



UNIVERSITI PUTRA MALAYSIA

**ANTIMICROBIAL ACTIVITIES OF ESSENTIAL OILS
AGAINST *Escherichia coli* and *Salmonella* spp.**

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It is hereby certified that we have read this project paper entitled “Antimicrobial Activities of Essential Oils against *Escherichia coli* and *Salmonella* spp.”, by Tan Ee Wen and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 – Project.

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek.

**AKTIVITI ANTIMIKROB MINYAK PATI
TERHADAP *Escherichia coli* DAN *Salmonella* spp.**

Oleh

Tan Ee Wen

2021

Penyelia: Prof. Dr. Zunita Zakaria

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Kegunaan agen antimikrob yang tidak bijaksana telah menyebabkan kemunculan bakteria yang rintang kepada pelbagai jenis antimikrob merupakan satu ancaman terhadap sektor kesihatan awam dan veterinar. Perkara ini mendesak penelitian antimikrob alternatif bagi memelihara keberkesanan antibiotik terhadap penyakit. Ekstrak herba semulajadi seperti minyak pati tumbuhan yang menunjukkan aktiviti antimikrob telah menarik minat ramai. Dalam projek ini, aktiviti antibakteria minyak pati oregano, taim (thyme) dan kayu manis (cinnamon) telah dikaji. Kepekatan minyak pati yang berlainan telah digunakan untuk menguji aktiviti antibakteria terhadap 15 isolat lapang

Escherichia coli dan *Salmonella* spp. masing-masing yang diperolehi daripada ayam ternakan menggunakan kaedah penyebaran cakera. Enam jenis antibiotik komersial dari kelas yang berlainan telah dipilih untuk menguji kepekaan antibiotik terhadap isolat tersebut. Enrofloxacin didapati menghasilkan zon perencatan purata yang terbesar berbanding dengan antibiotik komersial yang lain. Semua bakteria yang diuji didapati peka terhadap semua minyak esensial yang dikaji. Zon perencatan minyak esensial telah dibandingkan dengan zon perencatan enrofloxacin. Keputusan telah menunjukkan bahawa minyak esensial yang telah dikaji mempunyai sifat antibakteria yang lebih baik terhadap isolat *E. coli* E 203 dan isolat *Salmonella. spp* S 133 berbanding dengan enrofloxacin. Kajian ini telah memutuskan bahawa minyak pati yang dikaji menunjukkan aktiviti antimikrob yang ketara.

Kata kunci: minyak pati, aktiviti antimikrob, penyebaran cakera, *Escherichia coli*, *Salmonella spp.*

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 – Project.

ANTIMICROBIAL ACTIVITIES OF ESSENTIAL OILS

AGAINST *Escherichia coli* AND *Salmonella* spp.

By

Tan Ee Wen

2021

Supervisor: Prof. Dr. Zunita Zakaria

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Imprudent use of antimicrobial agents has caused the emergence of multiple antimicrobial resistance bacteria, posing a threat to both the public health and veterinary sectors. This urged the search of alternative antimicrobial sources to preserve the commercial antibiotics efficacy. Natural herbal extracts such as plant's essential oils that exhibit antimicrobial activity have sparked many interests. In this project, the antibacterial activities of essential oils of oregano, thyme, and cinnamon were studied. Different concentrations of the essential oils were tested for antibacterial activities against 15 field isolates of *Escherichia coli* and *Salmonella* spp. from broiler chickens respectively using the disc diffusion method. Six different classes of commercial antibiotics were chosen to be tested for their sensitivity against the clinical isolates. Zones of inhibition produced were

measured. Enrofloxacin was found to produce the largest inhibition zone compared to other commercial antibiotics. All the test bacteria were found sensitive to all the essential oil evaluated. The zones of inhibition of the essential oils were compared to the zones of inhibition of enrofloxacin. The results showed that the essential oils tested were exhibiting better antimicrobial property against isolate E 203 of *E. coli* and isolate S 133 of *Salmonella* spp. each compared to enrofloxacin. This study concludes that the plant essential oils tested have significant antimicrobial activity.

Keywords: essential oils, antimicrobial activity, agar disc diffusion, *Escherichia coli*, *Salmonella* spp.

INTRODUCTION

The World Health Organization (WHO) is urging everyone to stop overuse and misuse of antibiotics to combat resistance with World Antibiotic Awareness Week since 2015. Resistance arises when there is frequent and inappropriate use of antibiotics by all health professionals, including veterinary services which involve livestock production. Antibiotics for sub therapeutic or prophylactic purposes in animals were practiced in livestock production. This results in the existing portfolio of antimicrobials for food animal productions getting depleted rapidly, which could have an impact on not just animal health but also public health. Immediate actions are deemed to be taken to preserve the efficacy of antimicrobials that are important in human medicine by restricting the usage of commercial antibiotics in animal production.

Thus, interests developed in the scientific community to search for alternatives, such as using herbal medicines with antimicrobial properties in the livestock industry. Natural sources like the herbs constitute a wide range of structurally complex compounds. Herbal extracts and essential oils have been shown to exhibit antibacterial, antifungal, antiviral, and even antioxidant properties. These could be potential sources of novel antimicrobial compounds that can be beneficial in food preservation, and as alternatives as prophylaxis and

treatment of infectious diseases. Therefore, essential oils can be developed as an alternative to commercial antibiotics.

1.1 Hypotheses

- a) Essential oils of Oregano, Thyme, and Cinnamon possess antimicrobial activities against *Escherichia coli* and *Salmonella* spp.

1.2 Objective

The objective of this study is to determine the antimicrobial activities of essential oils against *Escherichia coli* and *Salmonella* spp. and to compare the antimicrobial activities of essential oils against commercial antibiotics.

2.0 LITERATURE REVIEW

2.1 Essential Oil

Essential oils are termed as “volatile oils or essences derived from vegetation and characterized by distinctive odors and a substantial measure of resistance to hydrolysis” according to Gooch (2011). Generally, essential oils are also called aromatic oily liquids obtained from distinct anatomic parts of plant materials such as leaves, flowers, and wood by steam or hydro-distillation method (Chouhan et al., 2017). The major components present in essential oils are terpenes, whereas aldehydes, alcohols, and esters are also present as minor components (Zuzarte & Salgueiro, 2015). However, even within the same species of plant, the proportion of the components in the plants might differ. According to Lopez and others (2017), essential oils are very complex mixtures of compounds, where the highest proportion of the component in comparison to other trace components will determine the chemotype of the essential oils. This might be due to factors such as climatic, geographic, growth conditions, drying, and extraction method of the plant. It is stated by Mariod (2016) that the weather, in particular temperature, soil quality, the altitude, and country where the plants are grown are the major causes for variations between essential oils.

2.2 Essential Oil of Oregano (*Origanum vulgare*)

Oregano (*Origanum vulgare*), also called wild marjoram from the mint family- Lamiaceae, originates from the Mediterranean. It is a perennial herb (up to 80 cm in height), with dark oval fragrant leaves and white, pink, or purple flowers formed in spikes. Oregano has a long history of use as food flavorings and a medicinal plant in ancient Egypt, also frequently used by Greeks and Traditional Chinese healers to treat a variety of illnesses (Kumar et al., 2016). The characteristic flavor produced by oregano essential oils is due to its high content of carvacrol, a noncrystallizable phenol (Kokkini et al., 2003).

