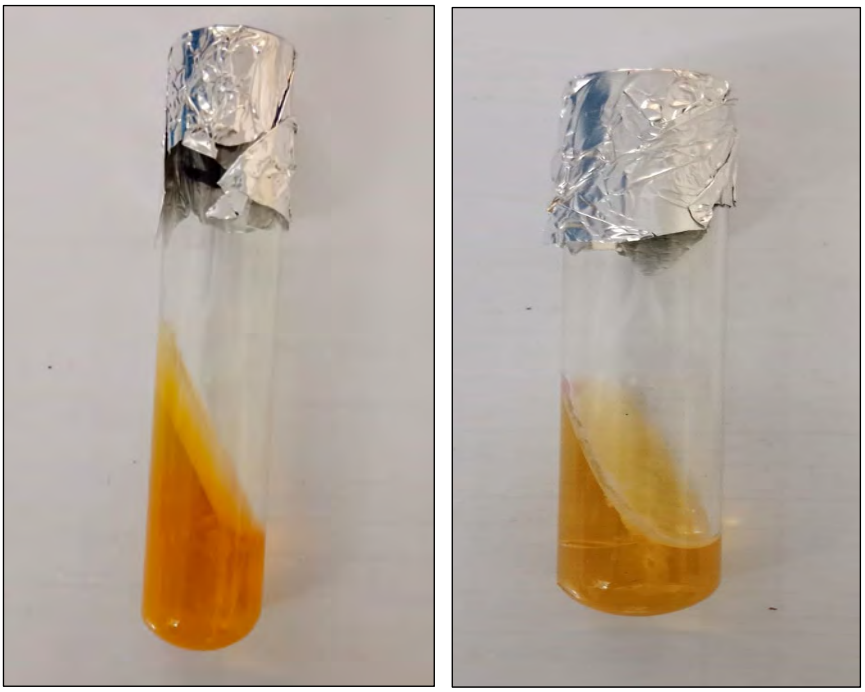

Figure 4.8: Represents E. coli results for the Triple Sugar Iron test

4.2.2.6 Simmons Citrate Test

The Simmons citrate test is used to determine the utilization of citrate and the capacity of bacteria that use citrate as a carbon source. The slants' color changed from green to

blue, suggesting that bacteria were using citrate as a carbon source. If the color remained the same, however, the organisms were not producing the citrate permease enzyme, which is necessary for citrate breakdown.

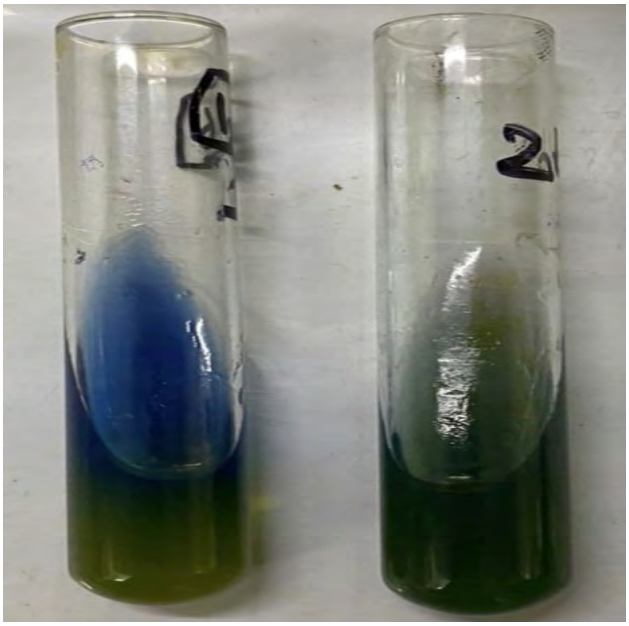

Figure 4.9: Represents E. coli results for the Citrate test

4.3 Stock Preservation

Following confirmation, all isolates of *E. coli* stock were prepared. And preserved in -20C^{o} in the labeled Eppendorf containing half of 40% glycerol and the other half of tryptic soy broth with 2 to 3 colonies from freshly prepared MacConkey agar plates. After that for any experiment, isolates were refreshed from the stock.

4.4 Antimicrobial Susceptibility Testing (AST) Profile

Kirby Bauer's “disc diffusion” test was performed to generate an antibiotic susceptibility profile. The result was interpreted by measuring the inhibition zone around the antibiotic discs. Bacteria were classified as sensitive, intermediate, and resistant which is based on the zone of inhibition by interpreting zone size provided in CLSI guidelines.

Table 4.3: Antibiotic Susceptibility Testing of *E. coli*

Class	**Antibiotics**	**Symbol**	**(µg)**	***E. coli* n%**		
				Sensitive	**Intermediate**	**Resistant**
Polymyxin class	Colistin Sulphate	CS	10ug	48.31%	0%	51.68%
Cephalosporin (Beta-lactams)	Cefepime	FEP	30ug	73.03%	4.49%	22.47%
	Cefixime	CFM	5ug	76.4%	0%	23.5%
Penicillin (Beta-Lactams)	Amoxicillin	AML	25ug	5.6%	1.1%	93.2%
Tetracycline	Doxycycline	DXT	30ug	29%	28%	43%
	Oxytetracycline	OA	30ug	0%	0%	100%
Fluoroquinolones	Enrofloxacin	ENR	5ug	11.23%	3.39%	85.39%
	Ciprofloxacin	CIP	5ug	11.23%	1.12%	87.64%
	Norfloxacin	NOR	10ug	15.7%	13.48%	70.78%
	Oxalinic Acid	OA	2ug	0%	0%	100%
Phenicol	Florfenicol	FFC	30ug	14.6%	3.37%	82.02%
Phosphonic Acid	Fosfomycin	FOS	200ug	60.6%	2.24%	37.07%

