INVESTIGATE THE BACTERICIDAL EFFICACY OF CARVACROL FROM OREGANO ESSENTIAL OIL AND EXTRACT COMPARED WITH COMMERCIAL DISINFECTANT.

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TABLE OF CONTENTS

ABSTRACT

Herbal extracts and oils are potential alternatives to chemical-based disinfectants due to concerns on antibiotic resistance and adverse consequences of synthetic antimicrobials on human health. A native Mediterranean spice herb, Oregano harbors bioactive compounds Carvacrol and Thymol which possessed outstanding bacteriostatic effect on broad community of microbes. A comparative analysis of Oregano plant-based extracts, their oil and commercial synthetic antimicrobial was focused on this research. Solvent extraction method for Oregano extracts synthesis and microdilution assay was performed to determine the minimum inhibitory concentration of derived product against Escherichia coli ATTC®15597 strain. Four products viz: ethanol extracts from freshly produced Oregano, Carvacrol rich essential oil of Oregano, Benzalkonium chloride and Sodium hypochlorite-based disinfectant were assessed for their efficacy as antimicrobial agent in invitro condition. Data analysis was performed using SPSS and MS-EXCEL to calibrate the bacterial growth curve based on optical density at 600nm. All the disinfectants killed bacteria at different concentration at different time and a significant bactericidal effect was observed between plant based and chemical based biocide. Oregano essential oil at 25% concentration, Bleach at 100%, 50%, 25% and 12.5% concentration, Pine-o-Cleen® at 25%, 12.5%, 6.3% and 3.2% dilution were highly effective to suppress bacterial growth for 24 hours. Oregano ethanol extracts depicted moderate antibacterial effect until 10-15 hours of exposure but fails for a complete growth inhibition. Oregano-based antimicrobials exhibited a promising substitute against conventional disinfectants and inherent challenges of antibiotic resistance offering reduced toxic effects on human health.

DECLARATION

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ABBREVIATION

AIHW : Australian Institute of Health and Welfare

ATCC : American Type Culture Collection

BAC : Benzalkonium Chloride

BHI : Brain Heart Infusion

CAGR : Compound Annual Growth Rate

CFU : Colony Forming Unit

DMSO : Dimethyl Sulfoxide

Eos : Essential oils

GC-MS : Gas Chromatography-Mass Spectrometry

HPLC : High Performance Liquid Chromatography

LB : Luria Bertani Broth

MBC : Minimum Bactericidal Concentration

MDR : Multi Drug Resistant

MN : Micro nucleus

MRSA : Methicillin Resistant Staphylococcus Aureus

MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

OECD : Organization for Economic Commerce and Development

OEE : Oregano Ethanol Extract

OEO : Oregano Essential Oil

PA : Pseudomonas Aeruginosa

POC : Pine-o-Cleen®

QACs : Quaternary Ammonium Compounds

STEC : Shiga Toxin Producing Escherichia Coli

TPC : Total Phenolic Content

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: INTRODUCTION

Cleaning and sanitization are basic prerequisites to maintain and minimize the microbes within safe level for human being. The economically feasible way to combat the spread of infections from public spaces can only be possible from used of disinfectants. Increasing number of people with low immune response and higher susceptibility to microbial infections is major driving factor for development of effective disinfectants. Recent trend of immunisation and vaccination had lowered the burden of severe infections, but sanitisation has always been considered as initial strategy to overcome the detrimental consequences (Australian Institute of Health and Welfare, 2022). While chemical disinfectants are widely used as cleaning agent reasonable concern aroused about its potential harm to human health and its role in antimicrobial resistance. Over a prolonged period, this chemical exposure from disinfectants to food and human could have compromising health effects to be concerned. Moreover, increasing concentration of benzalkonium chloride had been correlated with bacterial adaptive resistance and development of cross resistance of Listeria monocytogenes like bacteria to Ethidium bromide, Ciprofloxacin's and Cephalosporins (Yu et al. 2018).

Oregano essential oil (OEO) has emerged as potential alternative antimicrobial agent. Its constituents, Carvacrol and thymol, had been studied for their antimicrobial properties and have shown effectiveness against various pathogens (Chami et al. 2005, Siroli et al. 2014).Other than this, Oregano oil's had been used in feed additives for its immunomodulating and antioxidizing nature (Alagawany et al. 2020). While some research had assessed Oregano oil's efficacy against bacterial biofilms, gaps remain in understanding its effectiveness, that needed to be confirmed through repetitive studies (Valeriano et al. 2012). The biotechnological significance of the study is to develop an alternative antimicrobial by evaluating the potential of Carvacrol rich Oregano oil and fresh Oregano ethanolic product as an environmentally friendly alternative of chloride-based

disinfectants using Escherichia. Coli (ATTC 15597) clinical strain as an indicator in ambient growth environment. The global demand of the synthetic disinfectant was reported to be inclining along with the associated threats of antibiotic resistance, prolonged persistence of disinfectants in environment and threats of bioaccumulation in food chain therefore the study will be helpful to address need of novel biocides through antibacterial efficacy test of Oregano based products in oil and extract forms. Similarly, Australia grown Oregano spices had been observed with significant amount of total phenols (140.59 ± 9.52 mg GAE/gm) and antioxidants (DPPH: 23.24 ± 1.23 mg AAE/g) but research work on antimicrobial properties for these culinary spice was limited therefore this study will insights on antimicrobial value for this product (Ali et al. 2021).