Essential oils of oregano (OEO) are well known for their antimicrobial activity according to Mellencamp et al. (2011) and are amongst the most effective antimicrobial agents (Fernández-Pan et al., 2012). According to the Encyclopedia of Food and Health, “the major constituents of *O. vulgare* leaves essential oil are carvacrol, p-cymene, c-terpinene, limonene, terpinene, ocimene, caryophyllene, β -bisabolene, linalool, and 4-terpineol” (Kumar et al., 2016, p. 332-337). The main antibacterial compounds identified in the different oregano essential oils are carvacrol and thymol, which are also responsible for the characteristic aroma of the plant (Garcia et al., 2015). Mechanistic studies have shown that carvacrol altered the permeability of the cell membrane of bacteria causing leakage of essential cations which eventually kills them (Arfa et al., 2006).

2.3 Antibacterial Activity of Oregano Essential Oil

The essential oil of oregano is reported to exhibit an inhibitory effect against *B. cereus*, *B. subtilis*, *E. coli*, *S. typhimurium*, and *S. aureus* as shown in the study by Cattelan et al. (2013). Several studies mentioned by Hulánková and Bořilová (2011) have shown essential oil from *Origanum vulgare* to be highly efficacious showing inhibitory effect on gram-negative bacteria, such as, salmonella. Earlier studies (Hammer et al., 1999; Dorman and Deans 2000) also demonstrated the efficacy of essential oil of oregano (*Origanum vulgare*) against *E. coli*.

2.4 Essential Oil of Thyme (*Thymus vulgaris*)

Thyme belongs to the same family as Oregano, Lamiaceae and is distributed throughout Europe, Northern Africa, Asia, Southern America, and Australia (Nabavi et al., 2015). It is an aromatic and medicinal perennial herb of increased economic interest, with representative species *T. serpyllum* (wild thyme) and *T. vulgaris* (common thyme) (Sakkas & Papadopoulou, 2017). The full-grown plant can reach 25 cm in height, with quadrangular stem and branches. The stalkless leaves are 6–12 mm in length and are oval to oblong in shape. *Thymus vulgaris* has been used since ancient times for medical purposes, antimicrobial agents; while recently more as a flavour enhancer, food preservatives, and even in the cosmetic industry (Mandal and DebMandal, 2016).

There are different chemotypes available in *Thymus* such as thymol, carvacrol, linalool, geraniol, thujanol, α -terpineol, borneol, and *p*-cymene but varies according to the dominant constituents in different species as shown in a study by Nabavi et al. (2015). According to several studies (Mota et al., 2012; Asbaghian et al., 2011), the major components of *Thymus vulgaris* include thymol (43.8%) and *p*-cymene (15.2%). *T. vulgaris* essential oil components, either alone or in combination, can produce a broad spectrum of antimicrobial activity attributed to thymol as a result of synergism between other main components in the oil (Mandal & Debmandal, 2016). Like carvacrol, thymol also disrupts the permeability of the cell membrane of bacteria resulting in leakage of essential cations that could kill the bacteria (Arfa et al., 2006).

2.5 Antibacterial Activity of Thyme Essential Oil

Thymus vulgaris essential oils (TEO) exhibit broad-spectrum antibacterial activity against foodborne pathogens such as *E. coli*, *Salmonella*, *Shigella flexneri*, *B. cereus*, and *S. aureus* (Dobre et al., 2011; Miladi et al., 2013). Whereas according to Sheeladevi (2012), TEO also produces inhibitory effects on other foodborne bacteria such as *Campylobacter* sp., *Listeria* sp., *Yersinia* sp., and *Pseudomonas* sp. One recent study by Oulkheir et al (2017) also shows that TEO exhibits antibacterial activity towards *E. coli*, *Salmonella* spp, *Streptococcus* group D, and *S. aureus*.

2.6 Essential Oil of Cinnamon (*Cinnamomum verum*)

According to Cardoso-Ugarte et al (2016), Cinnamon is one of the most popular spices used by humans in the earliest days. Cinnamon essential oil is obtained from the different species of the genus *Cinnamomum*, from the family Lauraceae. *Cinnamomum* is a small, evergreen tree about 10–15 m tall (Leela, 2006). The bark is widely employed as a spice, its leaves are ovate-oblong in shape, and 7–18 cm long. The flowers, arranged in panicles, have a greenish color, and have a rather disagreeable odor. The fruit is a purple 1 cm berry containing a single seed. *Cinnamon verum* is native to Southern India and Sri Lanka (Hamidpour et al., 2015). The cinnamon's tree inner bark and leaves can be made into oils. The essential oil is mainly used in the pharmaceutical, confectionery, perfumery, and food industries. (Leela, 2006).

The main chemical compound of cinnamon leaf essential oil is Eugenol (70%), whereas in cinnamon bark, it is cinnamaldehyde and both contain antimicrobial property. Other compounds in cinnamon include, cinnamic acid and cinnamyl acetate, α -thujene, α -terpineol, α -cubebene, eugenol, β -caryophyllene, and terpinolene (Paranagama et al., 2001; Knauth et al., 2018). Eugenol has been reported to exhibit antimicrobial property in a recent study (Millezi et al., 2019). Wendakoon and Sakaguchi (1993) said that eugenol contains hydroxyl groups

that might combine with proteins, preventing enzyme action of the bacteria that can result in cell death.

2.7 Antibacterial Activity of Cinnamon Essential Oil

The cinnamon essential oil has been reported to inhibit both gram-positive and gram-negative bacteria (Prabuseenivasan et al., 2006). The antibacterial mechanisms of cinnamon essential oils were reported as leakage of the intracellular potassium ion or cell contents such as lipids and protein from the cells of the bacteria and cell membrane damage by the lipophilic compounds (Bouhdid et al. 2010; Silveira et al., 2012) that lead to eventual death and reduction in the metabolic activity together with the replication capacity. Several studies (Duan and Zhao, 2009; Silveira et al., 2012) also demonstrated that cinnamon essential oil can exhibit antibacterial effect against *Escherichia coli* O157:H7, *Salmonella enterica*, *Staphylococcus aureus* and *Yersinia enterocolitica*.

2.8 Escherichia coli and Salmonella spp.

According to several recent studies (Brower et al., 2017; Yassin et al., 2017; Manageiro et al., 2017; Davis et al., 2018), there is an increase in the prevalence of antimicrobial resistant *E. coli* due to the increased usage of commercial antibiotics for subtherapeutic or prophylactic purposes in broiler farms. Findings

of *E. coli*'s resistance to multiple antibiotics are concerns to public health as most of the same antibiotics are medically important to human health (FDA, 2016) as well. Whereas some new studies also suggest that *Salmonella* spp. found on contaminated broiler meat or eggs with multiple drug resistance are causing foodborne diseases in humans and losses to the poultry industry (Moultotou et al., 2017; Kim & Lee, 2017). Therefore, alternative antibacterial agents such as essential oils could be beneficial for both humans and the livestock industry.