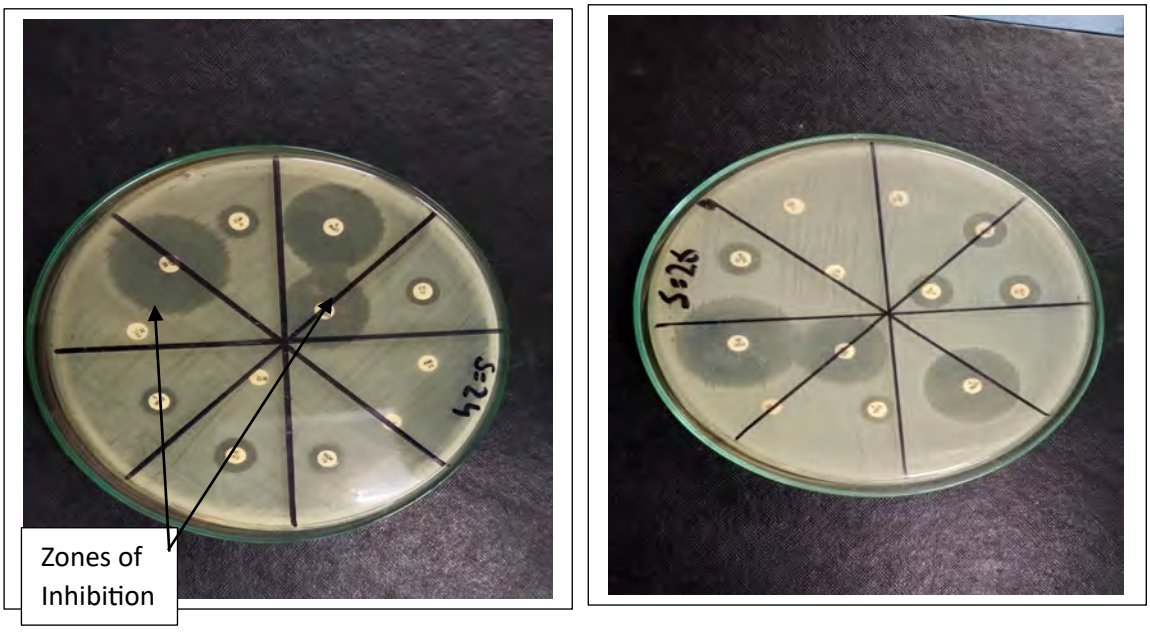

Figure 4.10: Represents Kirby Bauer (disc diffusion) test

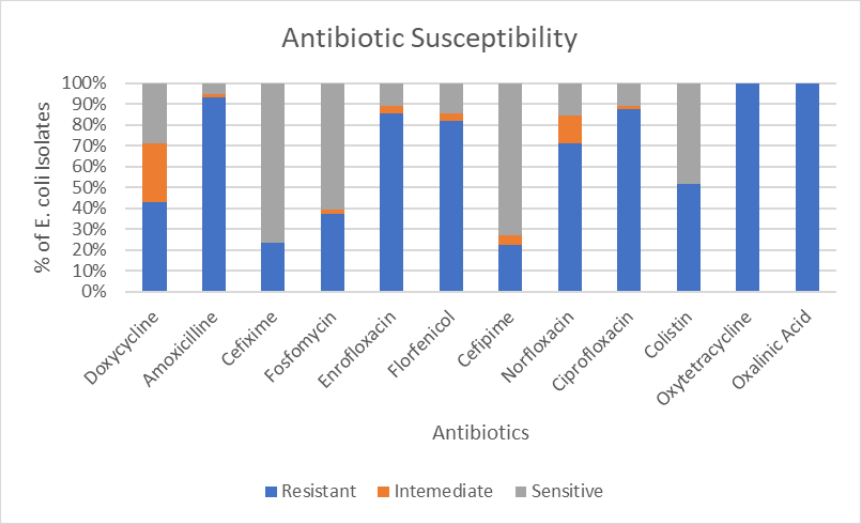

Figure 4.11: Represents Antimicrobial Susceptibility Pattern by Disc Diffusion Method

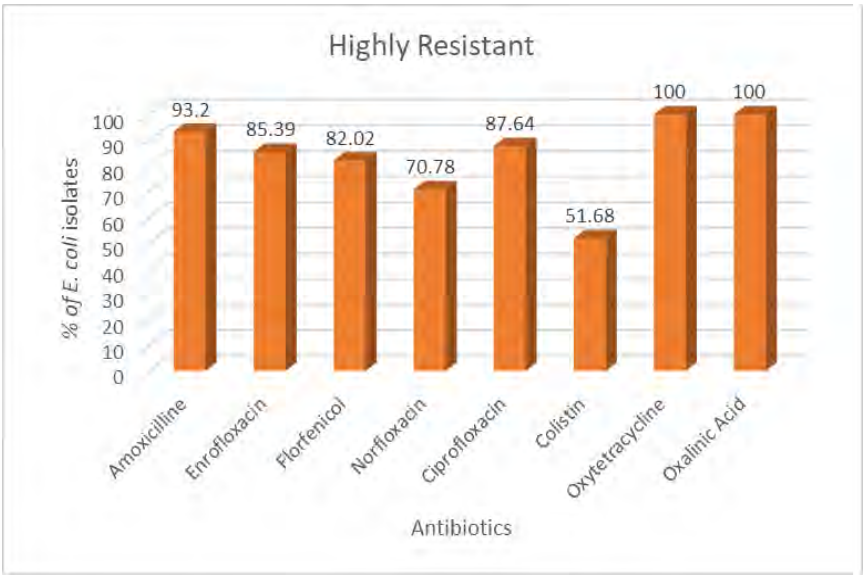

Figure 4.12: Represents a higher resistant pattern of *E. coli* against Amoxicillin**,** Enrofloxacin, Florfenicol, Norfloxacin, Ciprofloxacin, Colistin, Oxytetracycline, Oxalinic Acid.

4.5 Antibacterial Activity of Pine Essential Oil

The zone diameter was measured of the triplicates and the mean value was noted and observed. The results for the antibacterial activity of Pine Essential Oil were interpreted according to (S. Keeratirathawat et al., 2013) and (Vasireddy et al., 2018).

Table 4.4: Interpretation of Antibacterial Activity of Pine oil

Antibacterial Activity	**Weak**	**Moderate**	**Strong**	**Extreme Active**
Zone Diameter	<8mm	8-14mm	14-19mm	>19mm