The introductory section will emphasized on review papers related to Oregano oil, their antimicrobial nature, meachanism of action of essential oils (EOs) constituents, underlying safety concerns, methods employed for the extraction and antimicrobial assessment.

1.1 Trend of chemical disinfectant use.

Disinfectants are a germicidal agent which is non-specific to microbes and can be applied on non-living surfaces that ensure no traces of vegetative spores. Various mode of microbial inhibition including alkylation, oxidation or protein denaturation has been expected from these agents. (Kozmenko et.al 2014). Quaternary Ammonium Compounds (QACs) are widely used supplements for domestic cleaning products. Benzalkonium Chloride (BACs) was expected to be highly produced QACs (20-25million pounds) with majority (approximately 80%) of use in disinfectants or sanitizers and biocides whereas minor amount in hair conditioners in shampoos and cream rinses, emulsifying agents, and deodorizers.(Boethling 1984). Alcohol based disinfectants as an effective disinfectant exhibited a promising market growth because of the biocidal property and easy vaporisation after use. Global market on alcohol-based disinfectants attributed a market size of 1.78 billion (USD)in 2019 and has been projected a CAGR of >11% to attain a market value of 3.78

billion(USD) by 2026.In an industrial setting these disinfectants are used for sanitizing municipal and streets areas, air craft and industrial equipment whereas higher demand for food storage sanitation, pest elimination, household disinfection, industrial and commercial floor cleaning had been expected to be significant drivers for incremental consumption in future. (Global Market Insights, 2020).

1.2 Antimicrobial efficacy of Oregano Essential Oil

An antimicrobial enhancement with 0.5%v/v Oregano essential oil (OEO) proved to be an efficient detergent solution for handwashing and surface decontamination. Inoculation of Salmonella containing wipes with 0.2%OEO solution decreased bacteria vole by 1log Colony Forming Unit (CFU)/wipe. Minimal effects of 0.2% OEO solution was observed for *E. coli* whereas reduction of 3log CFU/wipe was noticed with 0.5% OEO solution. At a higher concentration (1% OEO solution),no pathogens were detected and the bacterial counts were below 2.7 log CFU/wipe.(Rhoades et al. 2013)

Similarly, stainless steel surface decontamination for *E. coli* after wiping with water only produce 2.4 log CFU, plain shop was also not effective whereas combined effect of soap and OEO removed all the detectable microbes(<0.95logCFU). Antimicrobial wiping of wooden surface using water, plain soap, and soap with OEO influenced bacterial survival rate of 6.9, 4.2 and 2.3 log CFU respectively while plastic surface were detected with visible count of 4.9, 1.8 and 1.2 log CFU respectively (Rhoades et al. 2013).

Uropathogenic *E. coli* biofilm inhibition has been investigated and 79 essential oils were screened for their effectiveness in 96-well plates under crystal violet assay, scanning electron microscopy, and confocal laser scanning microscopy. Biofilm producing ability of Uropathogenic *E. coli* was inhibited at sub lethal concentrations below 0.01% of Oregano oil and thyme red oil. Carvacrol and

thymol were expected to subdue fimbriae development and swarming motility of the bacteria. (Lee et al. 2017).

The MIC and MBC estimation of Oregano oil (diluted in ethanol 96%) for incremental inoculation of 2log CFU/ml, 4log CFU/ml and 6log CFU/ml each of Listeria monocytogenes ScottA and *E. coli* 555 shows higher level of MIC and MBC. The MIC value of essential oil for L. monocytogenes lies between 125-275mg/ml whereas for *E. coli* it ranged between 250-350mg/ml. Higher MIC value was estimated in gram negative bacteria due to the presence of outer membrane that is selectively permeable and impervious to hydrophobic macromolecules. (Siroli et al. 2014)

1.3 Plant part as source of Oregano oil compounds.

Origanum vulgare subsp. *gracile* was observed for essential oil constituents in various plant parts including leaves, stem, roots, and flowers. Carvacrol were extracted in the essential oils from flowers (79.2%), roots (70%), and the early vegetative growth (67.34%) (Morshedloo et al. 2018). Substantial amounts of Carvacrol were also found in both the leaves (45.92%) and stems (18.1%) of the plant. Among the stem components, the essential oil contained the significant concentrations of p-cymene (15.8%), g-terpinene (13.19%), (Z)-b-ocimene (6.82%), b-pinene (5.82%), (E)-b-ocimene (5.17%), and myrcene (4.8%). Fifty-two compound was observed in stem/leaf of Oregano richly harbouring sesquiterpene hydrocarbons (33.2%) and oxygenated sesquiterpenes (25.6%), Caryophyllene oxide (18.1%) and germacrene D (13.5%). Similarly, the flower parts from shaded region supplied up to 51 compounds of chiefly oxygenated sesquiterpenes (76.1%), Caryophyllene oxide (25.5%), germacrene D (14.5%), and (E)-caryophyllene (10.8%) (Ilić et al. 2022).