3.0 MATERIALS AND METHODS

3.1 Bacteria

All test bacteria were field isolates from broilers obtained from Dr. Rezaul Karim, Bacteriology laboratory (PhD candidate). A total of 30 different isolates comprising 15 isolates of *Escherichia coli* and 15 isolates of *Salmonella* spp. were subcultured onto selective media; MacConkey agar (Oxoid Ltd.) and Xylose Lysine Deoxycholate agar (Oxoid, Ltd.). The isolates were re-identified using Gram-staining and series of biochemical tests which include Triple Sugar Iron Test, Sulfur Indole Motility Test, Citrate Utilization Test, and Urease Test.

3.2 Essential Oils

The essential oils of Oregano (*Origanum vulgare*), Thyme (*Thymus vulgaris*), and Cinnamon (*Cinnamomum verum*) were purchased from Plant Therapy supplied by OilyPod.my. These essential oils are 100% pure essential oils with detailed Gas Chromatography-Mass Spectrometry (GC/MS) reports for compounds identification by reputable third-party testers for their products (<https://www.planttherapy.com/why-plant-therapy>).

3.3 Antibacterial Susceptibility Testing

The antibacterial activities of the selected antibiotic and essential oils were determined using standard Agar Disc Diffusion Method, following procedures stated in the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol (American Society for Microbiology, 2009).

3.3.1 Control Experiment Using Selected Antibiotics

The sensitivity of the test bacteria was done against six commercial antibiotics (Oxoid, Ltd) from different classes - amoxicillin/clavulanic acid (AMX) 30 μ g, erythromycin (E) 15 μ g, enrofloxacin (ENR) 5 μ g, streptomycin (S) 25 μ g, sulfamethoxazole/trimethoprim (SXT) 25 μ g, and tetracycline (TE) 25 μ g. The tests were done using the Agar Disc Diffusion Method. The zones of inhibition were measured using callipers and were compared to the zones produced by the essential oils.

3.3.2 Preparation of Oil Dilutions

Five different concentrations of all three types of essential oils which include 100%, 80%, 60%, 40%, and 20% were prepared. Pure concentrated essential oils were predetermined as 100% concentration. The other concentrations were essential oils diluted with dimethyl sulfoxide (DMSO) solvent

(Friendemann Schmidt), and vortexed for 10 seconds each. (Joshi et al., 2010).

One hundred percent DMSO was used as control.

3.3.3 Preparation of Essential Oil Impregnated Discs

Sterile 6mm diameter blank discs were impregnated with 10 μ l of all the essential oils of the different concentrations (100%, 80%, 60%, 40%, and 20%). All the impregnated discs were then left to air dry on the petri dishes for approximately one hour.

3.3.4 Preparation of Inoculums

Each test bacteria was inoculated onto nutrient agar and incubated at 37°C overnight to obtain freshly isolated colonies. Five different isolates of *E.coli* (E 202, E203, E 208, E 250, and E 272) and *Salmonella* spp. (S 133, S 145, S 196, S 240, and S 244) were further chosen randomly to compare among different treatments on the same plate. A sterile inoculating loop was used to take a few of the isolated colonies, suspended into sterile saline (0.85% NaCl) and shaken thoroughly to create a smooth suspension in the sterile test tube. The turbidity was adjusted to a 0.5 McFarland standard by positioning the bacteria suspension and standard solution in front of a card similar to the Wickerham card as a visual aid.

3.3.5 Inoculation of Test Plates

The bacterial suspensions were immediately used to inoculate onto the Mueller-Hinton agar (MHA) plate. The sterile swab was dipped into the inoculum test tube and rotated against the side of the tube with pressure to remove excess fluid. The swab was streaked over the entire agar surface three times, by rotating the plate approximately 60 degrees each time to ensure an even distribution of the inoculum. Finally, the edge of the plate was swabbed entirely to remove excess liquid. The inoculated agar was left to be allowed to dry for 15 minutes at room temperature. Each isolate was tested twice with the same essential oils.

3.3.6 Placement of the Discs

The antibiotic discs were placed using a multi-disc dispenser to dispense six types of the antimicrobial disc onto the MHA at once. The essential oil and DMSO impregnated discs were placed onto the MHA individually using forceps. Each type of essential oil disc was placed onto selected five *E. coli* and five *Salmonella* spp. inoculated plates respectively. The forceps were cleaned with 70% ethanol, dried, and flamed over the Bunsen burner to be sterilized. The discs were placed onto the agar with a template underneath the plate and pressed gently. Each time a disc was placed, the forceps were sterilized again. After all discs were in place, the lid was replaced, and the plates were not reverted until

after 30 minutes to ensure all the discs were nicely attached to the agar. Finally, all the plates were incubated at 37°C for approximately 18 hours. Each treatment was replicated using the same selected isolates three times.

3.3.7 Zones of Inhibition

The zone of inhibition of each plate was measured following the guidelines from The Clinical & Laboratory Standards Institute (CLSI, 2018). The diameter of zones of inhibition was measured using callipers. Only the zones with complete inhibition based on unaided eye were regarded as the margin. Partial inhibition zones with faint growth were not included for measurement. The plates were placed on top of a black paper to ease visual judgment.

4.0 RESULTS

All essential oils tested produced inhibition zones against all test bacteria at various concentrations (**Appendix 1, 2 & 3**). Thyme essential oil's stock solution contains the highest amount of Thymol (0.46g/ml); Oregano essential oil's stock solution with the highest amount of Carvacrol (0.68g/ml); Cinnamon essential oil's stock solution having the highest amount of Eugenol (0.75g/ml). The inhibition zones' diameter produced by each of the essential oils increased with increasing concentration followed by a plateau. The inhibition zones plateaued once the Thymol and Carvacrol reached 0.28g/ml in the Thyme essential oil (60% concentration) and Oregano essential oil (40% concentration) respectively, and when Eugenol reached 0.30g/ml in the Cinnamon essential oil (40% concentration).

From 5 different isolates of *E. coli* and *Salmonella spp.* tested (**Table 1 and Table 2**), there were 3 isolates out of 5 (60%) for *E. coli* and *Salmonella spp.* which were resistant to more than 1 type of commercial antibiotics and any resistance to more than 3 antimicrobial categories, was recorded as multidrug resistant (MDR) bacteria, according to CDC (2019). *E. coli* isolate E 250 is regarded as pan drug resistant bacteria as it is resistant to all antibiotics tested in all antimicrobial categories. All tested bacteria (100%) for both species were resistant towards Erythromycin. Enrofloxacin was chosen to compare with the essential oils based on its largest inhibition zones, at an average of 20.38 mm

Table 1: Diameter of Inhibition Zones Produced by Essential Oils (60%) and Commercial Antibiotics against *E. coli* (mean \pm standard deviation)