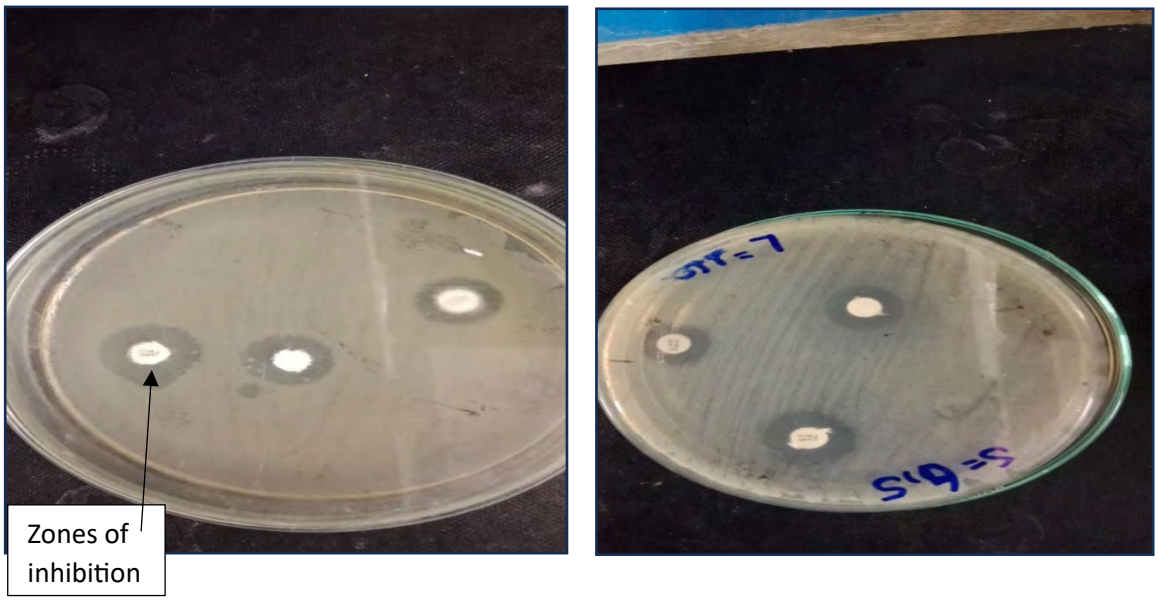

Figure 4.13: Represents Paper disc diffusion test of Pine Oil against *E. coli*

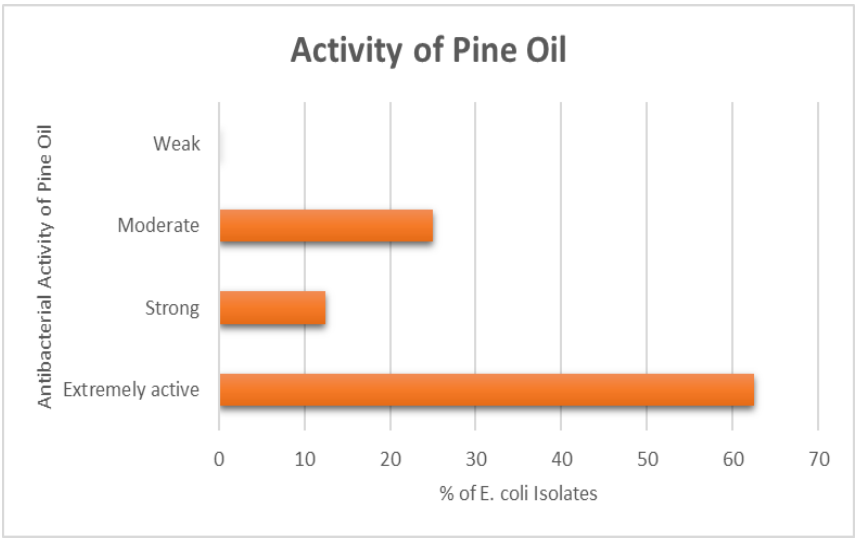

Figure 4.14: Graphical presentation of Pine Oil activity against *E. coli*

4.6 Biofilm Formation Assay by 96-well Microtiter Plate Method

Biofilm assay was performed to characterize the isolates as strong, weak, and moderate biofilm formers. The microtiter plate method is the most common and easiest method to put bacteria in these categories. By using this method, I have also characterized 89 isolates of *E. coli* as strong, moderate, weak biofilm former, and non-biofilm former. As mentioned in the methodology, (the OD) of each isolate was compared with the (OD) of negative control such as crystal violet (ODc) to rank these isolates into different categories. Results were based on OD values.

Table 4.5: Results of Biofilm Formation

***E. coli* Isolates**	**Strong Biofilm Former**	**Moderate Biofilm Former**	**Weak Biofilm Former**	**Non-Biofilm Former**
	0(0%)	1(1.12%)	12(13.42%)	76(85.39%)

Figure 4.15: Represents Microtiter Plate Biofilm formation assay

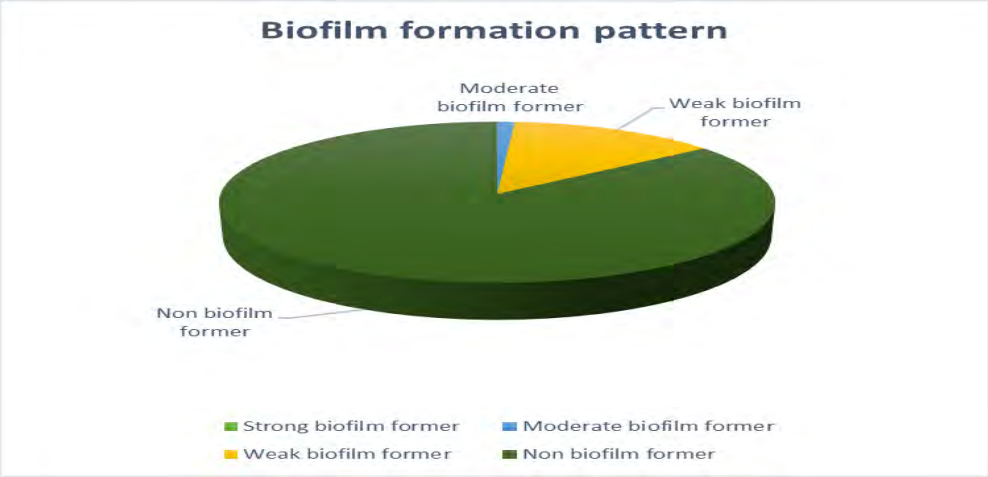

Figure 4.16: Graphical presentation of biofilm formation pattern of *E. coli* isolates

4.7 Minimum Inhibitory Concentration of Planktonic Cells (mic-p)

4.7.1 MIC through Microtiter Plate Method

The microtiter plate method is the easiest and most time-saving procedure to evaluate the minimum inhibitory concentration of antibiotics against tested strains. By using this methodology, we can perform MIC of seven isolates at one time due to which chances of human error are also decreased. MIC for Colistin, Oxytetracycline, Enrofloxacin, and Florfenicol against different MDR strains was evaluated through the microtiter plate method. Percentages of selected MDR isolates against their respective MIC values of the above-mentioned drugs were determined.

