UNIVERSITI PUTRA MALAYSIA

EVALUATION OF *IN VITRO* ANTIBACTERIAL AND SYNERGISTIC EFFECT OF GARLIC EXTRACT WITH ENROFLOXACIN AGAINST *ESCHERICHIA COLI*

RAVENIA SURESH

EVALUATION OF *IN VITRO* ANTIBACTERIAL AND SYNERGISTIC EFFECT OF GARLIC EXTRACT WITH ENROFLOXACIN AGAINST *ESCHERICHIA COLI*

RAVENIA SURESH

A project submitted to the
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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Evaluation of *in vitro* Antibacterial and Synergistic Effect of Garlic Extract against *Escherichia coli*", by Ravenia Suresh and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4901- Project.

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PROF. MADYA DR. ARIFAH BINTI ABDUL KADIR
DVM (UPM), PhD (UNIVERSITY OF LONDON),
Lecturer,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Supervisor)

____

DR. SHARINA BINTI OMAR
DVM (UPM), MVS (Massey University), PhD (University of Leicester),
Lecturer,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Co-Supervisor)

____

DR. NIK MOHD FAIZ NIK MOHD AZMI
DVM (UPM), MVS (UPM), PhD (NC State University)
Lecturer,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Co-Supervisor)

DEDICATION

I would like to dedicate this project to my late grandmother and my pet, Kiki.

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LIST OF ABBREVIATIONS

°C	=	degree Celsius
µg	=	microgram
g	=	gram
mm	=	millimeter
ml	=	milliliter
µl	=	microlitre
%	=	percentage
CFU	=	colony forming unit
CFU/ml	=	colony forming unit per milliliter
E.coli	=	*Escherichia coli*
Spp.	=	species
AGE	=	aqueous garlic extract
S. aureus	=	*Staphylococcus aureus*
FICI	=	fractional inhibitory concentration index

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4901- Projek

PENILAIAN *IN VITRO* ANTIBAKTERIA DAN KESAN SINERGISTIK EKSTRAK BAWANG PUTIH DENGAN ENROFLOXACIN TERHADAP *ESCHERICHIA COLI*

Oleh

Ravenia Suresh

Penyelia : Prof. Madya Dr. Arifah Abdul Kadir

Pembantu penyelia : Dr. Sharina Omar dan Dr. Nik Mohd Faiz Nik Mohd Azmi

Adalah amat penting untuk mencari sumber rawatan alternatif baharu dengan segera memandangkan perkembangan rintangan antimikrobial semakin meningkat dalam industri ayam. Bawang putih (*Allium sativum*) menunjukan pelbagai sifat antimikrob dan telah lama digunakan sebagai ubat tradisional pada manusia. Walau bagaimanapun, penggunaan tumbuhan ubatan dalam sektor penternakan agak terhad terutamanya di Malaysia. Kajian ini bertujuan untuk menyiasat aktiviti antimikrob dan kesan sinergistik ekstrak bawang putih apabila digunakan dalam kombinasi dengan enrofloxacin terhadap *Escherichia coli*. Sepuluh sampel najis ayam telah diperoleh dan disalut pada media terpilih dan pembezaan. *Escherichia coli* telah dikenal pasti melalui kaedah mikrobiologi piawai (n=4). Empat pencilan klinikal dan satu strain rujukan (ATCC 25922) *Escherichia*

Coli digunakan selanjutnya dalam kajian ini. Aktiviti antibakteria ekstrak bawang putih dan enrofloxacin telah disaring menggunakan kaedah *disc difusion* dan *Minimum Inhibitory Concentration* (MIC) mereka ditentukan dengan kaedah *broth microdilution*. Keberkesanan gabungan enrofloxacin dan ekstrak bawang putih telah diuji menggunakan kaedah penyebaran cakera dan dianalisis dengan statistik. Keputusan menunjukkan bahawa *Escherichia coli* adalah sensitif kepada ekstrak bawang putih dalam semua pencilan. Nilai MIC ekstrak bawang putih dalam sampel klinikal adalah dalam julat 12.5%-25% manakala *Minimum Bactericidal Concentration* (MBC) untuk ekstrak bawang putih ialah 25-50%. Kesan sinergistik ekstrak bawang putih telah diperhatikan dalam gabungan enrofloxacin pada sampel. Zon perencatan meningkat dengan ketara dalam kombinasi ekstrak bawang putih dengan enrofloxacin. Kesimpulannya, bawang putih mempamerkan sifat antibakteria yang kuat terhadap *Escherichia coli* dan sangat cekap dalam meningkatkan aktiviti antibakteria enrofloxacin.

Kata kunci: bawang putih; enrofloxacin; *Escherichia coli*; *Minimum Inhibitory Concentration* (MIC); *Minimum Bactericidal Concentration* (MBC)

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4901- Project.

EVALUATION OF *IN VITRO* ANTIBACTERIAL AND SYNERGISTIC EFFECT OF GARLIC EXTRACT WITH ENROFLOXACIN AGAINST *ESCHERICHIA COLI*

By

Ravenia Suresh

Supervisor: Associate Professor Dr. Arifah Abdul Kadir

Co-Supervisor: Dr. Sharina Omar and Dr. Nik Mohd Faiz Nik Mohd Azmi

It is of utmost importance to search for new alternative sources of treatment quickly as the development of antimicrobial resistance is on the rise in the poultry industry. Garlic (*Allium sativum*) exhibits a wide range of antimicrobial properties and has been long used as traditional medicine in humans. However, the use of medicinal plants for the animal husbandry sector is rather scarce especially in Malaysia. This study aimed to investigate the antimicrobial activity and synergistic effect of garlic extract when used in combination with enrofloxacin against *Escherichia coli.* Ten fresh chicken fecal samples were obtained and plated on selective and differential media. *Escherichia coli* were identified by standard microbiological method (n=4). These four clinical isolates and one reference strain (ATCC 25922) of *Escherichia Coli* were further used in this study. The antibacterial activity of garlic extract and enrofloxacin were screened using disk diffusion method and their minimum inhibitory concentration (MIC) was determined by broth microdilution method. Combined efficacy of enrofloxacin and garlic extract was tested using disk diffusion method and was analyzed statistically. Results show that *Escherichia coli* was sensitive to garlic extract in all isolates. The MIC value of garlic extract in the clinical samples was in the range of 12.5%-25% while minimum bactericidal concentration (MBC) value for garlic extract was 25-50%. The synergistic effects of garlic extract were observed in the combination of enrofloxacin on the samples. The zone of inhibitions were significantly increased in combination of garlic extract with enrofloxacin. In conclusion, the garlic exhibits strong antibacterial properties against *Escherichia coli* and is highly efficient in enhancing the antibacterial activity of enrofloxacin.