Treatment (μ g)	Diameter of Inhibition Zones (mm) on <i>E. coli</i>				
	E 202	E203	E 208	E 250	E 272
Thyme EO (10)	21.34 \pm 1.30	16.90 \pm 1.25	18.24 \pm 1.12	13.65 \pm 0.89	15.22 \pm 0.06
Oregano EO (10)	20.14 \pm 0.93	16.37 \pm 0.62	17.83 \pm 0.23	14.32 \pm 0.45	15.67 \pm 0.23
Cinnamon EO (10)	13.40 \pm 0.41	10.77 \pm 0.15	11.47 \pm 0.16	11.22 \pm 0.51	12.00 \pm 0.27
Sulphametoazole-trimethoprim (25)	31.26 \pm 0.84 (S)	6.00 \pm 0.00 (R)	26.16 \pm 0.23 (S)	6.00 \pm 0.00 (R)	6.00 \pm 0.00 (R)
Tetracycline (30)	25.88 \pm 0.24 (S)	6.00 \pm 0.00 (R)	21.41 \pm 0.67 (S)	6.00 \pm 0.00 (R)	6.00 \pm 0.00 (R)
Amoxicillin-clavulanic acid (30)	18.99 \pm 1.53 (S)	9.97 \pm 1.06 (R)	16.77 \pm 1.14 (I)	8.10 \pm 0.18 (R)	15.83 \pm 1.53 (I)
Erythromycin (15)	8.01 \pm 0.44 (R)	8.12 \pm 0.36 (R)	8.02 \pm 1.04 (R)	6.00 \pm 0.00 (R)	6.00 \pm 0.00 (R)
Enrofloxacin (5)	32.98 \pm 0.11 (S)	6.75 \pm 0.41 (R)	30.62 \pm 0.16 (S)	14.56 \pm 0.63 (R)	14.34 \pm 0.66 (R)
Streptomycin (10)	22.31 \pm 1.19 (S)	14.50 \pm 0.27 (I)	17.22 \pm 0.47 (S)	6.00 \pm 0.00 (R)	11.24 \pm 0.86 (R)

S = Sensitive, I = Intermediate, R = Resistant (CLSI, 2018)

Table 2: Diameter of Inhibition Zones Produced by Essential Oils (60%) and Commercial Antibiotics against *Salmonella spp.* (mean \pm standard deviation)

Treatment (μg)	Diameter of Inhibition Zones (mm) on <i>Salmonella spp.</i>				
	S 133	S 145	S 196	S 240	S 244
Thyme EO (10)	21.95 \pm 0.03	14.80 \pm 0.25	21.76 \pm 0.39	14.12 \pm 0.22	13.30 \pm 0.25
Oregano EO (10)	19.98 \pm 0.51	13.78 \pm 0.54	20.76 \pm 0.60	13.57 \pm 0.24	12.58 \pm 0.40
Cinnamon EO (10)	12.26 \pm 0.43	12.12 \pm 0.03	12.12 \pm 0.03	11.78 \pm 0.48	11.03 \pm 0.09
Sulphametoxazole-trimethoprim (25)	6.00 \pm 0.00 (R)	27.67 \pm 1.57 (S)	27.67 \pm 0.52 (S)	25.51 \pm 0.70 (S)	24.31 \pm 0.76 (S)
Tetracycline (30)	6.48 \pm 0.30 (R)	16.03 \pm 0.68 (S)	22.47 \pm 0.59 (R)	6.00 \pm 0.00 (R)	6.00 \pm 0.00 (R)
Amoxicillin-clavulanic acid (30)	8.79 \pm 0.51 (R)	26.25 \pm 1.19 (S)	23.59 \pm 0.88 (S)	9.36 \pm 1.25 (R)	19.67 \pm 0.68 (S)
Erythromycin (15)	6.00 \pm 0.00 (R)	6.10 \pm 0.35 (R)	8.15 \pm 0.41 (R)	6.00 \pm 0.00 (R)	6.00 \pm 0.00 (R)
Enrofloxacin (5)	16.20 \pm 0.43 (R)	27.54 \pm 0.70 (S)	28.72 \pm 1.03 (S)	15.18 \pm 0.48 (R)	16.58 \pm 0.24 (R)
Streptomycin (10)	13.85 \pm 1.16 (I)	17.77 \pm 1.18 (S)	17.27 \pm 0.65 (S)	15.79 \pm 0.85 (S)	15.02 \pm 0.13 (S)

S = Sensitive, I = Intermediate, R = Resistant (CLSI, 2018)

towards all the selected tested bacteria compared to other antibiotics tested and its significant usage in broiler chicken production (Mohamed-Yousif et al., 2019).

At 60% concentration, all essential oils tested exhibited antimicrobial activity towards all the tested bacteria, even those multidrug resistant *Escherichia coli* and *Salmonella spp.* (**Table 1, Table 2**). However, all the essential oils reacted towards all the selected test bacteria differently.

Thyme and Oregano essential oil generally did not show significant difference in inhibiting five different isolates of *E. coli* (**Figure 1**). Thyme and Oregano essential oils also produced significantly larger inhibition zones compared to the commercial antibiotic, enrofloxacin ($p < 0.01$) against *E. coli* isolate E 203. Whereas for *E. coli* isolates E 250 and E 272, the inhibition zones produced by Thyme and Oregano essential oils have no significant difference compared to the inhibition zones produced by enrofloxacin. However, for *E. coli* isolates E 202 and E 208, the inhibition zones produced by Thyme and Oregano essential oils were significantly smaller compared to those produced by enrofloxacin. Overall, inhibition zones against all selected *E. coli* produced by Cinnamon essential oils were significantly smaller compared to other treatments.

As for *Salmonella spp.*, all 3 of the essential oils generally produced inhibition zones which are significantly smaller ($p < 0.05$) than enrofloxacin (**Figure 2**) *except* for isolate S 133. For isolate S 133, it displayed a significant difference in inhibition zones produced by all the treatment tested, whereby

Thyme essential oil was significantly better than Oregano essential oil, Cinnamon essential oil and enrofloxacin. Thyme and Oregano essential oils did not produce any significant difference in inhibition zones against most isolates of *Salmonella* spp. (S 145, S 196, and S 240). Generally, for Cinnamon essential oil, the inhibition zones produced against *Salmonella* spp. were all significantly smaller compared to other treatments.

Overall, Cinnamon essential oil produced the smallest inhibition zone against all the tested bacteria compared to other essential oils and enrofloxacin. However, it is the cheapest in cost among all the essential oils, where Cinnamon essential oil costs around RM3.80/ml while Thyme essential oil, is the most expensive essential oil among all, at RM4.90/ml. This shows that Thyme essential oil is 28% more expensive than the same amount of Cinnamon essential oil.

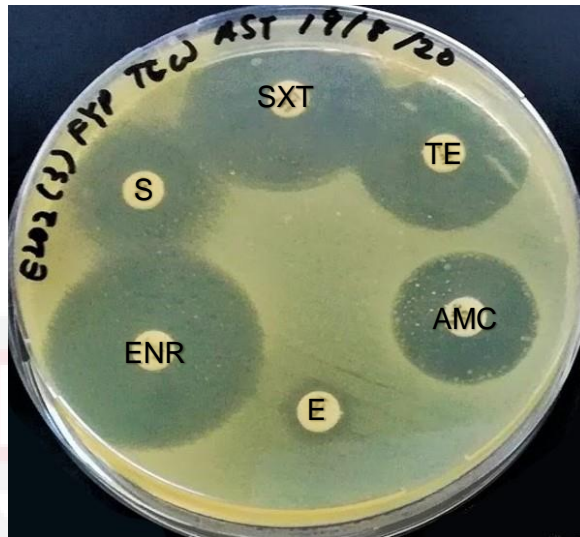


Plate 1: Inhibition Zones Produced by Commercial Antibiotics on *Escherichia coli*

SXT= Trimethoprim/Sulfamethoxazole, TE = Tetracycline, AMC= Amoxicillin/Clavulanate Acid, E= Erythromycin, ENR= Enrofloxacin, S= Streptomycin)