1.4 Chemical composition of Oregano oil.

The variation in the *O. vulgare* essential oil composition has been associated with distinct ecological conditions, collection periods and methods, origins, plant populations, stage of plant growth, and extraction and quantification methods.(Bisht et al. 2009). Oregano has been assessed for its qualitative and quantitative properties at different phenological stages which is expected to vary accordingly with growth and plant parts. Overall, 41 metabolites had been extracted from *Origanum vulgare* subsp. *gracile* essential oil extract comprising highest amount of *monoterpenoids* Carvacrol (18.1–79.2%) followed by p-cymene (3.9–18.0%), γ-terpinene (0.9–15.8%), Carvacrol methyl ether (0.8–9.9%), and (Z)-α-bisabolene (0.5–8.7%). (Morshedloo et al. 2018).

O. vulgare species hirsutum essential oil from Greek origin was assessed for deriving their antioxidant behaviour against lard oxidation. 16 compounds accounting for 97.6% of Oregano essential oil composition, with thymol (40.4%), Carvacrol (24.8%), and p-cymene (16.8%) as the primary constituents, and γ-terpinene (1.7%), 1-octen-3-ol (2.1%), borneol (1.2%), and terpinen-4-ol (2.1%) in minor quantities were observed for their anti-oxidising effect.(Miloš et al. 2000)

Oreganum vulgare essential oil from Portuguese origin was found to be composed of 64 compounds, collectively constituting 92.3% of its composition, with a predominant presence of oxygenated monoterpenes (53.8%) and monoterpene hydrocarbons (26.4%) while assessing their invitro antioxidant and antimicrobial properties. Major compounds examined by Agilent 6890 gas chromatograph within the oxygenated monoterpenes included Carvacrol (14.5%), thymol (12.6%), β-fenchyl alcohol (12.8%), and δ-terpineol (7.5%), while γ-terpinene (11.6%), α-terpinene (3.7%), and 1-methyl-3-(1-methylethyl)-benzene (6.8%) were the main monoterpene hydrocarbons (Teixeira et al. 2013).

1.5 Mechanism of action of Oregano essential oil

Essential oils have been found to be higher radical scavengers and this antioxidant property varied according to the plant parts and growth stage. *Origanum vulgare* subsp. *gracile* flowers essential oil exhibited the maximum antioxidant activity (EC_{50} =50.68mL/mL) at flowering stage whereas the stem oil had the minimum antioxidant activity in their vegetative and seed set period of growth. (Morshedloo et al. 2018). Investigation of mRNA expression of hilA, prgH, invA, sipA, sipC, sipD, sopB, sopE2 genes from *Salmonella typhimurium* cultures alone (CTR), and with either thymol 0.47mM or Carvacrol 0.47 mM at invitro condition exhibited a statistically significant ($p < 0.05$) inhibition of these genes with Carvacrol and thymol. Moreover, except sipC gene comparatively higher level of mRNA expression was limited by Carvacrol 0.47mM than thymol 0.47mM.(Giovagnoni et al. 2020). Oregano oil was effective in controlling MRSA (Methicillin Resistant Staphylococcus Aureus) bacteria where it ruptured the bacterial cell membrane leaking the cell content. Tri-carboxylic acid (TCA) cycle inhibition had been noticed to affect the production of its metabolites. The Carvacrol in essential oil also limited the expression of PVL gene and production of PVL toxins.(Cui et al. 2019).

1.6 Safety and toxicological aspect of essential oil

Oregano essential oil had been in the list of generally recognized as safe products for general provisions by Code of Federal regulations Title 21 (U.S. Food and Drug Administration, 2024).The examination of genotoxicity of *Origanum vulgare L. virens* essential oil (OEO) in a 90-day repeated dose oral assay (micronucleus (MN) test and comet (standard and enzyme-modified assays)) in 40 male and 40 female Wistar rats at various doses of 50, 100 and 200mg/kg body weight as per OECD guideline 408 indicate that OEO did not prevail mortality or any forms of oxidative impairment in tested specimens. Even at the highest tested dosage of oral feeding (200mg/Kg), no significant observed effect was noticed. The study concluded OEO as a safe food packaging

alternative because of its non-toxic nature even at significantly higher level (330-fold) than expected consumer exposure levels..The chemical composition of tested OEO as observed in gas chromatograph was Carvacrol (55.82%), ρ-cimene (16.39%), thymol (5.14%), ϒ-terpine (4.71%) and β-cariophyllene (2.40%) (Llana-Ruiz-Cabello et al. 2017).