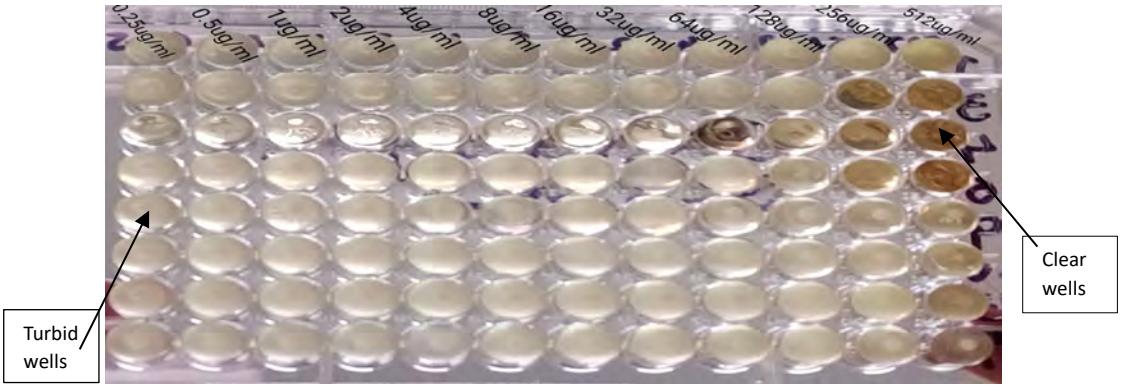

Figure 4.17: Represents MIC through a 96-well microtiter plate

Following MIC values are found after performing a microtiter plate against antibiotics

MIC values for Colistin

MIC Values	Number of Isolates
>512ug/ml	3
512ug/ml	4
256ug/ml	1
128ug/ml	3
64ug/ml	2
32ug/ml	4
16ug/ml	10
8ug/ml	10
4ug/ml	2
2ug/ml	6
1ug/ml	2

MIC values for Oxytetracycline

MIC Values	Number of Isolates
>512ug/ml	74
512ug/ml	7
256ug/ml	5
128ug/ml	1
32ug/ml	1
16ug/ml	1

MIC values for Enrofloxacin

MIC Values	Number of Isolates
>512ug/ml	15
512ug/ml	20

256ug/ml	7
128ug/ml	25
64ug/ml	8
32ug/ml	4

MIC values for Florfenicol

MIC Values	**Number of Isolates**
>512ug/ml	61
512ug/ml	10
256ug/ml	3
16ug/ml	1
8ug/ml	2

Table 4.6: Percentages of MDR isolates against their respective MIC values of Drugs

MIC (µg/ml)	**Colistin**	**Oxytetracycline**	**Enrofloxacin**	**Florfenicol**
>512	6.3%	83.1%	18.9%	79%
512	8.5%	7.8%	25.3%	12.9%
256	2.1%	5.6%	8.86%	3.8%
128	6.3%	1.1%	31.6%	
64	4.2%		10.1%	
32	8.5%	1.1%	5.6%	
16	21.2%	1.1%		1.2%
8	21.2%			2.5%
6				
4	4.2%			
2	12.7%			
1	4.2%			

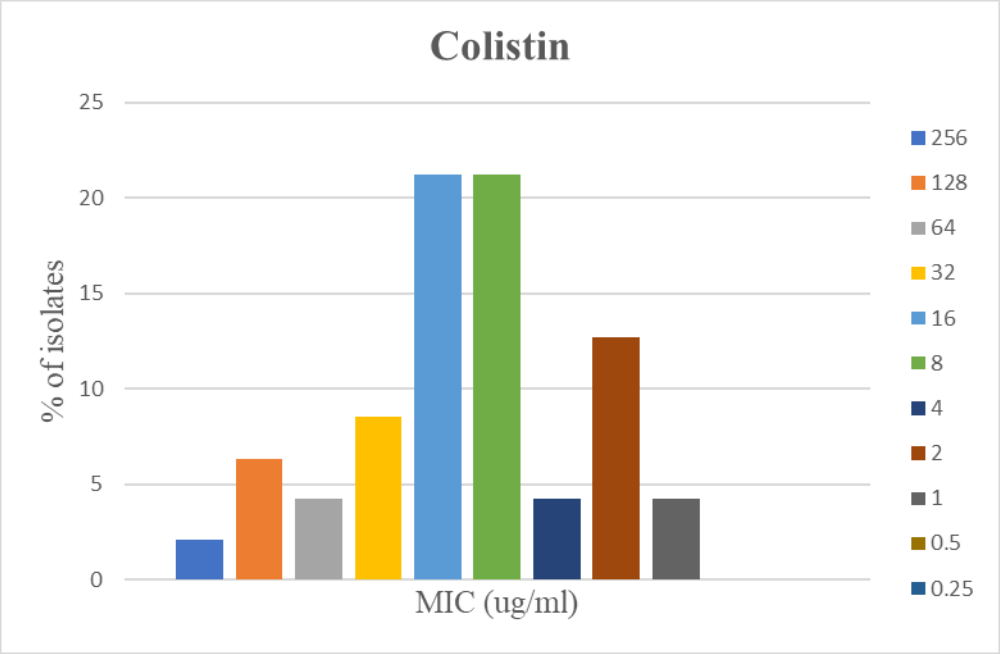

Figure 4.18: MIC values of Colistin through Graphical presentation

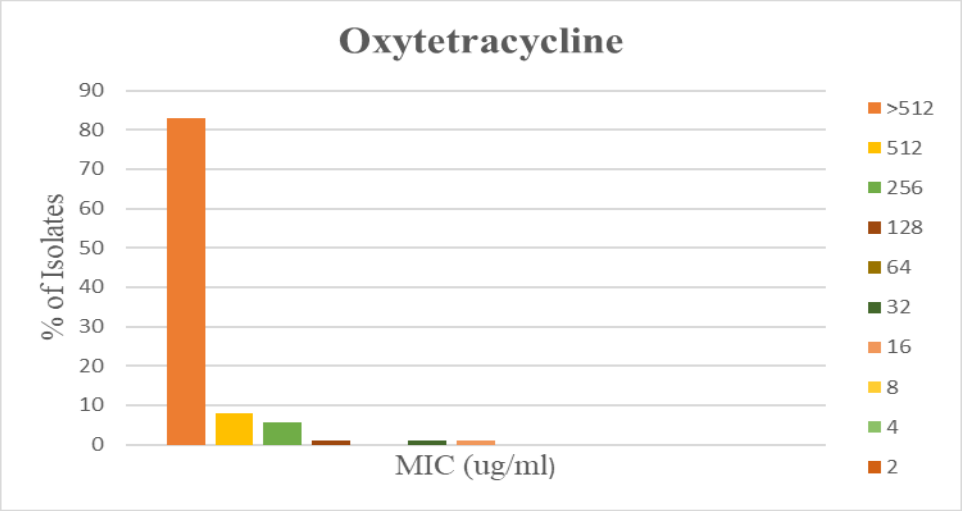

Figure 4.19: MIC values of Oxytetracycline through Graphical presentation

4.8 MIC and MBC of Pine essential Oil

Table 4.7: MIC and MBC values of Pine Oil against eight MDR isolates of *E. coli*