Keywords: garlic; enrofloxacin; *Escherichia coli*; minimum inhibitory concentration (MIC); minimum bactericidal concentration (MBC)

1.0 INTRODUCTION

Antimicrobial resistance (AMR) has grown significantly over the years, and Malaysia is no exception. The rapid emergence and spread of AMR present a serious challenge in healthcare systems and threaten the ability of antibiotics to effectively treat severe infections and pose a hazard to people and livestock. The excessive reliance on antibiotics has contributed to favorable conditions for selection, persistence and spread of AMR bacteria (Barbosa & Levy, 2000). The main drivers of AMR include the misuse and overuse either in humans or in food animals such as poultry and livestock. Spread of residue of these antibiotics in soils, crops and water also worsens the situation. In Malaysia, 81% of *Escherichia coli* that has been isolated showed multidrug resistance (MDR) (Roseliza *et al*, 2016). Besides that, in the year 2021, 51.8% of *E. coli* were isolated from poultry litter, 100% *E. coli* showed multidrug resistance. With this trend it demonstrates that *E.coli* is resistant to many antibiotics and it is an alarming situation to the nation.

Due to antimicrobial resistance and the public health risk of antibiotic growth promoters, finding viable alternatives for sustainable chicken production is critical (Muhammad Abdul Basit *et al*, 2019) This is due to poultry flocks are frequently reared under harsh circumstances, with huge doses of antimicrobials used to prevent and treat disease as well as promote growth. Antimicrobial-resistant poultry infections can cause treatment failure, resulting in financial crisis, but they can also be a source of resistant bacteria that can be harmful to humans. In order to battle bacterial infections, novel antibacterial compounds are being sought after due to the alarming rise in bacteria that are resistant to antibacterial and the challenges associated with their treatment. Consequently, due to their extensive history of usage in traditional medicine for the treatment of infectious disorders, medicinal plants have emerged as a viable new source of antibacterial compounds. Plant-derived substances may have direct antibacterial effects or indirect effects by altering antibacterial resistance, which when combined with antibiotics enhances their efficacy (Stefanovic, 2012). Garlic (*Allium sativum*) is a herbal plant that exhibits a wide range of antimicrobial properties and has been long used as traditional medicine in humans and many *in vitro* studies have been conducted on gram positive bacteria (Leyla, *et al.*, 2014).

However, in Malaysia the use of herbal plants in the poultry industry is still lacking and little is known about the potential of herbal plants to improve the susceptibility of *Escherichia coli* to antibiotics. Besides, the most previous study has only focused on the antimicrobial activities of garlic and its derived organosulfur compounds; allicin with other antibiotics such as gentamicin, neomycin and penicillin G and has not been tested with enrofloxacin. Therefore the objective of this study was to determine the antimicrobial activity and synergistic effect of garlic extract when used in combination with enrofloxacin against *E.coli.*

1.1 Hypothesis

a) Null hypothesis: There is no significant difference between the antimicrobial activity of garlic extract when used in combination with enrofloxacin against *Escherichia coli*.

b) Alternative hypothesis: There is a significant difference between the antimicrobial activity of garlic extract when used in combination with enrofloxacin against *Escherichia coli*.

2.0 LITERATURE REVIEW

2.1 Garlic (*Allium sativum L.*)

Garlic, *Allium sativum L.*, a member of the Alliaceae family, has long been valued as a valuable spice and popular treatment for a variety of diseases and physiological problems. It is also known as the king of medicinal plants. The name garlic may have originated from the Celtic word 'all' meaning pungent. Garlic appears to have originated in central Asia and moved to China, the Near East, and the Mediterranean region before spreading west to Central and Southern Europe, Northern Africa (Egypt), and Mexico. Garlic has been used medicinally for thousands of years. Sanskrit records show its medicinal use about 5,000 years ago, and it has been used for at least 3,000 years in Chinese medicine. The Egyptians, Babylonians, Greeks, and Romans used garlic for healing purposes (Tattelman, 2005). Besides, it has antibacterial, antiviral, antifungal and antiprotozoal properties (Ankri, *et al*., 1999). Moreover, it boosts the immune system, improves body weight gain, and heightens the digestibility. It is declared that garlic as an antibacterial agent is effective against bacteria (Gongcagul, *et al.*, 2010).

Garlic contains at least 33 sulfur compounds, several enzymes, 17 amino acids, and minerals such as selenium. It contains a higher concentration of sulfur compounds than any other Allium species. The sulfur compounds are responsible both for garlic's pungent odor and many of its medicinal effects. Allicin (allyl 2-propenethiosulfinate or diallyl thiosulfinate) is the principal bioactive compound present in the aqueous extract of garlic or raw garlic homogenate (Londhe, et al., 2011). Allicins are responsible for most of garlic's pharmacological activities (Stoll , 1948). When garlic is chopped or crushed, alliinase enzyme is activated and produces allicin from alliin.

2.2 Antimicrobial activity of garlic

This is due to the presence of Allicin, an organosulfur chemical which is one of the active ingredient of garlic. Some of garlic's medicinal properties could be explained by a molecular mechanism. This is made from the nonprotein amino acid alliin by the enzyme alliinase, which catalyses the conversion of alliin to allicin. This only happens when garlic cloves are crushed or broken, and it is also responsible for the intense scent of garlic. Stoll and Seeback, 1948 stated that allicin has antibacterial action and is thought to be responsible for the majority of garlic's pharmacological properties. Using allicin, garlic's major physiologically active component, the researchers were able to analyse how garlic operates at the molecular level.

Garlic is a natural antibiotic that prevents amoebic dysentery by inhibiting two kinds of enzymes: cysteine proteinases and alcohol dehydrogenases. Infection is mostly caused by cysteine proteinases enzymes, which provide infectious organisms with the ability to harm and penetrate tissues. Alcohol dehydrogenase enzymes are critical for the survival and metabolism of these dangerous organisms. As a result, groups of enzymes can be found in a wide range of infectious organisms, including bacteria, fungi, and viruses. This study gives scientific support for the idea that allicin is a broad-spectrum antibacterial capable of preventing various sorts of illnesses. Because this would require altering the enzymes that enable their action, it is conceivable that bacteria would evolve resistance to allicin. Allicin inhibits the enzymes by reacting with one of their crucial components, self-hydyl (SH) groups, or thiols, according to research. Since sulfhydryl groups are an essential part of various enzymes involved in the manufacture of cholesterol, the conclusion that garlic lowers bad cholesterol. (Onanuga, *et al.*, 2011; Sovova, *et al.*, 2004; Chauhan, *et al.*, 2006; Groppo. *et al.,* 2007).

2.3 *Escherichia coli*

Escherichia coli is a gram negative, non-spore forming bacillus and it is a common inhabitant in the intestinal tract of poultry (Francesco Castellone, 2020). Though many E. coli are not harmful, some have gained virulence factors, increasing their pathogenicity significantly. The majority of cases of colibacillosis appear to be due to *E coli* that have acquired a number of virulence genes clustered together in plasmid-borne pathogenicity islands (PAIs). These PAI-containing plasmids are said to be the defining feature of the APEC pathotype . Other cases are due to infection with commensal *E coli* that gain access to birds weakened by some predisposing condition such as mycoplasmosis, infectious bronchitis, Newcastle disease, haemorrhagic enteritis, turkey bordetellosis, poor air quality, or other environmental stresses (Nolan, 2019). Besides, pathogenic *E. coli* from animals, birds and humans can cause a variety of diseases, ranging from self-limiting gastrointestinal infections to bacteremia. Antimicrobial agents have been used for various veterinary and agricultural purposes, including animal husbandry and poultry production where poultry feed is supplemented with antibiotics (Chah, *et al.*, 2001). Moreover, antibiotics are widely utilized to control infectious illnesses and as growth promoters in poultry production. Application of antimicrobials and their misuse is considered to be the most important selecting influence for the spread of antimicrobial resistance in bacteria both in human and veterinary medicine (Moreno, *et al*., 2000). Indeed, antimicrobial resistance developed in pathogens colonizing animals can cause the emergence and distribution of resistant *E. coli* that are subsequently transmitted to humans by contact with infected animals or derived products (Schroeder, *et al.*, 2002). Even wild migrating and resident birds can act as carriers and transmitters of multidrug-resistant (MDR) *E. coli* and *Escherichia vulneris* (Shobrak, *et al*., 2014). MDR but non-pathogenic *E. coli* in the gastrointestinal tract could be a significant reservoir of resistance genes (Osterblad, *et al.*, 2000).