Similarly, during in vivo study of Oregano essential oil opposed to 11 MDR clinical isolates comprising 4 *Acinetobacter baumannii*, 3 *Pseudomonas aeruginosa*, and 4 methicillin-resistant *Staphylococcus aureus* (MRSA) and 2 luminescent strains of PA01 and MRSA USA300 higher level of microbial inhibition was observed. In female BALB/c mice model, administering 5 or 10mg/ml of grape seed oil diluted Oregano essential oil (Carvacrol (72.25%), thymol (6.62%), p-cymene (5.21%), γ-terpinene (4.12%), α-pinene (1.21%)) topically on third degree burn wounds for three times in three consecutive days corresponding to control groups reported no negative side effect on the skin histology. Tunnel assay for genotoxicity also revealed similar non-toxic effect of Oregano oil (Lu et al. 2018). The results derived from treatment of Candida albicans in oral candidiasis of rats also show an excellent inhibition of the fungal isolates along with absence of severe toxicity of Carvacrol and eugenol at approximated dose of 20µg/kg and 10 µg/kg respectively. (Chami et al. 2005). Also, invitro cytotoxicity evaluation by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in HEL 12469 human embryonic cells lines exhibited 50% cell death at 0.058 µL/mL for Essential oil of Oregano. The cytotoxicity assessment for wide range of essential oil from Oregano, *Thymus vulgaris, Lavandula angustifolia*, *Eugenia caryophyllata*, *Thuja pelicata* and clary sage had a lowest cytotoxic effect on HEL12469 cells as assessed within 24 hours period. The Carvacrol content in Oregano oil was not mentioned in the research paper (Puškárová et al. 2017).

1.7 Selection of growth media.

Different growth media has been focused for their favourable response on *E. coli* survivability. Luria broths are suited for rapid growth of *E. coli* plasmids and higher yield of inoculum. Terrific broth on the other hand constituted glycerol as major energy source supporting multiple microbial loads and counteracts the acidity problems with potassium phosphates (Lessard 2013). Trypticase Soy Broth (TSB) supported the growth of *E. coli O157:H7* in both acidic pH 4.5 induced by lactic acid and alkaline pH 9 from 6.5% NaCl. But fermentation conditions, drying and temperature below 4°C was not favourable for *E. coli* growth in TSB (Glass et al. 1992). The viability assessment test for *E. col ATTC* 8739 has confirmed that three growth media Nutrient Broth (NB), Brain Heart Infusion (BHI) and Luria-Bertani Broth (LB) could be suitable media even up to 11 weeks for *E. coli* growth if it is supplemented with optimal glucose level (Low et al. 2013). Moreover, the comparative analysis of TSB, MacConkey, and Gram-negative broth for enrichment of 52 isolates consisting of O157 and non-O157serotypes of Shiga Toxin producing *Escherichia coli* (STEC) depicted TSB as the more suitable initial enriching medium than rest of two. MacConkey and Gram-negative broth, being a selective media failed to support wide range of STEC isolates compared to the general media (Bording-Jorgensen et al. 2021).

1.8 Streak plate method for isolation of pure culture:

It is one of the important aseptic techniques for isolation and propagation of bacteria from mixed culture to a pure culture. Source for the bacterial inoculum could either belong to cell suspension broth or original agar plate of source culture in this method. Streaking in the form of quadrant could facilitate for robust single colony identification and isolation (Sanders 2012).

1.9 Microdilution assay:

Microtiter plate dilution method is considered as convenient and rapid way for a large-scale bacterial screening. Some literatures have used microtiter plates for assessment of MIC value of Oregano oil. Oregano stock solution is recovered in DMSO (40mg/ml) followed by two-fold dilution in brain heart infusion medium. Well plates were run with 20 µL of the suspensions containing 10^8 CFU/ml of bacteria and 180 µL diluted Oregano oil at different concentrations. Control wells are supplied with DMSO and media only. MIC estimation is based on the turbidity of wells, produced by bacterial cell growth after microplates are incubated at 37°C for 24 h period. (Lu et al. 2018). The use of microtiter plate in immunoassay is found to be very effective for detecting sensitivities for low drug concentration.(Kroener et al. 2003). Since surface attached bacteria has been shown to be more resistant to disinfectant compared to cells in planktonic broth. Therefore, further confirmation of efficacy of disinfectant should also be checked in the surface test. For 18 commercial disinfectants under four different category (alcohol, mixed, chlorohexidine) microtiter plate assay follows similar 2-fold dilution of disinfectants in mineralized water. 100 µL of disinfectants added to 100 µL mineralized water was serially diluted for its concentration by adding 100 µL of mineralized water for up to 7 times (A-G rows). Each well was subjected with 100 µL of different concentration of disinfectants and 100 µL of bacterial inoculum. Positive control is composed of sterile water and bacterial inoculum whereas negative control contains only tryptone soy broth and sterile water. MIC was assessed in terms of visible bacterial growth across the well.(Alajlan et al. 2022).

1.10 Extraction, drying and storage of Oregano oil.

In an invitro assessment of antioxidant and free radical scavenging capacity, a powdered Oregano (25 grams) was stirred continuously for 2 hours in 150ml ethanol (95%) at 60-degree Celsius temperature followed by drying using Büchi Rotavapor/water bath under vacuum at 40 degrees Celsius and stored at 4 degrees Celsius.(Amarowicz et al. 2009). The confirmation of ethanol concentration has been related to TPC (Total Polyphenol Content) for which 60% and 80% ethanol was best suited producing higher (ABTS and DPPH) antioxidant values respectively in *Centella asiatica* (Chew et.al., 2011). Carvacrol content in Oregano oil show considerable volume ranging from 56.2 to 81.4% when six different drying methods (shade drying at 25°C, hot air convective drying, microwave assisted drying in 3 different conditions and Osmotic treatment) was used. Assisted microwave heating/1150 W for 15 min show the highest Carvacrol yield under GC-MS and GC-FID chromatographic evaluation.(Caputo et al. 2022). *Thymus daenensis subsp. Daenensis* has been found with incremental volume of Carvacrol and thymol constituents in its essential oil when plant leaves were oven or microwave dried at 70°C temperature. Microwave drying produced relatively low essential oil but it has been found effective in terms of short drying time, colour quality and increment of major oil components.(Rahimmalek and Goli 2013).