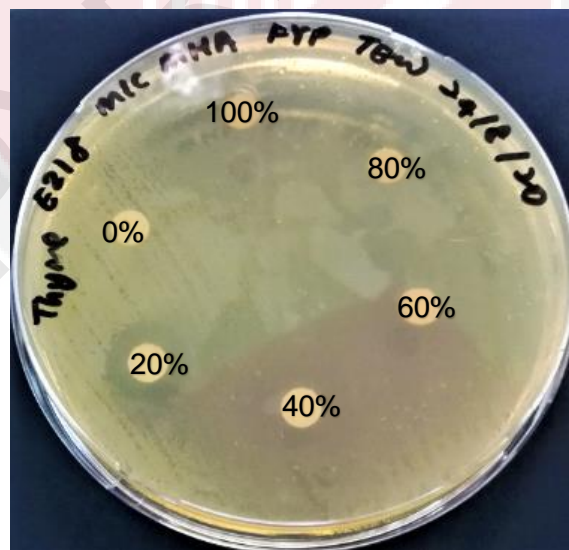


Plate 2: Inhibition Zones Produced by 6 Different Concentrations of Thyme Essential Oils on *Escherichia coli*

(0% = 100% DMSO)

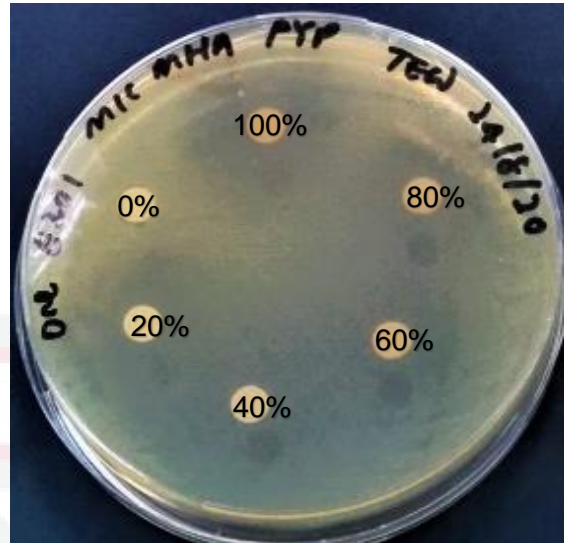


Plate 3: Inhibition Zones Produced by 6 Different Concentrations of Oregano Essential Oils on *Escherichia coli*
(0% = 100% DMSO)

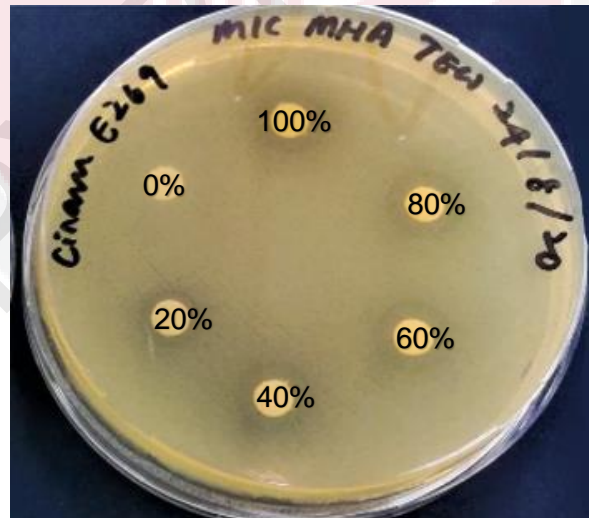


Plate 4: Inhibition Zones Produced by 6 Different Concentrations of Cinnamon Essential Oils on *Escherichia coli*
(0% = 100% DMSO)

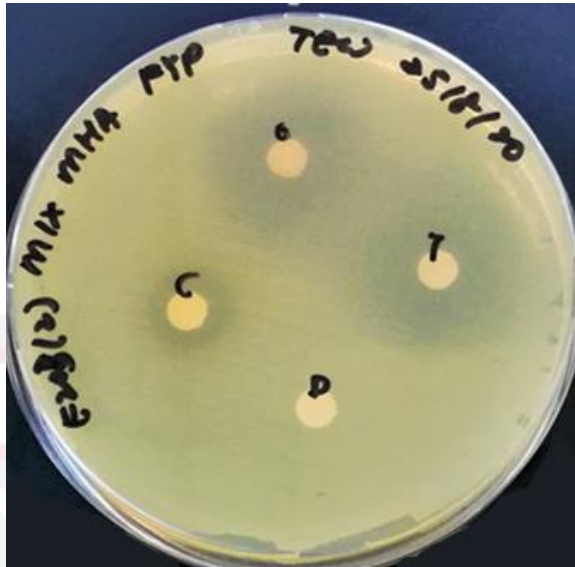


Plate 5: Inhibition Zones Produced by 3 Different 60% Essential Oils on *Escherichia coli*

(O= Oregano, T= Thyme, C= Cinnamon, D= DMSO)



Plate 6: Inhibition Zones Produced by 3 Different 60% Essential Oils on *Salmonella spp.*

(O= Oregano, T= Thyme, C= Cinnamon, D= DMSO)

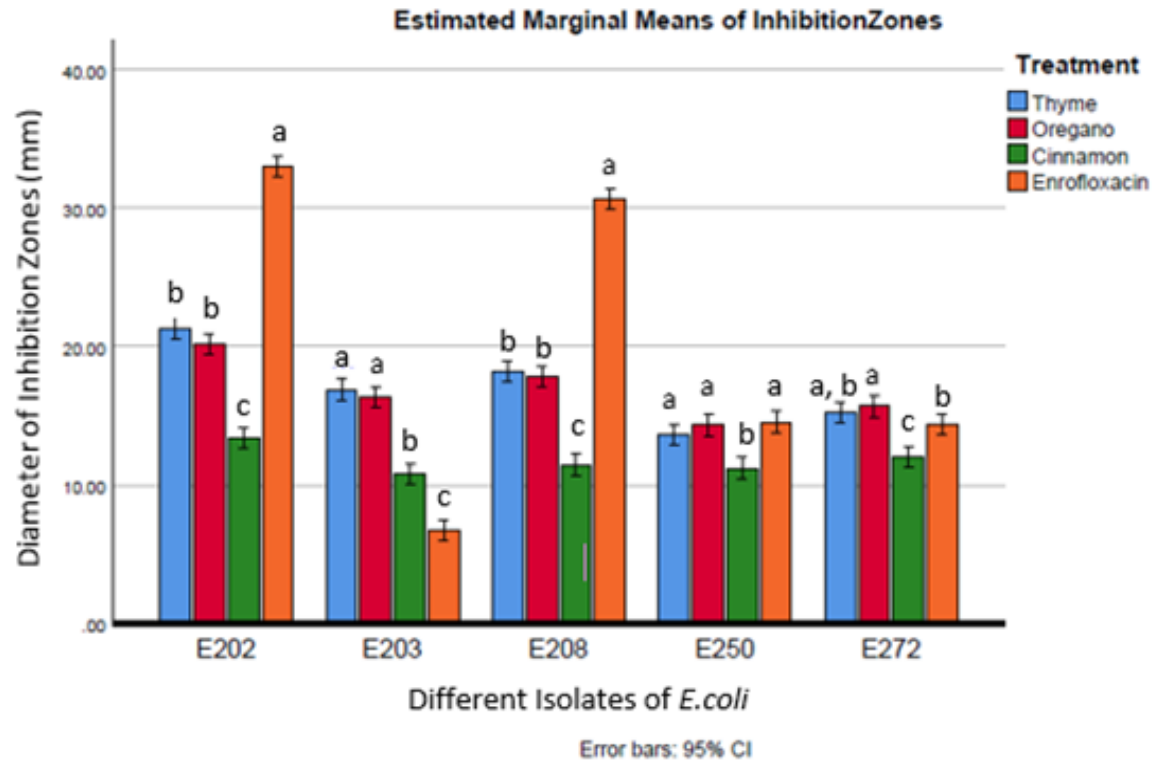


Figure 1: Effect of 60% essential oils and enrofloxacin on growth of *E. coli*.