These bacteria survive for long periods outside their host and are present in all bird environments, particularly the litter, and in poultry house dust. Antibiotic resistance towards *E.coli* is rapidly increasing especially to the fluoroquinolone drugs. Another study done by Johnson *et al.,* 2009 revealed that these drug resistant isolates are mainly found on retail poultry products as the results of indiscriminate use of antibiotics in poultry.

2.4 Enrofloxacin

Enrofloxacin (ENRO) is an antimicrobial agent of the fluoroquinolone group approved only in veterinary medicine, with a broad antimicrobial spectrum and high bactericidal activity (Walker *et al*., 1992). Sensitive bacteria include *Staphylococcus, Escherichia coli, Proteus, Klebsiella*, and *Pasteurella* (Cellini *et al*., 1996). ENRO was approved in Europe in the 1990s and is still widely used in poultry for colibacillosis treatment due to its remarkable efficacy against multidrug-resistant avian pathogenic

Escherichia coli (Bass, *et al*., 1999; Lutful Kabir, 2010). Due to the poor economic value of individual birds in avian species, single therapy is prohibitively expensive, therefore drinking water is the most common method to deliver medication. Individual therapy by oral or parenteral route is reserved for high-value breeders or small flocks for practical reasons. Fluorinated 4-quinolones were introduced to the market in the 1980s and were the top of the line antibiotics, offering a broad spectrum of activity and high efficacy in a wide range of infections both orally and parentally (Piddock,. *et al*., 1990).

Nevertheless, history has demonstrated that the extensive use of new antibiotics is eventually shadowed by the appearance of resistance to those chemicals that have become a major global problem. Besides, this antibiotic has been widely overused in poultry. As a result, *E. coli* resistance to fluoroquinolone antibiotics has significantly increased (Francesco Castellone, 2020). Furthermore, they exhibit a similar mode of action with garlic which inhibits DNA gyrase and thus interferes with DNA transcription (Prescott, *et al.*, 2005; Feldberg, *et al*., 1988).

2.5 Synergistic antibacterial activity of plant extracts and antibiotics

Many scientists were driven to investigate and appraise the relevance of synergistic acting of plant-derived chemicals and standard antibiotics, in which one agent improves the effect of the other and together they operate more efficiently than as independent agents (Wagner, *et al*., 2009; Hemaiswarya, 2008). Plant extracts are widely known for their antibacterial qualities, as well as their capacity to improve the action of an antibiotic when combined with it. The results of a combination assay between grape pomace extract and antibiotics revealed that the extract combined with representatives of various antibiotic classes such as -lactam, quinolone, fluoroquinolone, tetracycline, and chloramphenicol acted synergistically in all *S. aureus* and *E. coli* strains tested, with FICI values ranging from 0.031 to 0.155. Antibiotic MICs were lowered 4- to 75-fold (Sanhueza, 2017). In addition to the synergistic effects shown with plant extracts, in vitro studies have shown that isolated substances can increase the effectiveness of antibiotics. The major bioactive ingredient in *Rosmarinus officinalis* extracts, carnosic acid, was found to work synergistically with gentamicin against *S. aureus* clinical isolates (Vázquez, *et al*., 2016). Curcumin, a flavonoid extracted from the rhizome of the plant *Curcuma longa L.*, significantly lowered the MICs of the MRSA antibiotics oxacillin, ampicillin, ciprofloxacin, and norfloxacin. The combination activity of curcumin and antibiotics resulted in MIC values that were reduced by 2 to 128-fold (Mun, *et al*., 2013).

Allicin, an antibiotic component derived from garlic *(Allium sativum)*, enhanced the antibacterial activity of cefazolin (4- to 128-fold) and oxacillin (32- to 64-fold) against *Staphylococcus sp.*, as well as cefoperazone (8- to 16-fold) against *Pseudomonas aeruginosa* (Cai, *et al*., 2007). It is thought that active chemicals from plants change and decrease acquired resistance mechanisms in bacterial cells, exhibiting a synergistic impact with antibiotics (Abreu, *et al.,* 2012). The mechanism of synergistic action is explained by (i) modifying active sites on bacterial cells, (ii) inhibiting enzymes that catalyse antibiotic breakdown or modification, (iii) increasing membrane permeability, and (iv) inhibiting efflux pumps (Stefanovic, 2017).

3.0 MATERIALS AND METHODS

3.1 Sample collection and bacterial isolation

Fresh chicken faecal samples were collected from 10 chickens using clean gloves and transferred it into separate zipper bags at Animal Research Facility, Faculty of Veterinary Medicine, University Putra Malaysia. The faecal samples were then directly transferred onto Mac Conkey agar and labelled as A01, A02, A03, A04, A05, A06, A07, A08, A09, and A10. Then, it were incubated for 24 hours at 37 °C. After that, secondary culture was done on nutrient agar for 24 hours at 37°C in order to retrieve a single colony to proceed with the biochemical tests. Meanwhile, gram stains were performed after retrieving a single colony from the nutrient agar. Beside that, *Escherichia coli* ATCC 25922 was isolated from stock culture at Microbiology Laboratory of Faculty of Veterinary Medicine, Universiti Putra Malaysia and it was subcultured onto nutrient agar.

3.1.1 Biochemical test

Biochemical tests such as TSI, Urease, Citrate and SIM were performed to the samples in order to confirm the bacteria that was targeted. Targeted bacteria is then transferred to bijou bottles and kept at room temperature. A01, A04, A08, A10 are isolates that showed *E. coli* organisms.

3.2 Preparation of garlic extracts

Garlic was purchased from the local market. Garlic bulbs were peeled, weighed 100g and washed thoroughly. Clean cloves were blended and crushed using a commercial blender and then the mixture was filtered through a sterile cheesecloth. Eighteen ml of garlic juice was obtained from 100g of garlic cloves. This garlic juice was considered as

100% concentration. The garlic juice was prepared as 100%, 50%, 25%, 12.5% and 6.25% (Appendix 1).

3.3 Antibacterial susceptibility testing

Disc diffusion methods were used to test the antimicrobial activity of garlic extracts by following procedures stated in the CLSI, 2006. The Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of the garlic extracts were determined by the Broth Microdilution method with the reference of CLSI, 2010.

3.4 Disk diffusion method (Kirby-bauer method)

This procedure was used with reference to the CLSI, 2006.