The phenolic component for Origanum vulgare Extraction using 2L distillation unit at various time frames from 1.25, 2.5, 5, 10, 20, 40, 80, 160, 240, and 360 min were studied for which the highest concentration was found after 240 minutes of distillation. 2.312gm Oregano oil per 100gm of Oregano leaves was the highest volume extracted at 240 minutes. (Zheljazkov et al. 2012). In a comparative assessment of conventional hydro distillation with microwave assisted hydro distillation method (MAHD) the duration for complete extraction differed among applied techniques and conditions. Depending on the heat application of 600, 360 and 180W in microwave assisted hydro distillation method total extraction time was 24, 29, and 45 min whereas hydro

distillation method took 136 min. The commencement of extraction also varied from 4, 9, and 25 min for MAHD at 600, 360, and 180 W, respectively, while in case of hydro distillation 16 min was required. Heat transfer in hydro distillation was expected to occur gradually in contrast to volumetric dispersion of radiated heat in MAHD method (Drinić et al. 2020). Hydro distillation method has been assessed with higher production of monoterpene hydrocarbons whereas supercritical extraction was fruitful for oxygenated monoterpenes (Busatta et al. 2017). Components with higher molecular weight was expected in the retention time of 30-40 min from hydro distilled essential oils. Modified Clevenger method for extraction of peppermint and lemon grass essential oil from the fresh vegetal mass was able to produce the hydrolate within 2.5hours at approximately 100°C (de Oliveira et al. 2010). Clevenger apparatus has been used for extraction of light-dark yellow oil from finely ground aerial parts of Oregano within a time of 4 hour. Moisture content in the essential oil were dried using anhydrous Sodium sulphate (Özkalp et al. 2010).

Organic solvents show a higher yield of total phenolic content and greater scavenging activity for Mediterranean and Mexican Oregano in the order of Acetone>Methanol>Ethanol>Water. Also, among the various techniques of extraction including shaking, sonication, and vortex method, vertexing the specimen was assessed to be best method with higher yield of phenols from both type of Oregano (Karimi et al. 2015). Phenolic compound Carvacrol had been assessed with no polymerization or oxidation loss at higher temperature of 80 ℃ thus expressed no significant changes in colour or smell (Gandova et al. 2023). The heat stability for Carvacrol had been related to the aromatic ring structure in Carvacrol thus preventing the loss of its antimicrobial functions during extraction. process. Thermal stability of Carvacrol in nanofibers had been assessed to found Carvacrol degradation around 200 ℃-250 ℃ thus representing itself as thermostable encapsulating agent (Fonseca et al. 2019). Terpenes recoveries was found to be 70-80% at 100 ℃ and the highest Carvacrol was obtained at 150 ℃ but higher temperature exposure for prolonged

period(300min) induced poor stability of terpenes during subcritical water extraction test (Yang et al. 2007).

Qualitative and quantitative assessment of Oregano oil constituents has been done using HPLC chromatogram (Fraj et al. 2019) or Gas Chromatography coupled to Mass Spectrometry (de Oliveira et al. 2010) and GC-FID (Morshedloo *et.al* 2018). Hewlett Packard gas chromatograph 6890 GC with an autosampler [carrier gas helium, 40 cm·sec−1, 11.7 psi (60 °C), 2.5 mL·min−1 constant flow rate; injection: split 60:1, 0.5 µL, inlet 220 °C; oven temperature program: 60 °C for 1 min, 10 °C/min to 250 °C] has been used to analyse the constituents of distilled Oregano oil. The column used for detection was HP-INNOWAX (crosslinked polyethylene glycol; 30 m × 0.32 mm × 0.5 µm) and the flame ionization detector temperature was 275 °C (Zheljazkov et al. 2012).

Storage related changes has been assessed for *Leonurus cardiaca L.* (motherwort) essential oil where two-fold decrease in initial hydrocarbon concentrations was found at the end of 7 month. The critical factor affecting the essential oil constituents with low boiling temperature such as mono and sesquiterpene hydrocarbons were evaporation and oxidation (Mockutë et al. 2005).