Strain. No	**MIC** **% (v/v)**	**MBC** **% (v/v)**
1	0.125	0.25
21	0.0625	0.125
7	0.25	0.5

21	<0.0625	0.0625
14	0.0625	0.125
19	0.0625	0.125
27	0.0625	0.125
29	0.0625	0.125

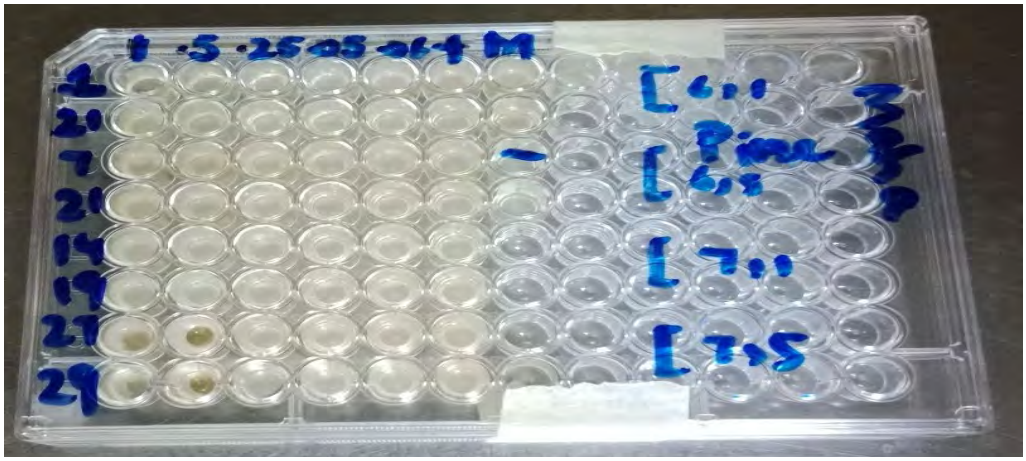

Figure 4.20: Represents MIC of Pine oil through the microtiter plate method

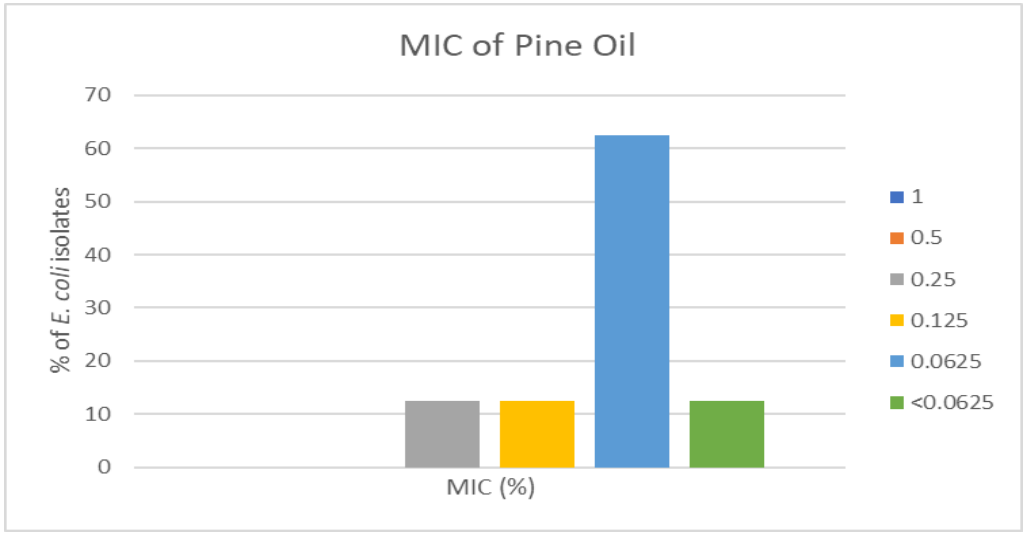

Figure 4.21: MIC values of Pine Oil through graphical presentation

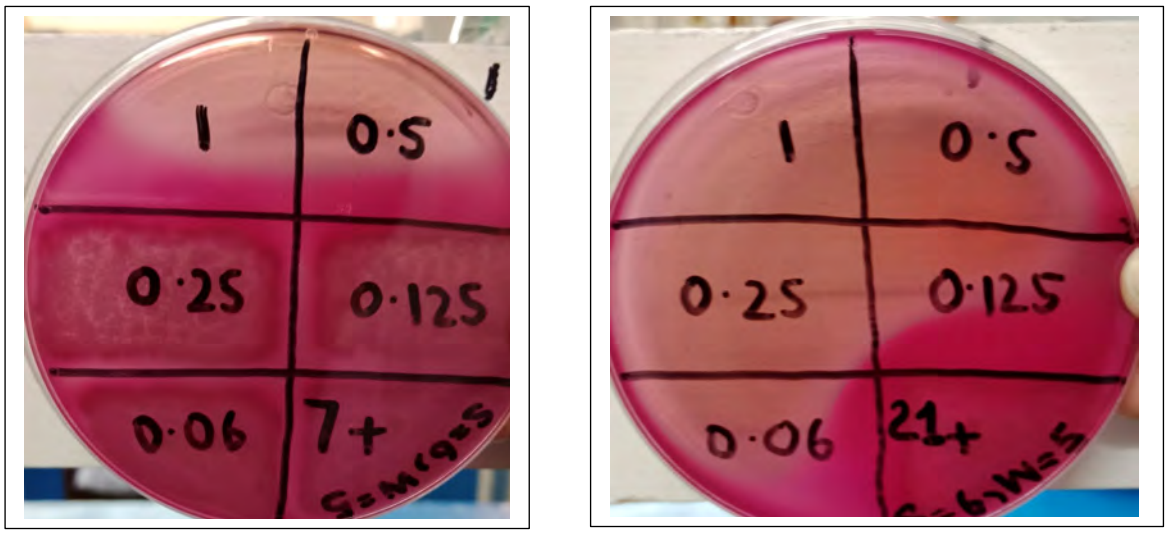

Figure 4.22: Represents MBC values of Pine oil on media plates

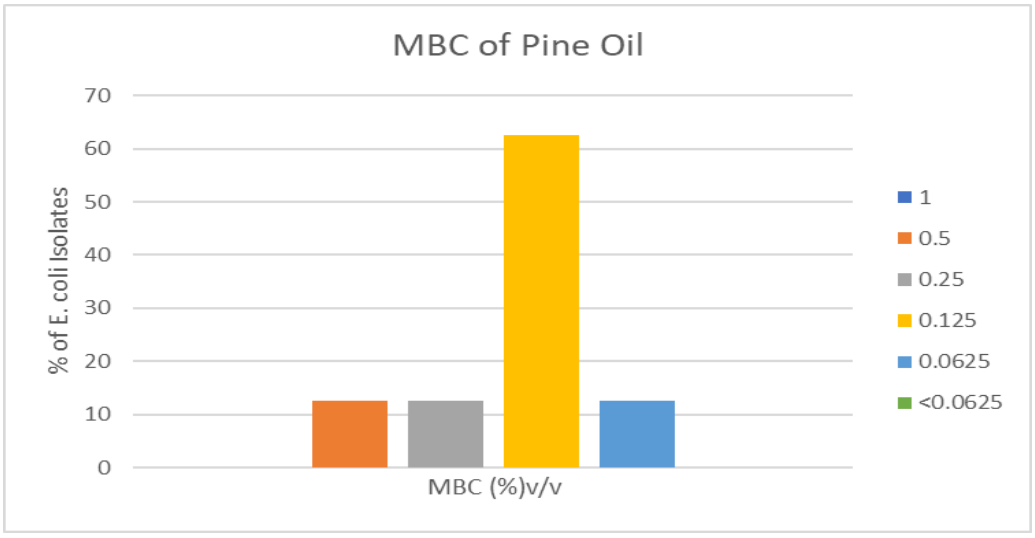

Figure 4.23: MBC values of Pine Oil through graphical presentation

4.9 Synergistic Antibiotic Assay (Pine Oil with Colistin)

A synergistic antibiotic assay was performed to assess the combinational effect of antibiotics with pine oil. So, in this study, Colistin was combined with Pine essential oil against colistin-resistant strains. The results of this combination were interpreted according to (Saquib et al., 2021).

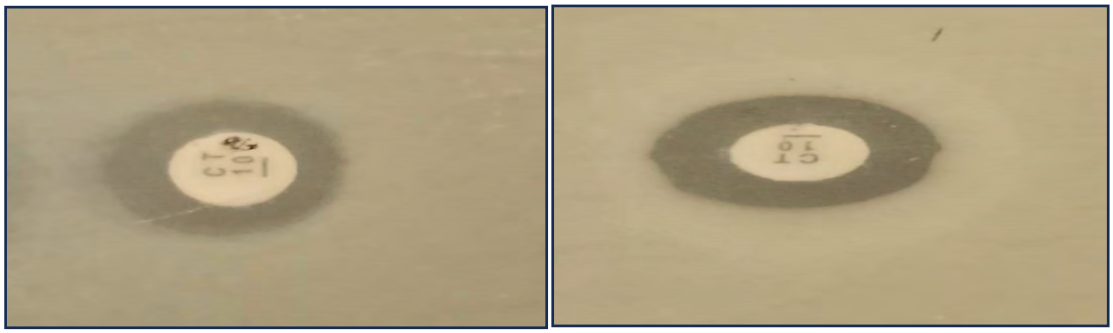

Figure 4.24: Represents the Combination of Colistin and Pine Oil against *E. coli*

Table 4.8: Results of the Combinational Effect of Colistin with Pine Oil

Strain. No	ZOP+ZOC	CZ	Combinational Effect
1	29.5mm	16.75mm	Antagonistic
21	36.5mm	20mm	Antagonistic
7	27mm	17.5mm	Antagonistic
21	29.75mm	16.5mm	Antagonistic
14	23mm	20mm	Antagonistic
19	20.5mm	13mm	Antagonistic
27	30.25mm	25.5mm	Antagonistic
29	24.5mm	21.5mm	Antagonistic

ZOP=zone of pine oil, ZOC=zone of colistin, CZ=combinational zone

4.10 Combination of antibiotics through Checkerboard Assay

To check the synergistic activity of antibiotic combinations in MDR isolates of *E. coli*, MIC of Colistin, and Oxytetracycline individually and in combination, a checkerboard assay was performed. Four *E. coli* isolates that were resistant to both drugs were tested by this method to check the combination of both drugs.

4.8.1 FICI INDEX VALUE