3.4.1 Agar medium

Mueller Hinton agar (MHA) was used as the susceptibility test medium in this experiment. The agar was prepared according to the manufacturer's instructions.

3.4.2 Preparation of the garlic extracts impregnated discs.

Sterile blank discs were impregnated with 5 ul of the various concentrations of garlic extracts. Enrofloxacin 5 ug was used as the positive control and the sterile distilled water was used as the negative control.

3.4.3 Preparation of inoculum

Clinical samples which are A01, A04, A08 and A10 that are known to be *Escherichia coli* and also ATCC *E. coli* were streaked onto the nutrient agar to obtain isolated colonies. The plates were incubated at 37°C for 24 hours. Using an inoculating loop, around 4-5 well isolated colonies were selected and suspended into sterile distilled water and vortex thoroughly. The turbidity of bacterial suspensions were then compared with the 0.5 McFarland standard visually to get the bacterial suspension turbidity equivalent to approximately 1 to 2 x 10^8 CFU/ml. The tubes were compared side by side with the black lines. The suspension was used within 15 minutes after adjustments of the turbidity.

3.4.4 Inoculation of test plates

The sterile cotton swabs were dipped into the inoculum tube. The swab was rotated against the side of the tube using firm pressure to remove excess fluid as it should not be dripping wet. The dried surface of Mueller Hinton Agar plate was inoculated by streaking the swab three times over the entire agar surface and rotate the plate approximately 60° each time to ensure an even distribution of inoculum. The lid left slightly ajar, allowing the plate to sit at room temperature for at least 3-5 minutes. The impregnated discs and control discs were applied onto agar plates using sterile forceps. Each clinical sample was inoculated onto two agar plates;

1. Various garlic concentrations and
2. Enrofloxacin as a positive control and distilled water as a negative control.

Each agar plate was incubated at 37°C for 24 hours.

3.4.5 Zone of inhibition

To enhance the viability of zones of inhibition zones, the plates were placed inverted against a dark background. Following incubation, the zone sizes were measured to the nearest millimeter using a ruler or caliper, including the diameter of the disk in the measurement. The results were subjected to the dose response study using Pearson's Correlation Analysis (two tailed tests of significance) and linear regression analysis.

3.5 Broth Micro dilution Method

The method was used with reference to the CLSI, 2010.

3.5.1 Preparation of inoculum

The method used was similar to the method prepared for disk diffusion test; explained on the page 11.

3.5.2 Broth Micro dilution Testing

The 96-well plates were prepared by dispensing 100 ul of Mueller Hinton Broth (MHB) into each well from well 1 to well 12. Well 11 is considered as growth control and well 12 is sterility control. Then, 100 ul of bacteria inoculum were added to each well except at the negative control. The turbidity of each well was determined before and after incubation period. The 96-well plates were incubated at 37°C for 24 hours. Tests were run in duplicates.

3.5.3 Minimal Inhibitory Concentration (MIC)

Minimal Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in broth microdilution susceptibility test. (CLSI, 2010).

3.5.4 Minimal Bactericidal Concentration

Minimal Bactericidal Concentration (MBC) is the lowest concentration of an antimicrobial agent that prevents bacterial growth on Mueller Hinton agar following the spread plate method.

3.5.5 Spread plate method

A drop of 24 hours culture from three wells from a 96-well plate was lawned individually onto nutrient agar plate using sterile wire loops. The agar plates were incubated at 37°C for 24 hours to observe the presence of bacterial growth.

3.5.6 Interpretation of results

As for MIC, the activity of the garlic extracts towards the bacteria is bacteriostatic. While the MBC, the activity of the plant extracts towards the bacteria is bactericidal.

3.6 Synergistics test effect

The synergistic effects of garlic extract with enrofloxacin were tested using Disk Diffusion method with reference to Rawat (2015).

3.6.1 Agar medium

Mueller Hinton agar (MHA) was used in this experiment and was prepared according to the manufacturer's recommendation.

3.6.2 Preparation of Enrofloxacin

In a synergistic test, the garlic extracts were tested with Enrofloxacin discs (5 ug).

3.6.3 Selection of plant extracts concentration

The concentration of the garlic extracts exhibiting maximum inhibition zones in the disc diffusion method were used to test for synergistic effect. In this study, 50% concentration of garlic extracts exhibited maximum inhibition zones.

3.6.4 Preparation of garlic extracts impregnated antibiotic discs

Sterile blank disc was placed on the petri dish prior to placement of it onto sterile agar plates. 2.5 ul of 50% concentration of the garlic extract and 2.5 ul enrofloxacin were impregnated into sterile discs. As for the negative control, sterile blank discs were impregnated into 5 ul of sterile distilled water. While the positive control, enrofloxacin of 5 ug were used.

3.6.5 Preparation of inoculum and inoculation of test plates

The method used was similar to the method described on the page 11.

3.6.6 Zone of inhibition

The method used was similar to the method described in the page 11. The results were subjected to One Way Analysis of Variance (ANOVA) to compare the differences of inhibition zones with or without plant extracts at 95% confidence level. Significantly different means were then elucidated using Tukey's multiple comparison test.

4.0 RESULTS

Ten chicken faecal samples were taken to isolate *E. coli*. Out of 10 chicken faecal samples only four were identified as *E. coli* (40%) by using conventional biochemical test. These four isolates were then further studied in this project. Mean zones of inhibition were expressed in mm ± standard error of mean. Mean zones of inhibition of different garlic extract concentration were measured by disk diffusion and compared with the control.

Garlic extract and enrofloxacin exhibited good antimicrobial activity towards all the clinical isolates of *Escherichia Coli* (Table 1). The 100% garlic exhibited the largest mean diameter of inhibition zones of isolate A08 with 33.35 ± 0.48 mm when compared with enrofloxacin which exhibited 28.83 ± 3.26 mm. A01, A04 and A08 isolates were more sensitive to 100% garlic extract compared to enrofloxacin which is 32.04 ± 0.24 mm, 32.05 ± 0.21 mm and 33.35 ± 0.48 mm respectively. While enrofloxacin in A01, A04 and A08 isolates, the zones of inhibitions are 26.65 ± 0.30 mm, 26.86 ± 0.76 mm and 28.83 ± 3.26 mm respectively. Besides, A10 isolate recorded a similar zone of inhibition with enrofloxacin which is 31.02 ± 0.52 mm and 31.22 ± 0.07 mm. The reference strain illustrates vice versa where enrofloxacin exhibited higher zones of inhibition (38.94 ± 0.07 mm) when compared with 100% garlic extract (33.14 ± 0.51 mm). Nevertheless, all isolates show that *Escherichia coli* is sensitive to garlic extract (Figure 1).

Garlic extracts exhibited clear inhibition zones at various concentrations against all the clinical isolates and also the reference strain (ATCC 25922) of *Escherichia coli*. The diameters of inhibition zones were all concentration-dependent, with significant high positive correlation. The correlation between the diameter of inhibition zones and the garlic extract were in the range of 0.95 to 0.92 ($p<0.05$) based on Pearson's correlation test (Appendix 4). The linear regression analysis suggests that the increase in the diameter in inhibition zones was due to increase in the concentration of plant extracts, with R^2 values ranging from 0.90 to 0.85 ($p<0.05$).(Appendix 4)

Based on the broth micro dilution testing, the minimal bactericidal concentration for garlic was in the range of 25 - 50% while the inhibitory concentration was 12.5%-25% (Table 2). In A10 isolates, it was observed that garlic at 25% concentration illustrates both bacteriostatic and bactericidal effects (Table 2).