1.11 Biofilm development

The ability to develop biofilm varied across the human pathogenic Shigatoxigenic *E. coli* exhibiting higher level of biofilm growth by seropathotype A (O157:H7 and O157: NM) compared to B and C. (Vogeleer et al. 2016). Exopolysaccharide (ATCC 4385 EPS) and curli production (mutant strain ATCC 43895) by *E. coli* was reported to increase the biofilm resistance against chlorine to some extent. Curli production by *E. coli* was associated to the biofilm formation rather than on bacterial adhesion on surface (Ryu and Beuchat 2005). The nutrient concentration has been found to be directly correlated to volume of bacterial growth on liquid media and contact surfaces exhibiting significant bacterial colonization. Bacterial adhesion behaviour has also been guiding factor for

stable colony formation across these media (Cowan et al. 1991). An optimal temperature of 37°C, proper aeration, and pH 7 was found to double the growth of E coli within 20 minutes in Luira-Bertani (LB) broth and further attain overnight cell growth of more than 10^9 CFU/ml.(Son and Taylor 2012). In relation to contact surfaces studies showed that the growth of *E. coli O157:H7* significantly increased by $3\log_{10}$ CFU/coupon after 1 day incubation at 25 °C and the pathogenic volume was maintained for consecutive 7 day. Among the three different broth PBS, DW and TSB, five microbes produced a bacterial count of approximately 3–4, 3–5, and 3–6 $\log_{10}$ CFU/coupon after 2h of initial inoculation at 25°C. Significantly higher pathogenic level (7-8 $\log_{10}$ CFU/coupon) was obtained for TSB after 1 day of storage at RH 100%. While Relative humidity of 100% was suitable to maintain the increased level of 3 $\log_{10}$ CFU pathogen until 21 days in steel coupons (Bae et al. 2012).

1.12 Bacteria Culture for Viability Assessment

Viability assessment of bacterial film has been done done by various methods including permeability assessment by hydrophobic probe, conventional cultivation, resistivity measurement by BacTrac 4100 instrunment, epifluorescence analysis of acridine orange stained sample and so on (Wirtanen et al. 2001). In this report *Pesudomonas species* are detected in conventional method by scraping the biofilms with cotton swab, transferred into Maximal Recovery Diluent, stirred for 1minutes, serially diluted and finally incubated at 30°C on plate count agar for 3 days. Conventional method despite being tedious and time consuming is convenient method for initial detection of bacteria (Nocker et al. 2007). The challenges for molecular assay and polymerase chain reaction despite being rapid method is the over estimation of cell volume during DNA amplification by dead cells and extracellular DNA within the test (Rudi et al. 2005).

CHAPTER 2: AIMS AND HYPOTHESIS

2.1 Aims

The study addresses the questions related to efficacy of plant derived product as an alternative to chemical based bactericidal solutions. This study comprised a fresh Australia grown Oregano herb and a food-based Oregano essential oil for assessment of their potential to be used as an antimicrobial solution.

Aim 1: To extract the Oregano phenolic content through ethanolic extraction method.

Aim 2: To investigate the antimicrobial inhibition of novel disinfectant from different origin against Escherichia coli.

2.2 Hypothesis

From among the disinfectants and plant derived biologicals, Oregano extracts and oil will have greater antimicrobial control than the chlorine-based sanitizing agents.

CHAPTER 3: MATERIAL AND METHODS

This section dictates on the materials, media preparation, method development for extract preparation and the experimental design followed.

3.1 Biosafety training

The microbial strain of Escherichia coli had been used as an indicator organism to assess the microbial response for which PC2 level risk was realized. Biosafety training to excess PC2 facility was completed in initial weeks. This biosafety training certificate was renewed in October 2024 by Flinders University Institutional Biosafety Committee as shown in appendix 1.

3.2 Choice of chemical disinfectant

Two commercially used disinfectant irrespective of their active compounds for bactericidal action had been comparatively assessed with each other and Oregano-based extracts and EO. The widely used hospital grade disinfectant containing 1.6% w/w concentration of Benzalkonium chloride as an active ingredient in 1.25 litre was chosen for this research. The disinfectant had been marketed by its trade name “Pine-o-Cleen”. This Lavender aroma infused product had been prescribed for disinfecting the hard surfaces with traces of *Escherichia coli*, *Streptococcus aureus* and viruses like *Polio virus, Corona 19 virus, Herpes Simplex Virus 1, and Influenza A Virus*.

The second chemical disinfectant selected was Australian manufactured and Woolworths marketed Bleach. This product had been reviewed with 4.8 ratings to be used for general purpose cleaning. An active ingredient for this product as specified in a label was Sodium hypochlorite 42gm/L with available chlorine of 4% w/v and sodium hydroxide 9gm/L. Subsequently, both products had been assessed for their minimum inhibitory concentration of bacteria in pure form and no stock solution has been made for these two disinfectants.

3.3 Oregano Essential Oil

The packaged Oregano essential oil was selected with an assumption that the Carvacrol content >80% was enough to inhibit the bacterial growth to greater extent. The dietary essential oil marketed by Zane Hellas' had been derived from *Origanum heracleoticum* supplemented with blend of 30% part extra virgin olive oil and & 70%-part Oregano essential oil. The certificate of confirmation in Appendix-4 proved that the oil has Carvacrol content >86% and less than 2% Thymol. Oregano essential oil bought in the month of February 2024 and used from February to May.