The FICI index value for the tested isolates is calculated by the sum of the FIC of drug A and the FIC of drug B, whereas the FIC value for each drug is calculated by dividing the MIC of drug A in combination with the MIC of drug A alone and the same for drug B. Based on this formula, results were interpreted for the combination of Colistin and Oxytetracycline. Four MDR strains were tested against this combination of drugs. Their combination shows a synergistic effect against all isolates.

Figure 4.25: Represents the microtiter plate for the combinational effect of Colistin and Oxytetracycline through checkerboard assay

Table 4.9: FICI values for Checkerboard Assay show different interactions against different isolates.

Isolate No	MIC of Drug A (Oxytetracycline) alone	MIC of Drug B (Colistin) alone	MIC of Drug A (Oxytetracycline) in combination	MIC of Drug B (Colistin) in combination	FICI Value Range	Combination effect
1	128	128	8	8	0.18	Synergistic
7	512	8	128	8	0.37	Synergistic
14	512	512	128	4	0.25	Synergistic
27	512	256	128	16	0.31	Synergistic

DISCUSSION

In our study, the total prevalence of *E. coli* in cloacal samples collected from chickens of a poultry farm in Pakistan, Punjab was recorded as 74.16% which is 10% more than the finding of (Sarker et al., 2019) reported the prevalence was 61.67%, whereas, research was directed on the isolation of *E. coli* from various chicken brands were 70%, 60%, 53.3%, and 50% which is dynamic to our study. (Crecencio et al., 2020). In a study performed in backward farming, cloacal samples generated 63.9% of *E. coli* isolates which showed a similar *E. coli* prevalence as mentioned in our study. (Al-Marri et al., 2021). A study conducted by (Noreen et al., 2022) presents the isolation rate of *E. coli* as 75%. The occurrence of *E. coli* found in a study held in Qatar was 52% (Eltai et al., 2017). The variability percentage (%) of *E. coli* may be attributed to varying geographical climate conditions.