The garlic extract at 50% showed synergistic effect with enrofloxacin (Figure 4, table 3). The inhibition zones were significantly increased ($p<0.05$) when combined with garlic and enrofloxacin ((Appendix 5). The percentage of increase in diameter of inhibition zones when garlic extract combined with enrofloxacin was in the range 16-20% of all isolates as shown in table 4.

Table 1: Diameter of zone inhibition of garlic extract at various concentrations on different *Escherichia coli* isolates

Isolate	100% (mm)	50% (mm)	25% (mm)	12.5% (mm)	6.25% (mm)	Enrofloxacin (mm)
A01	32.04 ± 0.2	27.89 ± 0.8	25.85 ± 0.6	21.48 ± 1.4	20.24 ± 0.4	26.65 ± 0.3
A04	32.05 ± 0.2	29.07 ± 0.4	26.37 ± 0.2	21.00 ± 1.4	18.42 ± 0.1	26.86 ± 0.7
A08	33.35 ± 0.4	29.04 ± 0.7	21.59 ± 0.7	18.54 ± 0.2	14.88 ± 0.8	28.83 ± 3.2
A10	31.02 ± 0.5	28.38 ± 0.5	25.91 ± 1.5	22.48 ± 2.1	19.20 ± 0.5	31.22 ± 0.1
ATCC 25922	33.14 ± 0.5	30.91 ± 0.6	24.55 ± 0.3	20.30 ± 0.4	18.20 ± 0.5	38.94 ± 0.1

Values are mean ± std deviation.

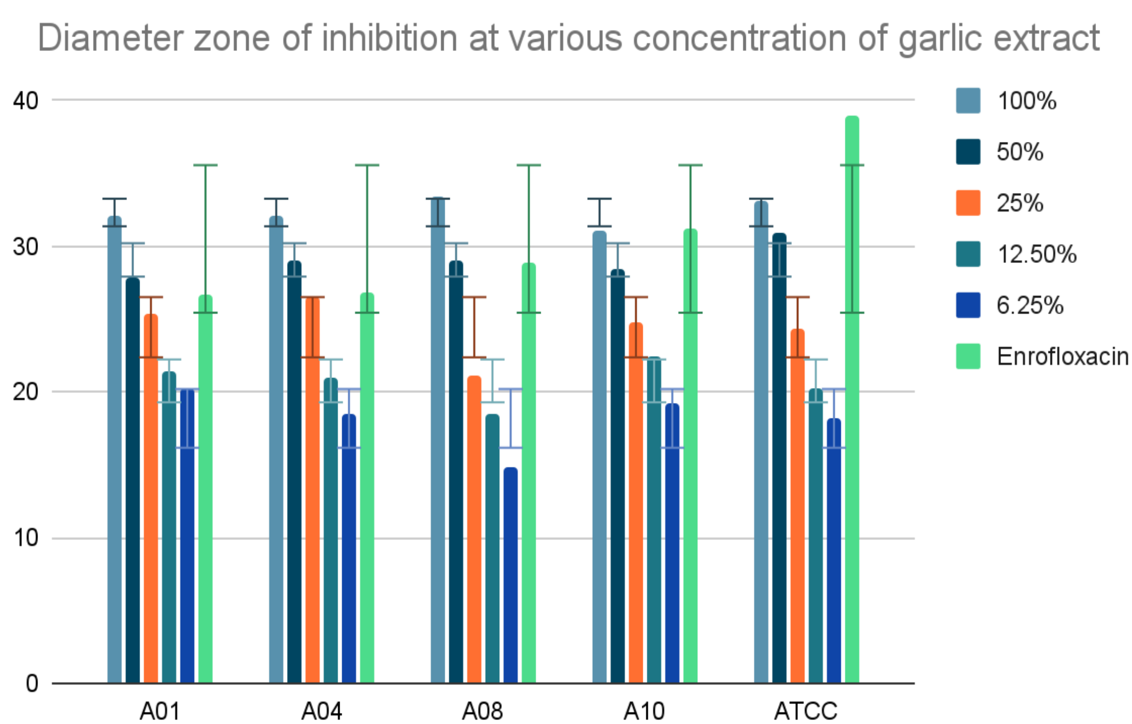

Figure 1: Mean diameter of inhibition zone of garlic extracts and enrofloxacin.