3.4 Oregano Extract Preparation

Australia grown fresh Oregano leaves available in market were dried at room temperature for three weeks. Once dried, mortar and pestle w ere used for manual grinding of all the leaves into fine powder and debris like stems were removed. After that, 10 g of finely ground Oregano powder was immersed and extracted with 100 mL of 70% ethanol using a magnetic stirrer at room temperature for 4 hours operated at 800 revolution per minute inside the fume hood. The initial extract at the end of 4 hour was filtered using Whatman 1 filter paper fitted in Buchner funnel under the 85kpa vacuum pressure. The residue was re-extracted in 100 mL of ethanol for 8 hours' time and filtration process was repeated. The ethanolic extract was then centrifuged (4000rpm) for 20 minutes, and the combined ethanol filtrates were collected and taken to dryness in a rotary evaporator. This process was optimized from (Chuang et al. 2018) Oregano ethanol extract preparation method. The rotary evaporation unit was operated inside the fume hood all the time to avoid harmful effect of ethanol vapour produced during the evaporation process. Firstly,

heating bath was filled ½ of its volume with water and temperature was maintained at 100-degree

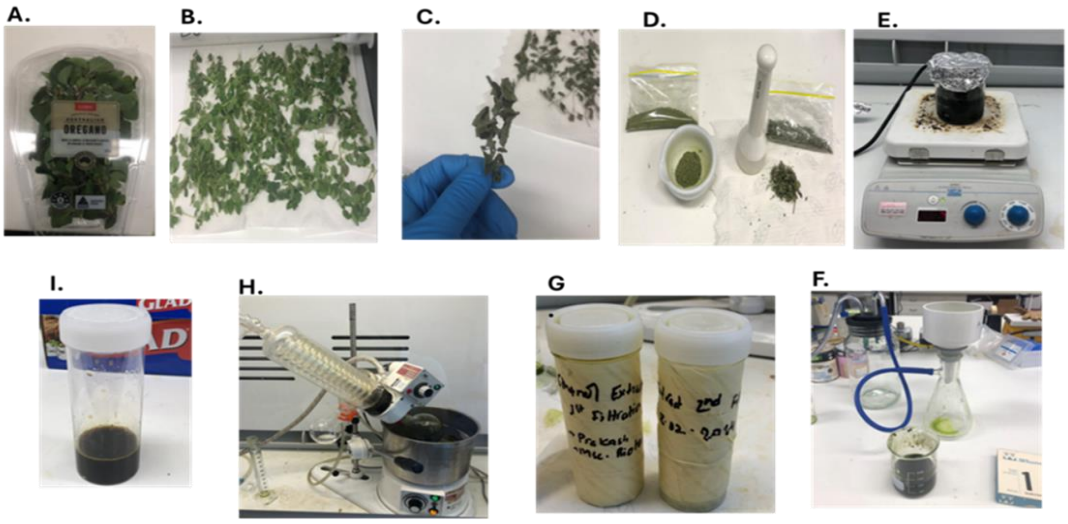


FIGURE A: represented a packaged 10gm of fresh Oregano as a source material, Figure B & C: represented a room temperature dried leaf for day 1 and day 20, Figure D: comprised manual grinding process with finely crushed powder(left) and coarse leaves(right), Figure E: represent ethanolic extraction using magnetic stirrer either at 4 or 8hrs period, Figure F & G: illustrates vacuum assisted filtration setup and filtrated product, Figure H is a step for concentration of extract in rotary evaporator, Figure I is the final ethanol extract of Oregano.

Figure 1: Ethanol extracts of Oregano prepared from rotary evaporation techniques.

Celsius at boiling point of water. The high temperature extraction process was expected to speed up the extraction process. 190 ml of Oregano filtrates was fed into the rotating evaporation flask. Condenser unit is adjusted with cold water flow throughout an experiment whereas vacuum pressure of 85kpa was created at rear end of condenser unit. The evaporation process was operated constantly for a period of approximately 1 hour until the extract was concentrated in to brown and greasy semi liquid form. To avoid the problem of overcooking from rapid evaporation the speed of rotary flask was adjusted, and extract was retrieved prior to sticking of brownish concentrate in the wall of flask. The final extract was recovered using 10ml pipette into a container as shown in figure 1(I). The container was completely sealed with the masking tape to prevent light exposure to the phenolic derivatives present in the extracts. The tube was labelled and sealed properly and stored inside a refrigerator at -20 ℃ until further use. Oregano extract