Values are mean \pm standard deviation. Means with different superscript within the same isolate differ significantly ($p < 0.05$).

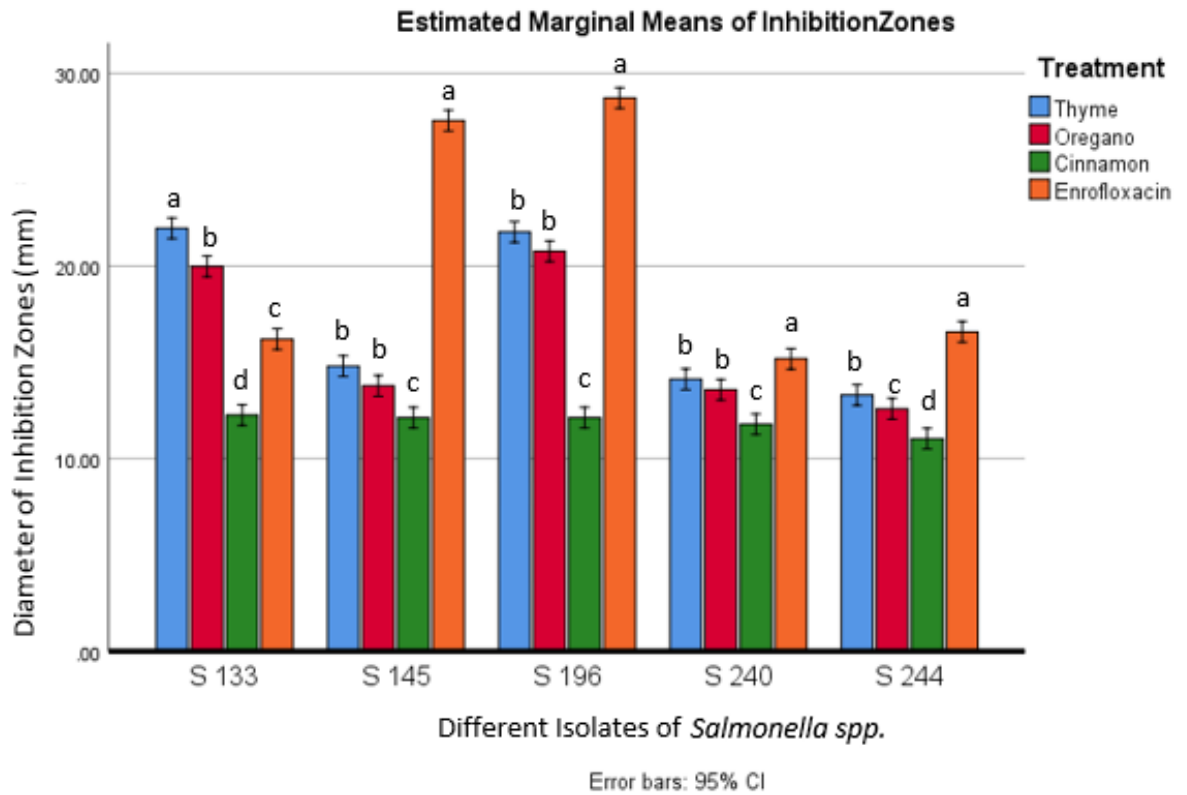


Figure 2: Effect of 60% essential oils and enrofloxacin on growth of *Salmonella* spp.

Values are mean \pm standard deviation. Means with different superscript within the same isolate differ significantly ($p < 0.05$).

5.0 DISCUSSION

Looking at the trend of the zones of inhibition produced by different concentrations of all essential oils, the partially diluted (40% to 60%) essential oils produced similar antibacterial effects against all the tested bacteria compared to the undiluted essential oils. This suggests that stock solutions of essential oils can be diluted to obtain the desired inhibitory effects in a more cost-effective way.

At 60% concentration, all of the essential oils produced inhibition zones against all the selected test bacteria, but all reacted differently, producing different diameter of zones of inhibition towards different isolates of test bacteria. These results are similar to several studies that were reviewed by Mandal and DebMandal (2016). The differences in the inhibitory effect might be due to different concentration of essential oils used, with different amounts of antimicrobial phytochemicals contained or simply due to different serotypes of the same species of bacteria.

The major phytochemicals of Thyme and Oregano essentials responsible for the antibacterial properties consist of thymol and carvacrol respectively. Both of these components have shown similar inhibitory effects against selected *E. coli* and *Salmonella spp.* which is in agreement with several studies (Helander et al., 1998; Xu et al., 2008) that have proven both thymol and carvacrol inhibited *E. coli* equally. The similarity between Thyme and Oregano is due to their major

component which is Thymol and Carvacrol that have a same precursor which is γ -terpinene (Rudolph et al., 2016), and this monoterpene has been said to have excellent antimicrobial properties (Kainat et al., 2019). The significant antibacterial effect of thymol and carvacrol also coincide with other studies (Memar et al, 2017, Sakkas et al., 2017) especially on multidrug resistant test bacteria (**Table 1, Table 2**).

Generally, the inhibitory effects produced by the essential oils, especially against different selected *Salmonella* spp. were significantly smaller than the tested commercial antibiotic, enrofloxacin. This result is in accordance with the study done by Dakhili and his colleagues in 2006, where thyme oil was less effective than enrofloxacin against *Salmonella typhimurium*. Although the efficacy may not be as good as enrofloxacin, it is suggested that these compounds can be developed and applied as an alternative to other commercial antibiotics for prophylaxis. This will be particularly useful when used as a feed additive to improve growth performance in poultry where there are already some positive results (Abdel-Wareth et al., 2013)

Eugenol, the antimicrobial phytochemical in Cinnamon essential oils have shown the weakest inhibitory effect against the tested bacteria compared to other treatments, is in accordance with other studies (Atki et al., 2019; Pei et al., 2009), in comparison to thymol and carvacrol on *E. coli* and other types of test bacteria.

This variation may be due to the different mechanism of action of different phytochemicals in different essential oils. Since the hydroxyl groups of eugenol are able to combine with cell cytoplasm protein, Pei et al. (2009) hypothesized that eugenol works better when the cell membrane is disrupted by Thymol and Carvacrol to enter the cytoplasm to inhibit enzymatic actions of the bacteria to kill them. Therefore, this also suggests that eugenol can be combined with other more effective phytochemicals or commercial antimicrobial agents to have a better inhibitory effect. This may help to improve the multidrug resistant bacteria sensitivity towards the antibiotic (Johny et al., 2010).