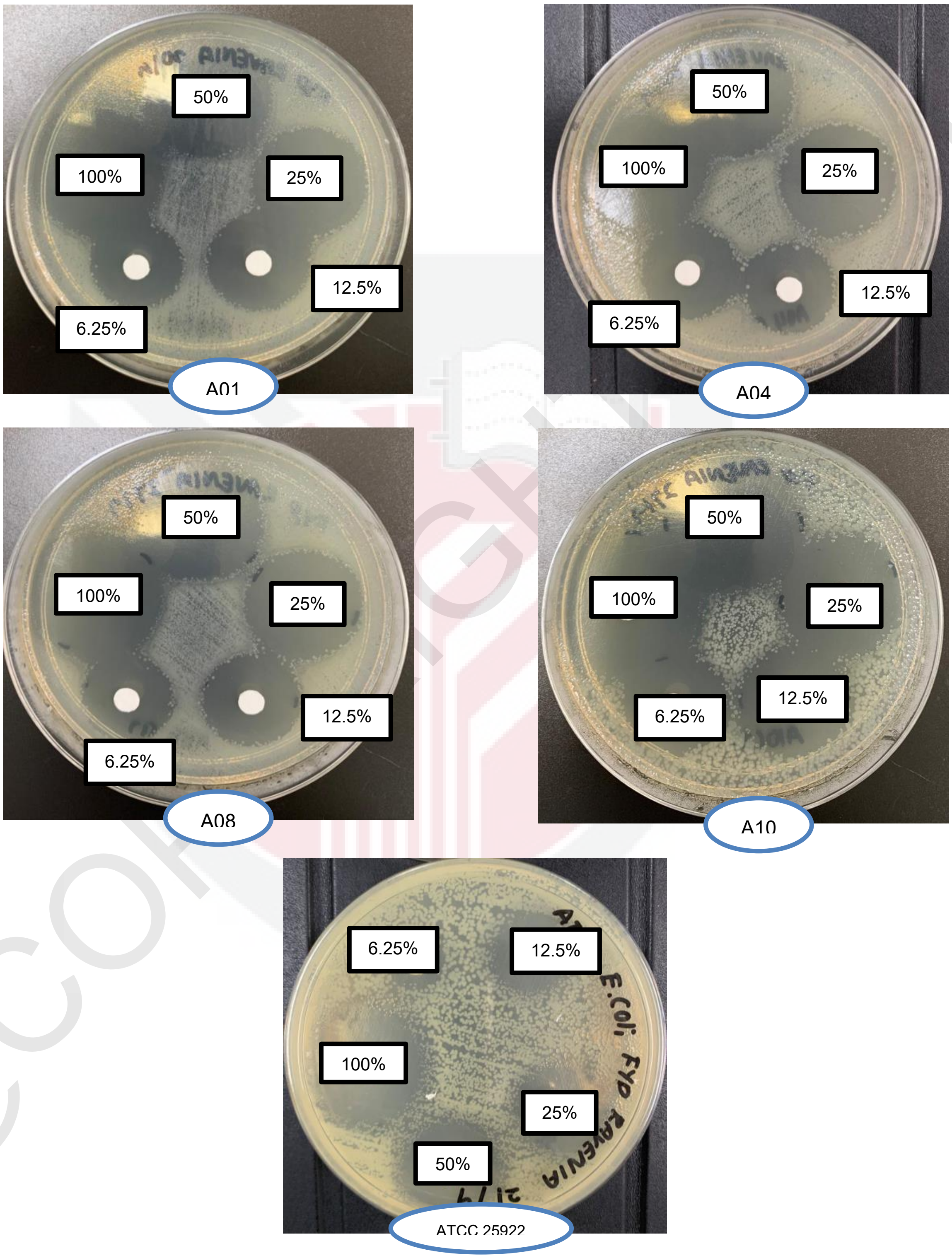

Figure 2: Inhibition zones produced by garlic extract at various concentrations on clinical *Escherichia coli* isolates.

Table 2: The minimal inhibitory concentration and minimal bactericidal concentration of garlic extracts

Isolates	Minimal Inhibitory Concentration (MIC) (%)	Minimal Bactericidal Concentration (MBC) (%)
A01	6.25	25
A04	6.25	25
A08	12.50	50
A10	25	25
ATCC 25922	12.50	50

Table 3: Diameter of inhibition zones produced by garlic extract (50%) in combination with enrofloxacin

Isolates	Enrofloxacin (mm)	Garlic extract 50% (mm)	Enrofloxacin + Garlic extract 50% (mm)	Distilled water (mm)
A01	26.65 ± 0.30	27.89 ± 0.81	31.13 ± 0.43	0
A04	26.86 ± 0.76	29.07 ± 0.45	32.47 ± 0.25	0
A08	28.83 ± 3.26	29.04 ± 0.74	31.35 ± 0.78	0
A10	31.22 ± 0.07	28.38 ± 0.52	37.55 ± 0.12	0
ATCC 25922	38.94 ± 0.07	30.91 ± 0.69	41.90 ± 0.32	0

Values are mean ± std deviation. $P < 0.05$ when compared to negative control.

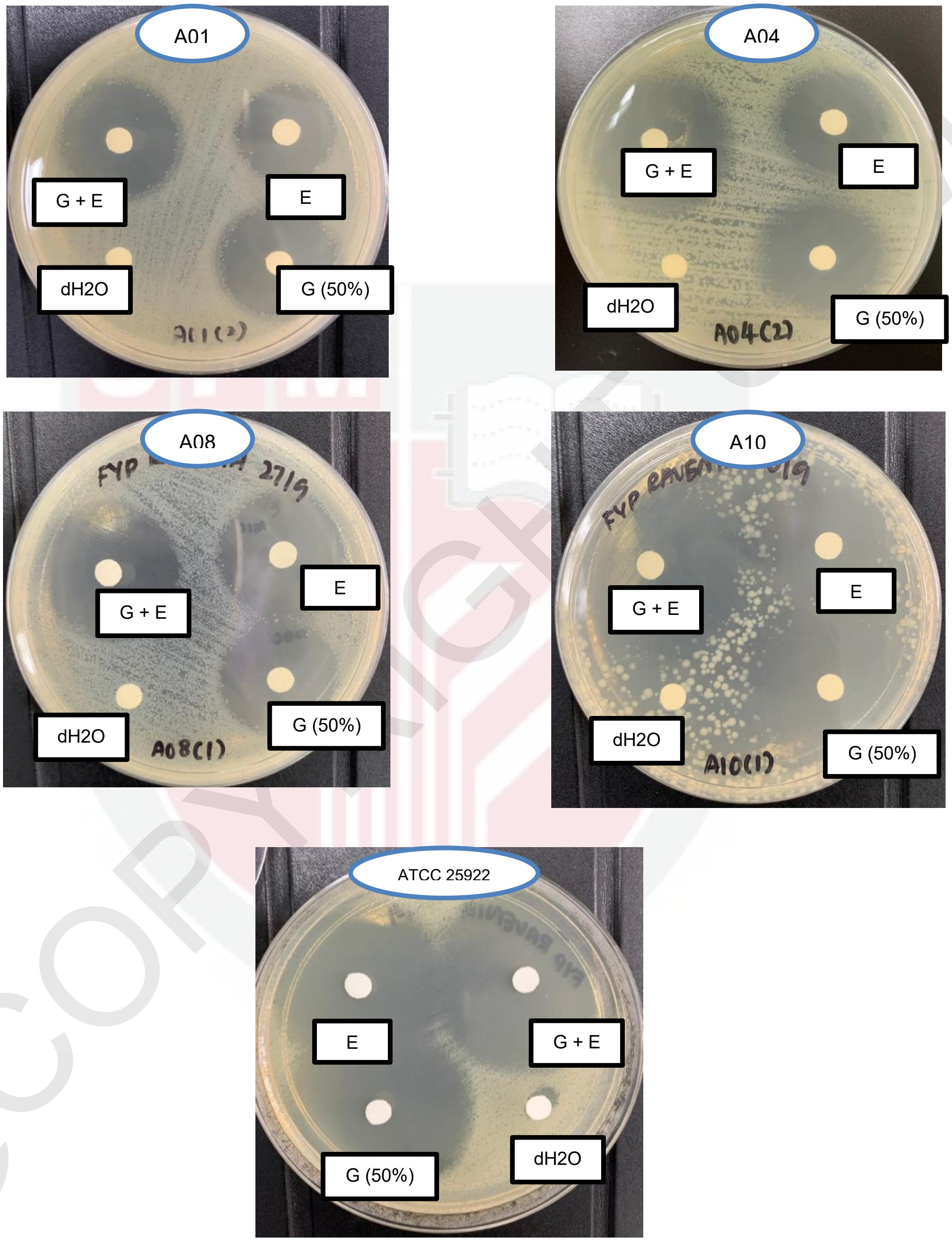

Figure 3 : Inhibition zones produced by garlic extract at 50%, enrofloxacin and synergistic between garlic and enrofloxacin on various *Escherichia coli* isolates.