Our study represented that 100% of the *E. coli* isolates were MDR. Similar findings were reported on 100% MDR isolates of *E. coli* from diverse studies performed in different countries (Sarker et al., 2019). Another study published a high percentage of MDR *E. coli* strains (99%) isolated from poultry fecal samples that almost matched our findings (Abo-Amer et al., 2018). Overall, 100% of the isolates showed resistance to at least one drug with 100% being resistant to at least three different classes of antimicrobials. A study of backward farming revealed only 34.9% of isolates as MDR strains. (Al-Marri et al., 2021). 33% MDR strains of *E. coli* were found in a study conducted in Qatar. (Eltai et al., 2017). The reason for this is the prevalent practice of utilizing antibiotics as preventative measures and growth stimulants in the poultry sector.

An international problem is the development of resistance in bacteria to fluoroquinolones like ciprofloxacin, Oxalinic acid, and Norfloxacin. In our study, all the tested E. coli isolates were 100% resistant to Oxytetracycline and Oxalinic acid followed by Amoxicillin (93.2%), Ciprofloxacin (87.64%), Enrofloxacin (85.39%), Florfenicol (82.02%), and Norfloxacin (70.78%). Antibiotics belonging to the same class cause cross-resistance, which further results in the development of high resistance rates. The broad application of these antibiotics to livestock is the reason for the development of resistance to them. According to a study conducted on ESBL- *E. coli* isolated from poultry accused of colistin resistance from poultry areas of Bangladesh

in 2017 to 2018 reported that 94% of *E. coli* were phenotypically resistant to colistin. As the plasmid-mediated colistin resistance mcr-1 has developed in bacteria that are already resistant to numerous antibiotics, this essential last-resort medication is losing its efficacy. But in our study, the colistin-resistant strains were 51.6% evaluated, and a similar finding to this was the resistant pattern of fluoroquinolones but there was a contrast to their finding was a resistant pattern of cephalosporins because according to our findings, very few strains were resistant to them while others were sensitive to the two cephalosporin antibiotics used in our study. (Amin et al., 2020). A study conducted in Saudi Arabia in 2018, showed 97% of resistant *E. coli* strains to Oxytetracycline from poultry nearest to our findings while contrast to the resistant pattern of Ciprofloxacin having only 59% resistant strains (Abo-Amer et al., 2018)

In 2020, a study was conducted on poultry chicken in Bangladesh which resulted in 100 percent resistant strains of *E. coli* against oxytetracycline and also revealed that all *E. coli* isolates that are resistant to Oxytetracycline include the tetA gene, which indicates that this gene is primarily responsible for Oxytetracycline resistance and may have been passed from the resistant strain to the susceptible one. similar to the results analyzed in our study which evaluate that all the strains of *E. coli* were resistant to Oxytetracycline. (Avijit Kumar Das et al., 2020)

According to the findings of this study, Fluoroquinolones such as Enrofloxacin, Ciprofloxacin, Norfloxacin, Oxytetracycline, and Oxalinic Acid, a common first-line antibiotic used for empiric therapy of severe infections, are no longer effective against MDR E. coli in poultry. As a result, there are relatively few antimicrobial drugs such as Cefepime, Cefixime, Fosfomycin, and Doxycycline that are consistently effective against the majority of MDR *E. coli* discovered in our investigation, which means that treating illnesses caused by these bacteria will continue to be difficult for veterinarians. This predicts negative clinical results in the production of chicken, leading to serious health and welfare issues and financial losses. Therefore, this study focused on improving the efficiency of drugs that are already in use by veterinary animals or developing some natural alternatives to drugs for the treatment of resistant pathogens along with some precautions such as the implementation of biosecurity measures, appropriate vaccination regimens, and the prudent use of antibiotics are becoming requirements in the chicken production pyramid. As mentioned in a prior study to limit

the usage of antibiotics in animals raised for food, it is also necessary to value current antibiotic alternatives, such as plant extracts and essential oils, and to continuously develop new ones. (Benklaouz et al., 2020)

A study conducted in Qatar presented that E. coli resistance against Colistin was 15.5%, 40% resistance against Ciprofloxacin, and Fosfomycin shows 3.33% resistance only. The resistant pattern of this study is very low as compared to our study (Eltai et al., 2017). The results of the study conducted in China in 2017 from poultry where the resistance rate of quinolones such as Ciprofloxacin and Enrofloxacin was over 50% similar to our finding showed the highest resistance of *E. coli* against the quinolone class of antibiotics showing that changes to molecular targets such as DNA gyrase and topoisomerase IV, result in the primary mechanisms of quinolone resistance being chromosome-encoded. (Yassin et al., 2017). A study conducted in 2022 on retail chicken meat showed a resistance pattern of *E. coli* as 90% Oxytetracycline, 90% Amoxicillin, 61% Ciprofloxacin, 60% Doxycycline, 26% Colistin whereas, this study finds 100%, 93.2%, 87.64%, 43%, 51.68% resistance respectively lower resistant pattern in cefepime 5%, and cefixime 7% as compared to our study which shows 22.47%, 23.5% resistance respectively. (Noreen et al., 2022). A previous study reported 100% resistant *E. coli* strains against Colistin, Doxycycline 63.63%, and Cefixime 80.51% which contrast our study's outcomes and shows a high rate of resistance to these three drugs. (Singh et al., 2019). The varying usage of antibiotics may be the reason for the differing rates of antibiotic resistance patterns in *E. coli*. The high levels of resistance are not surprising given the frequent use of these drugs in veterinary medicine. Unfortunately, farmers are not adhering to proper guidelines when administering antibiotics.

The MICs of antibiotics against studied MDR strains demonstrated a high level of resistance is worrying. This may be the result of unrestricted practice of antibiotics in chicken farms, where entire flocks are treated instead of individual birds, or the lack of testing for in vitro antimicrobial susceptibility before administering the antibiotics. Our study revealed that 39 isolates show resistance to Colistin (MIC≥4µg/ml) with a percentage of 82.9% which is very high compared to a study that mentioned 25% Colistin-resistant strains (Badr et al., 2022). The MIC of Colistin in this study ranges from 1µg/ml to 512µg/ml while a previous study reported 4µg/ml to ≥256µg/ml are

somehow matches our results showing an almost resistant range of Colistin. (Banik& Shamsuzzaman.,2021). A study conducted in 2014 reported 0% resistance against Colistin and Florfenicol. (Adelowo et al., 2014). The 100% resistivity recorded against Enrofloxacin in a study performed in Qatar, matched our study and also revealed 100% resistance of *E. coli* against Enrofloxacin. (Eltai et al., 2017).