As 60% of *E. coli* and *Salmonella spp.* from the selected test bacteria were resistant towards more than 3 different commercial antibiotics each in different categories, more than half of the selected test bacteria were regarded as multidrug resistant. The multidrug resistance *E. coli* and *Salmonella* in this study was lower in comparison to other studies (Geidam et al., 2012, Azad et al., 2019; Alam et al., 2020), where all their isolates were regarded as multidrug resistant. All tested bacteria possess maximum resistance towards erythromycin. In the present study, 100% of the tested *Salmonella* isolates were resistant towards erythromycin similar to the findings of Chahbi et al., (2020). Schwartz et al., 2006, similarly reported that *Salmonella* is naturally resistant towards erythromycin. In contrast, a 100% resistance *E. coli* isolates towards erythromycin in this study is higher than those reported by other studies (Kappel et al., 2015; Kibret & Abera,

2011). The variations in prevalence of multidrug resistant bacteria may be linked to different management factors of the farm, such as antibiotics usage, hygiene and sanitation, also the mobile resistance genes possessed by the bacteria that facilitate the emergence of multidrug resistant bacteria (Partridge, 2015).



6.0 CONCLUSION

Essential oils from thyme, oregano and cinnamon was proven to exhibit inhibitory effects towards all clinical isolates of *E. coli* and *Salmonella spp.* obtained from broiler chickens in this study. More than half of the selected test bacteria were found to be multidrug resistant against 6 different categories of commercial antibiotics. Enrofloxacin produced the largest inhibition zone on average against all the selected test bacteria. Antimicrobial properties of the essential oils have been seen towards, not one, but all, including multidrug resistant isolates. Thyme and oregano essential oils both equally produced the largest zones of inhibition towards selected test bacteria, followed by cinnamon essential oils which produced the smallest zones of inhibition compared to other treatments. The essential oils evaluated in this study have potential to be applied as alternatives to commercial antibiotics used for prophylactic purposes in poultry farming.

7.0 RECOMMENDATION

For future studies of the essential oils antibacterial activities, the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of the different major phytochemicals should be determined for better and more accurate efficacy comparison with the commercial antibiotics. Combination of the essential oils antimicrobial property could be done by using the checkerboard method to evaluate the combined effects using Fractional Inhibitory Concentration (FIC) and Effect of the Combination (EC) to determine if the combined essential oils have lower MIC compared to the single essential oils MIC (Pei et al., 2009).

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APPENDICES



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9.0 APPENDICES

Appendix 1

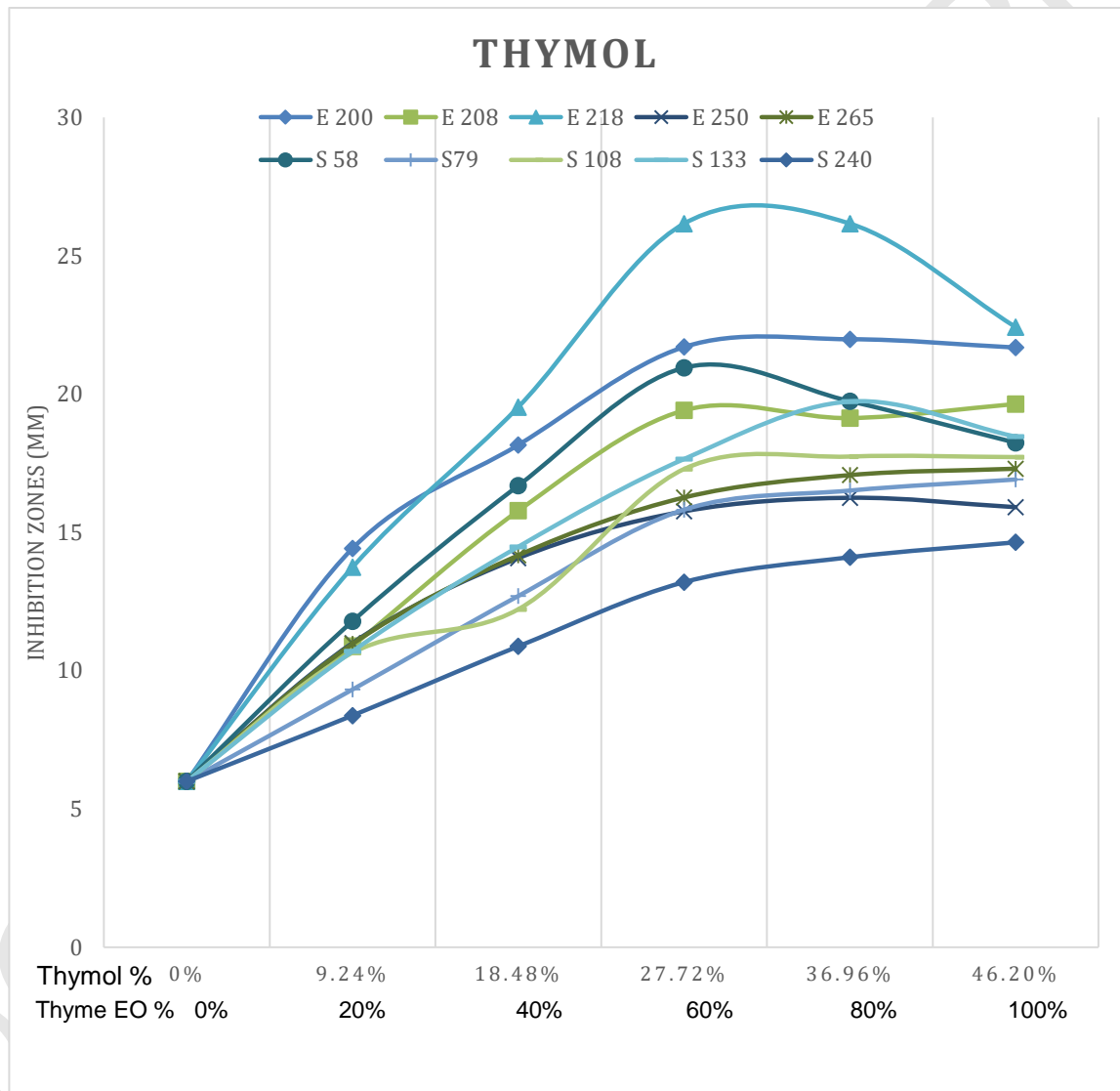


Figure 1: Inhibition Zones of Different Thyme Essential Oil Concentration against Different *E. coli* & *Salmonella* spp.