G + E = garlic with enrofloxacin , E= enrofloxacin, dh2O= distilled water, G= garlic

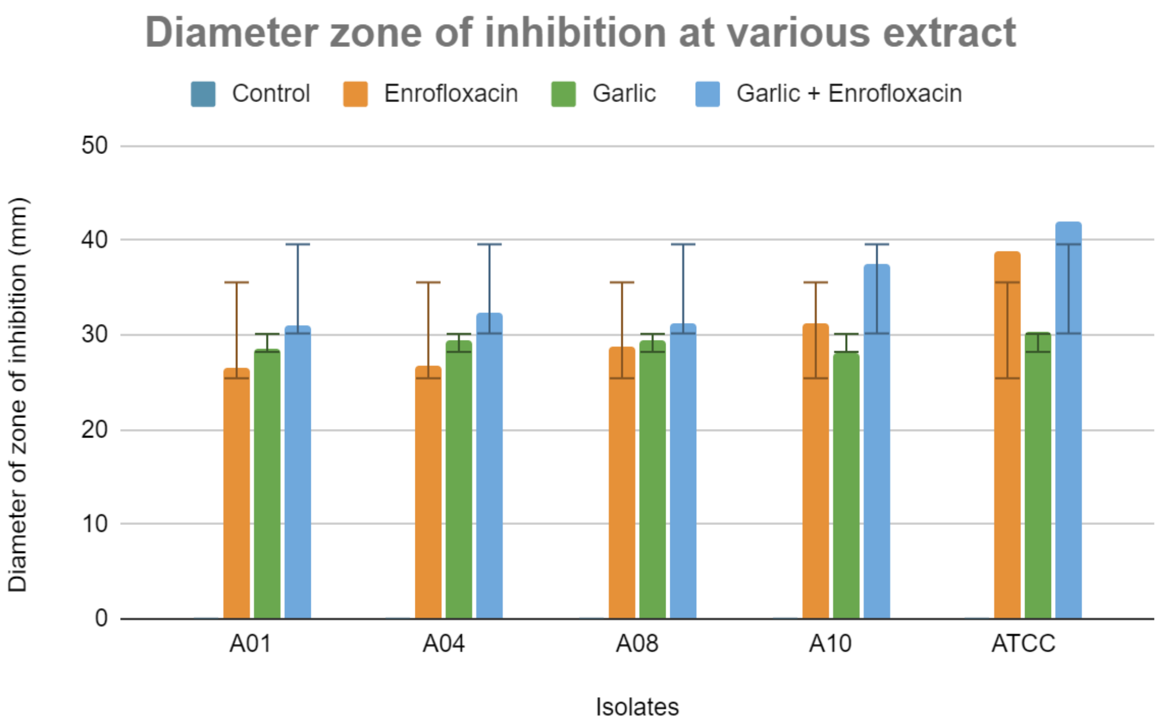

Figure 4: Diameter zone of inhibition of enrofloxacin with and without garlic extract, garlic extract and control on *Escherichia coli* isolates. (P <0.05)

Table 4: The percentage of increase in diameter of inhibition zones when enrofloxacin combined with garlic extracts

Isolates	Without extract (A) (mm)	With extract (B) (mm)	Garlic extract + Enrofloxacin (%)
A01	26.65 ± 0.30	31.13 ± 0.43	36.44
A04	26.86 ± 0.76	32.47 ± 0.25	46.13
A08	28.83 ± 3.26	31.35 ± 0.78	18.24
A10	31.22 ± 0.07	37.55 ± 0.12	44.66
ATCC 25922	38.94 ± 0.07	41.90 ± 0.32	15.78

Percentage of increase = (B^2 -A^2/A^2) x100, A= Mean diameter of inhibition zones without extract, B= Mean diameter of inhibition zones with extract

5.0 DISCUSSION

Garlic is known to have antibacterial, antifungal, and anti proteolytic activities (Ankri, & Mirelman, 1999). In this study we observed that the sensitivity of the bacteria was gradually increased with the increase in the garlic extract concentration. According to Cheesbrough (1984) an antimicrobial agent which the diameter of the zone of inhibition is above 3mm, the organism is said to be sensitive but if it is 2mm or less than that, the organism is said to have resistant against the particular agent. Therefore, considering the statement of Cheesbrough, and comparing the result obtained we can conclude that *E.coli* is indeed sensitive to garlic extract to all the concentrations on all clinical isolates. Other studies also reported similar results where the *E.coli* is sensitive to all garlic extract concentration but resistant to enrofloxacin (Appusamy, *et al.*, 2013). Another study also described similar results where Louis Pasteur said that Onion and Garlic juices (extract) possess antibacterial activities against both Gram positive and Gram negative bacteria (Whitemore and Naidu, 2000). In this study, we observed that *Eschericia coli* was more sensitive to the inhibitory activities of garlic extract compared to enrofloxacin using the disc diffusion method due to the chickens might be exposed or given to enrofloxacin before. Moreover, this result indicates that garlic has a broad spectrum of activity against *Escherichia coli*. As previously shown, garlic extract also had an influence on species of *Escherichia coli, Salmonella, Staphylococcus, Streptococcus, Klebsiella, Proteus, Clostridium, Mycobacterium,* and *Helicobacter* (Cellini *et al*., 1996).

Furthermore, the MIC and MBC values support the findings of the disc diffusion method. The antimicrobial activity of garlic extract against *E. coli* could be attributed to the presence of allicin in the garlic extract. More than 100 phytotherapeutic sulfur compounds contained in garlic in various quantities are thought to be responsible for its antibacterial effect. The odourless amino acid allicin undergoes metabolic activation by the enzyme alliinase when it is crushed, resulting in the formation of allicin and thiosulfinates (Alli, *et al*., 2011). It is clear that garlic extract may be useful as an antimicrobial agent against *Escherichia coli*. According to earlier studies, garlic is helpful against germs that have been found to be resistant to common antibiotics (Jezowa, *et al*., 1966). There are several reports of antibacterial activity of aqueous garlic extract (AGE) against a variety of bacteria that supports the current findings. Another study where the *Bacillus cereus* was completely inhibited in vitro by AGE (10%), and the activity varies depending on storage conditions and heat treatment of the aqueous extract (Saleem & Al-Delaimy, 1982). In vitro, AGE demonstrated antibacterial efficacy against a variety of pathogenic bacteria, including *Shigella* and *Salmonella species*, as well as enterotoxigenic *E. coli* (Arora & Kaur, 1999). According to in vitro antibacterial studies, AGE is effective against a variety of Gram-positive and Gram-negative oral bacteria, including periodontal pathogenic bacteria *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *S. mutans* (Bakri and Douglas, 2005; Fani, *et al*., 2007; Velliyagounder, *et al*., 2012). AGE was found to be active against a wide range of Gram-positive and Gram-positive pathogenic bacteria, including MDR strains and isolates such as MDR *M. tuberculosis*, demonstrating not only garlic's potency against drug-resistant bacteria but also its broad spectrum (Iwalokun, *et al*., 2004; Gupta, *et al.*, 2010; Gull, *et al.*, 2012; Meriga, *et al.*, 2012). All of these investigations suggest that allicin is the primary phytochemical responsible for garlic's antibacterial action. Although allicin is present in garlic ethanol extract, AGE is more powerful because to the presence of additional antibacterial compounds, which may result in a synergistic or additive action.