A study conducted in 2016 in China reported 25.39% strong biofilm, 31.25% moderate biofilm, 28.90% weak biofilm, and 18.36% non-biofilm forming isolates (Wang et al., 2016) compared to our study presenting strong, moderate, weak, and non-biofilm former as 0%, 1.12%, 13.48%, and 85.39% respectively. A study conducted in 2017 mentioned 70% of resistant *E. coli* isolates can form biofilm but, in our case, only 14.6% of MDR were able to form biofilm. (Pavlickova et al., 2017). The result of biofilm-forming *E. coli* from a backward poultry farm was 66.3%, which is greater than our finding. (Al-Marri et al., 2021). In contrast, our study a study conducted by (Noreen et al., 2022) showed 29% strong, 37% moderate, and 34% weak biofilm former *E. coli* isolates.

A significant public health issue is the spread of microorganisms that are multi-drug resistant in the environment such as animals as well as humans. There is a dire need to find innovative approaches to combat the issue of antibiotic resistance, particularly in Gram-negative bacteria that currently prevent the use of many medications. As a result, some researchers are concentrating on natural products made from unusual sources, such as plants. (Zhou et al., 2014). The major finding of our study was, that the Colistin-resistant strains that were exposed to Pine oil showed (62.5% extremely active), (12.5% strong), (and 25% moderate) activity while resistant to almost three classes of antibiotics. This may be due to the difference in modes of action of various compounds that constitute Pine Oil, to which the organism was never exposed before and hence never had a chance to develop resistance against them while the bacteria such as *E. coli* isolated from poultry were many times exposed to these antibiotics due their extensive use in poultry feed and therapeutic usage, resulting in the development of MDR strains of *E. coli* found in the fecal material of poultry chickens. The antibiotic properties of Pine essential Oil were superior as compared to different antibiotics used in poultry for those selected MDR strains of *E. coli* bacteria from the cloaca of chickens. Compared to the previous report published that the pine essential oil shows moderate antibacterial

activity against *E. coli* from human regions while our study represents the extremely high antimicrobial activity of Pine oil against MDR strains of *E. coli* isolated from poultry but one similar part of both of the research was inhibition of growth of *E. coli* by Pine oil. (Dzaferovic, 2017)

Our study revealed the strongest activity of Pine essential oil against gram-negative bacteria as 62.5% isolates show (MIC=0.0625%), 12.5% isolates show (MIC=0.125%), and 12.5% isolates show (MIC≤0.0625%) which is considered as the strongest activity of essential oils against gram-negative bacteria as shown in a study conducted in 2015 to evaluate the effects of some essential oils other than Pine oil which is a contrast to our study against foodborne pathogens (El-Shenawy et al., 2015), this may be due the less interaction of bacteria with Pine oil which results in less or no resistant against this natural antibiotic or it may be due to the diverse antibacterial action of essential oils that may be the result of their complex chemical structure. Our results showed that selected Pine essential oil exhibited different antimicrobial activity against the tested MDR strains of *E. coli* isolated from poultry as compared to previous studies. It may be a better option to treat the resistant pathogens of poultry, especially *E. coli* but after more investigations and studies regarding the in-vivo application of pine oil against *E. coli* and use at a batch level in limited and controlled quantities.

According to a report, a combination of two drugs shows effective synergy if the tested bacteria is resistant to at least one of them. (Haroun & Al-Kayali, 2016), as shown in the current study. Before entering the post-antibiotic era, we must develop novel approaches to combat antibiotic resistance in *E. coli*, especially those related to poultry environments. Combinational approaches to antibiotic usage provide a wider range of pharmacological therapy. (Zhou et al., 2014) because we did not have much time to wait for the development of new drugs. Combinations of antibiotics are frequently employed in medicine to broaden the antimicrobial susceptibility range and provide synergistic effects. To the best of our knowledge, this therapy has been successful in treating MDR bacteria. As mentioned in this study 4 isolates were tested for the combination of Colistin and Oxytetracycline, and these all showed synergistic effects against *E. coli*. Colistin, a cationic peptide that breaks bacterial cell membranes, may combine with other antimicrobials to increase their effectiveness whereas, Oxytetracycline inhibits growth by inhibiting translation. (Olszewska.,2006) In a

previous study colistin combination with imipenem, shows synergy against 3 of the 4 tested MDR *E. coli* strains and 1 gives the additive effect (Banik& Shamsuzzaman.,2021). Our findings of the synergistic effect of Colistin with Oxytetracycline constitute an alternative and promising therapeutic option for the cure of MDR strains of *E. coli* isolated from poultry.

The outcomes of our study present that poultry-related *E. coli* shows 100 percent antagonistic behavior for the combination of Colistin and Pine essential oil while 100 percent synergistic effect for the combination of Colistin with Oxytetracycline by using different methodologies such as disc diffusion assay and checkerboard microdilution assay respectively. However, the research regarding this combinational effect is very limited.

Conclusion

E. coli is a major challenge for the commercial poultry industry, causing colibacillosis and high mortality rates. To combat antibiotic resistance, new drug combinations and natural sources like essential oils should be used. In our study, 74% of isolates were identified as *E. coli* strains. All *E. coli* strains showed different patterns of susceptibility toward different antibiotics. The MDR prevalence rate was 100%. In this way, the MIC values were high. The main objective of this research was to investigate the antibacterial properties of Pine oil in combination with Colistin. The present study shows the positive antibacterial activity of Pine oil against *E. coli*. Moreover, Pine oil shows the highest activity with the lowest MIC and MBC values. The synergy test performed for the combination of Colistin with Pine oil shows 100% Antagonistic behavior while the combination of Colistin with Oxytetracycline presents 100% Synergistic behavior against *E. coli*. The study proposed the potential of utilizing the aforementioned synergistic approach of Colistin and Oxytetracycline for combating infections caused by *E. coli*. These findings indicate the need for immediate action to maintain antibiotic efficacy and lower the likelihood of the spread of highly resistant *E. coli*. Further research will find the best combinations of antibiotics with essential oils to overcome the difficulties of the post-antibiotic era.

Future Outlook

Future research will be needed to elaborate on the following implications of this work:

- It will be important to use a combination of Colistin and Oxytetracycline on animal models in future studies
- In the future, we need to create new combinations of antibiotics that are already being used in poultry. In the future, the effect of Pine oil can be investigated on other pathogens related to poultry
- The application of antibiotics, nanoparticles, antimicrobial peptides, and plant extracts such as essential oils other than pine oil, in combination with antibiotics, should be explored to combat the threat raised by infections caused by MDR *E. coli* strains.
- Molecular approaches should also be applied to find out the resistant genes in *E. coli* of poultry regions to evaluate the prevalence of their resistance to other bacteria and the human environment in the future.

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