Appendix 2

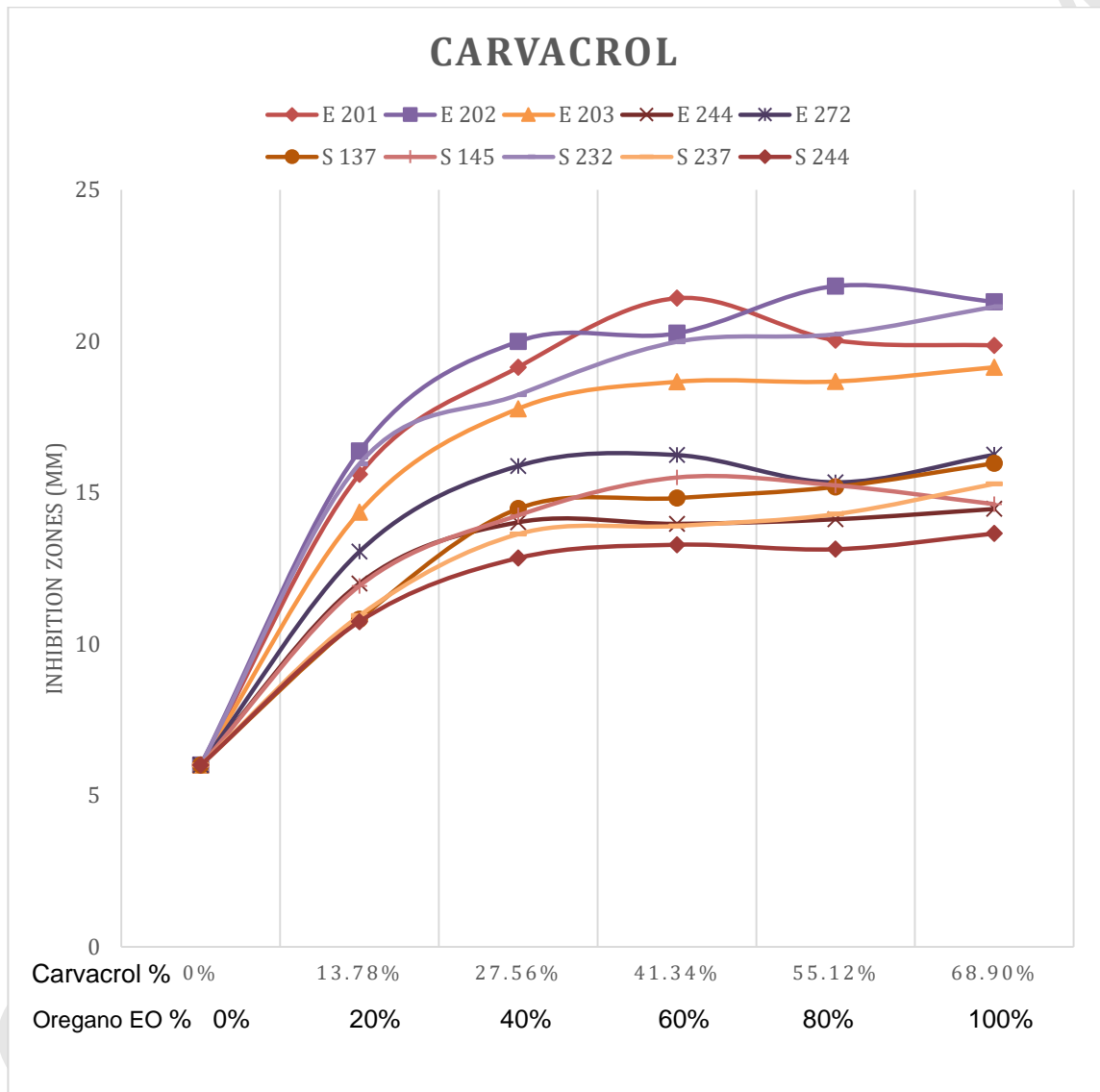


Figure 2: Inhibition Zones of Different Oregano Essential Oil Concentration against Different *E. coli* & *Salmonella* spp.

Appendix 3

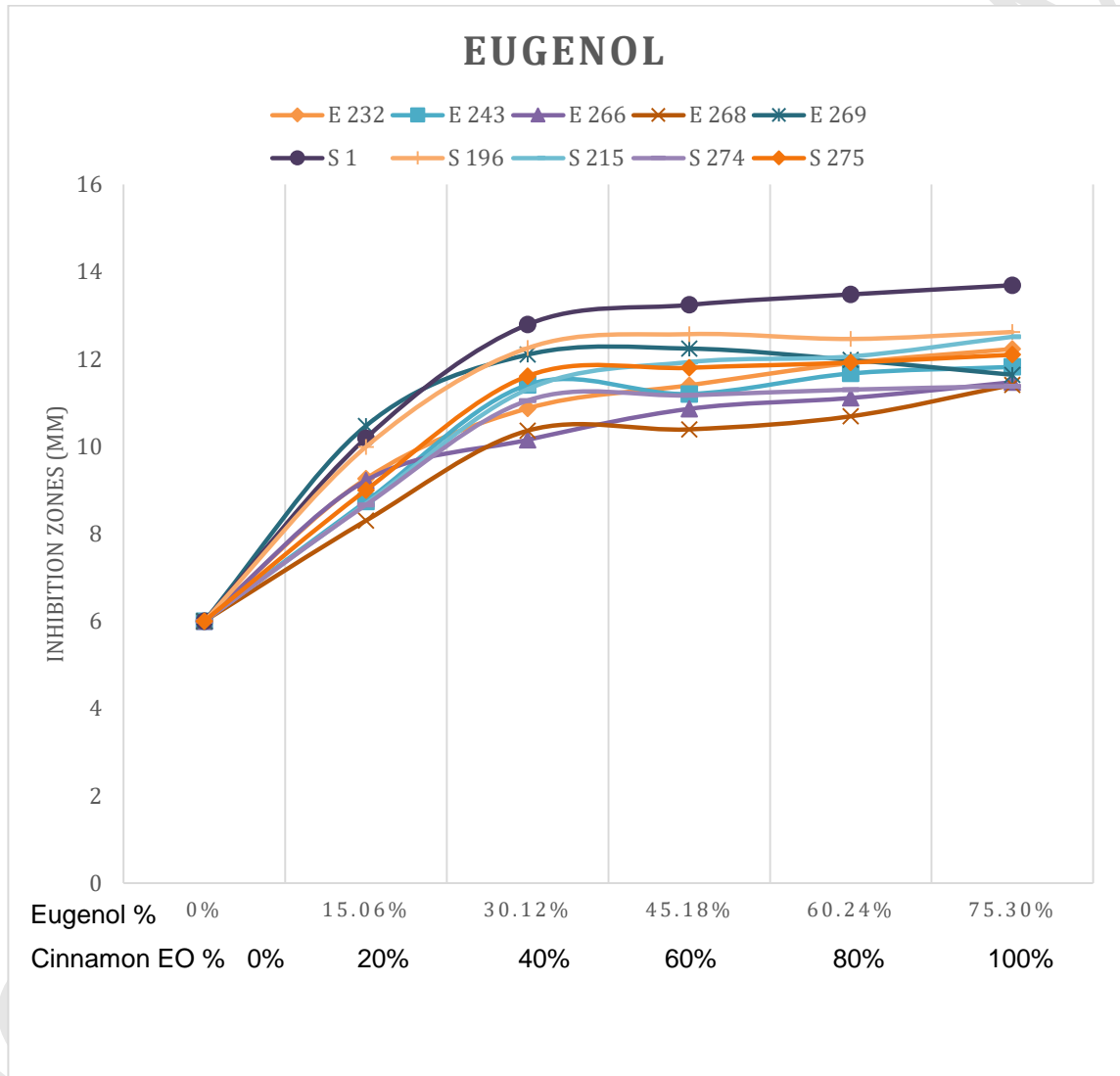


Figure 3. Inhibition Zones of Different Cinnamon Essential Oil Concentration against Different *E. coli* & *Salmonella* spp.