Furthermore, Allicin interferes with the formation of the phospholipid bilayer of the cell wall, the production of the cell membrane, and the synthesis of RNA in bacteria. As a result, bacteria cannot proliferate and have a bactericidal effect in the presence of allicin (Srinivasan, *et al*., 2009). Allicin's antibacterial effect is primarily demonstrated by fully and promptly blocking RNA synthesis, albeit DNA and protein synthesis are also hindered to some extent. This finding implies that allicin's primary target is RNA. The structural differences between the bacterial strains may influence how responsive the bacteria are to garlic components (Feldberg, *et al.*, 1988). An aqueous extract of garlic inhibited the development of *E.coli* isolated from infected chickens (Ziarlarimi, *et al.*, 2011). Garlic's resistance to resistant bacteria improves its ability to attack them. The current study's statistical significance was most likely due to this. As a result, introducing fresh garlic filtrate in the poultry and cattle industries may help to reduce the requirement for antibiotics (Safithri, *et al*., 2011). However, more research is needed to evaluate its effectiveness and any potential bad effects before the therapeutic application can be confirmed (Dikasso, 1999). In this investigation, we discovered that the garlic extract is concentration dependent, which means that the higher the dose, the larger the zone of inhibition. This validates Wallock-Richrad's study, which found that the AGE, allicin, has dose-dependent antibacterial action against *Bacillus spp* (Wallock-Richards, *et al*., 2014). Thus, increased activity is assumed to be owing to allicin's highly reactive sulfoxide group. However, the clinical use of allicin is complicated by its instability and solubility. Animal studies show that allicin administration causes decreased bioavailability and toxicity (Amagase, *et al.,* 2001). There is widespread worry about the rise in antibiotic resistance (Jonkers *et al*., 1999); consequently, using garlic to treat infections may be a solution to the drug resistance problem.

Aside from that, the cell wall structural structure of gram-negative enteric bacteria may explain why garlic extract has a larger zone of inhibition on *E. coli*. Gram-negative bacteria's cell wall, for example, includes 15-20% polysaccharides and 10-20% lipid, whereas gram-positive bacteria's cell wall has 35- 60% polysaccharides and just 0-2% lipid (Carpenter, 1968). It has been found that the cell membrane of *E. coli* contains 20% lipid (Sivam, 1998). The cell wall's polysaccharides and lipid contents influence the permeability of allicin and other garlic ingredients, and consequently the observed susceptibility to garlic by diarrheagenic microbes (Cellini, *et al*., 1996; Sivam, *et al*., 1997). Garlic has been reported that it easily suppress *Enterotoxic E. coli* strains and other pathogenic intestinal bacteria that cause diarrhoea in humans and animals (Caldwell, *et al.*, 1988). Garlic's lack of resistance improves its capacity to kill even highly resistant bacterial strains like *Enterococcus* and *Pseudomonas aeruginosa*. As a result, it appears appealing that antibiotics that inhibit DNA and RNA synthesis could create a successful combination with garlic. As a result, we investigated the synergistic effect of garlic extract and enrofloxacin further.

In the present study, the garlic extract at 50% concentration showed good synergistic effect with enrofloxacin. The inhibition zones were significantly increased ($p<0.05$) when combined with garlic and enrofloxacin. The percentage of increase in diameter of inhibition zones when garlic extract combined with enrofloxacin was in the range 16-20% of all isolates. This could be due to garlic treatment impairs the microorganism's quorum sensing and communication processes, *Escherichia coli* may be more vulnerable to antibiotic actions. This proposes a method for reducing virulence and controlling *Escherichia coli* infections (Bjarnsholt, *et al*., 2005). Furthermore, garlic appears to alter the exterior structure and integrity of microbial cells, as well as diminish their overall lipid content, making it easier for the antibiotics under study to access and inhibit their respective targets, such as proteins or nucleic acids (Iwalokun, *et al*., 2004). Another antibacterial feature of garlic is thought to be the prevention of bacterial adherence, which could be another possible explanation for the enhancement of antibacterial activity when coupled with garlic. An earlier study that demonstrated sub-MIC allicin could help decrease *Staphylococcus epidermidis* adhesion to microtiter plates provided support for this final mechanism (Perez-Giraldo, *et al*., 2003). Hence, the synergism suggests that garlic could be utilised as an adjuvant in medicines to boost their resistance against bacterial species in which we can see positive results from this study.

6.0 CONCLUSION

There is a lot of interest in creating new classes of antimicrobials to combat diseases especially in the poultry industry due to the rise in bacterial resistance to antibiotics. Since ancient times, garlic has been used as medicine due to its wide range properties such as antibacterial, antifungal, and even antiviral properties.

From this current screening test or study, garlic extract exhibits a strong antimicrobial activity and synergistic effect with enrofloxacin against *Escherichia coli.* Besides, the plant extract can be easily obtained due to wide availability in the local market. Garlic extract can be used as a replacement for enrofloxacin that is widely used in the poultry industry. With this AMR can be reduced by reducing the usage or dose of the antibiotics. It has also shown a significant difference when antibiotics are combined with plant extract. Hence, it can be concluded that garlic can be used in combination therapy or solely is fruitful by adding in poultry feed, given directly via oral and mixed with drinking water to combat with bacterial infections such as colibacilosis.

7.0 RECOMMENDATION

Further research is needed in order to support the findings of this study as it is only performed on four clinical isolates. The presence study is limited by the use of the general strain of *Escherichia coli*. Hence, molecular identification needs to be performed to know the exact strain of *Escherichia coli* because *e.coli* has various strains that are pathogenic and non- pathogenic. Known field/clinical strains need to be incorporated to determine the efficacy of garlic extract as antimicrobial agent. A broad study can be conducted by using more conventional antibiotics that are used in the poultry industry and understand mechanisms of synergy with garlic extracts. It is also important to understand the exact drug-plant ratio where maximum interaction with the combination of antibiotics and garlic extract.

Besides, the ability to assess a drug's properties, including physiological and biochemical processes like side effects and drug-drug interactions that cannot be seen *in vitro,* is made possible through *in vivo* investigations, which are crucial for drug development that is derived from plant extracts. Hence, future research can include the use of clinical trials on experimentally infected chickens.

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APPENDICES

Appendix 1

Preparation of Garlic Extract for Disc Diffusion Method

Desired concentration (%)	Volume of pure garlic extract (ml)	Volume of sterile distilled water (ml)
100	1	0
50	0.5	0.5
25	0.25	0.75
12.5	0.125	0.875
6.25	0.062	0.9375

Appendix 2

Preparation of 96-well plate

Column	Mueller-Hinton Broth (µl)	Concentration of garlic extract (%) (100 µl)	Bacteria inoculum (µl)
1	100	100	100
2	100	50	100
3	100	25	100
4	100	12.5	100
5	100	6.25	100
6	100	3.125	100
7	100	1.56	100
8	100	0	100
9	100	0	0

Appendix 3

Preparation of Garlic Extract Impregnated Antibiotics Discs

Antibiotics	Plate 1 (50% garlic extract)	Plate 2 (Control)
Enrofloxacin (2.5μg)	**2.5μl**	**0μl**

Appendix 4

Linear regression analysis of the diameter of Inhibition zones produced by garlic extract

Isolates	R value	R^2 value	Sig.
A01	0.9537	0.9096	0.0119
A04	0.9062	0.8212	0.0340
A08	0.9528	0.9077	0.0122
A10	0.9088	0.8259	0.0326
ATCC 25922	0.9243	0.8543	0.0247

Appendix 5

One-way ANOVA analysis of diameter of inhibition zones produced enrofloxacin, garlic extracts and with combination of garlic extracts and enrofloxacin

Antibiotics	Garlic extract, P value
Enrofloxacin (2.5 μg)	<0.0001