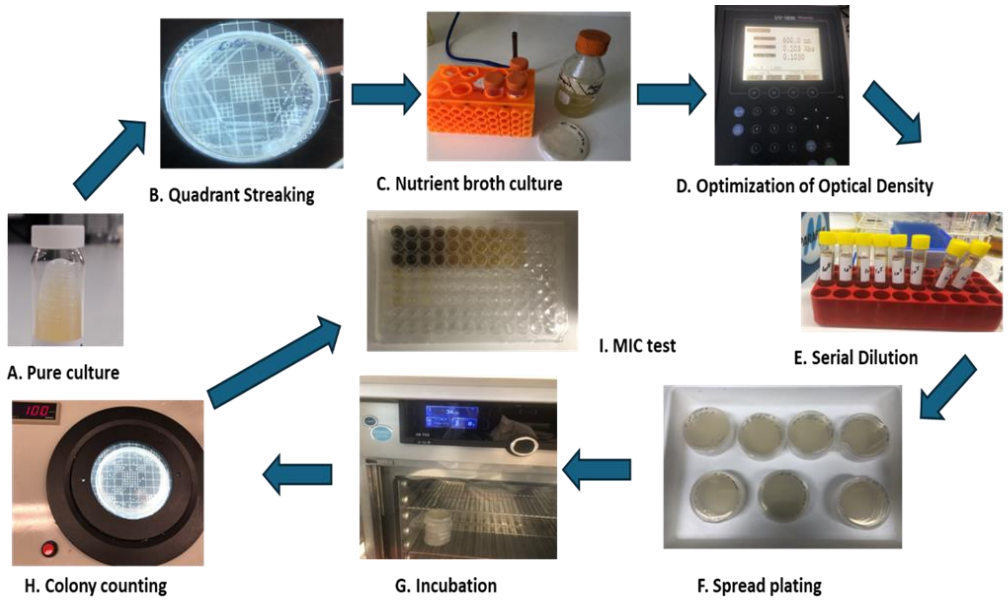
prepared in February 2024 and used for MIC assay from February to May for consistency and limit storability loss.

3.4.1 Trial for Oregano preparation

The source material for the trial experiment was dried leaves of Oregano obtained from the nearest market. The Oregano for this product had been imported and packaged in Australia and was marketed as Garden fresh each containing 10 grams per package. The Oregano used for trial was selected irrespective of their origin of Oregano plant during research during October,2023. The motive of the trial was to be familiar with the techniques used during extraction process and make necessary optimization. Crushing with small volume for (approx. 50 mg) creating the fine powder for extraction. It eased the manual grinding process and prevented yourself from hurting your fingers. 70% ethanol was supposed to be vaporized for prolonged period of stirring as observed during trial when left open inside the fume hood. The beaker was covered with aluminium foil throughout the 12-hour (first extraction: 4hrs and Second extraction: 8hrs) extraction process to minimize the loss. The size Glass beaker was minimized (from 400ml to 200ml) allowing uniform stirring of the powdered product. Large beaker had a disadvantage of escaping the powder mass to the sides and remained isolated throughout the mixing process. Majority of the refinement in techniques for rotavapor use was learnt. It was related with carefully maintaining water level in a water bath, proper fitting of the Keck clips in the joints of round bottom flask and distillation unit. Finalizing the rotating speed of the boiling flask as well as estimation of extract volume to be concentrated in the flask were done. The cooking time for the extract had been minimized to prevent overcooking and stickiness of the dried extract to walls of boiling flask. This step had been followed to prevent the loss of the extract which can’t be retrieved by scraping. The problem with scraping prevailed as result of round structure of flask that can’t be reached, adhesive nature of the extract and small opening (mouth) of flask.

3.5 Media preparation, *Escherichia coli* Culturing and Standardization.

The culture media of choice for growth of *E. coli ATTC®15597* strain was nutrient broth media which was optimized at the concentration of 13gm/litre of water. Moreover, nutrient agar at concentration of 28gm/litre of water was useful for streak plating as well as spread plating. The powdered mass of nutrient broth (3.25gm/250ml water) and nutrient agar (7gm/250ml water) were autoclaved at 121 degree Celsius for 15 minutes and used in experimental culture of *E. coli*.



"Figure-A" illustrated cryopreserved pure culture of ATTC 15597 strain of E. coli. "Figure-B" demonstrated 24hrs incubated streak plates prepared for pure colony isolation. "Figure-C" demonstrated the triplicated 50ml centrifuge tubes containing 20 ml nutrient broth and single *E. coli* colony prior to incubation. "Figure-D" represented bacterial broth standardisation at 0.1 McFarland standard using UV-1800 Spectrophotometer (SHIMADZU UV-1800). "Figure-E" represented 10-fold serial dilution of *E. coli* from 1 to 1:10^8 folds. Figure-F: represented triplicates of spread plated petri-plates each containing dilutions from 1 to 1:10^8 fold dilutions. "Figure-G" illustrated overnight incubation of spread plates at 37 degrees Celsius. "Figure-H" depicted a picture for manual colony counting for 30-300 isolated *E. coli* colony. "Figure I" represented finalization and use of optimized bacterial culture in MIC Test.

Figure 2: *E. coli* culture standardization for MIC test.

Clinical isolates of *E. coli* (ATCC 15597, 19/8/21 EK) pure culture, preserved at -80 ℃ was favoured. It was a commercial strain sourced from bacteriophage of *E.coli K-12* and considered as an indicator strain for disinfection test (Quek and Hu 2008). A sterile loop was used to extract cryo-

preserved *E. coli* from microcentrifuge tube and spread plating was performed on the petri-plates of nutrient agar. Initially, 15 plates were streak plated and incubated at 37 ℃ overnight to isolate pure *E. coli* colony in a Quadrant structure. Fresh sterilized nutrient broth was used to grow single colony of *E. coli* from spread plate to incubate it for 12-18 hours period in a shaking incubator (New Brunswick™ Innova® 44) at 37 ℃ at 100rpm. Triplicates of test tube each containing 20ml of nutrient broth were incubated in a shaking incubator. Bacterial broth was taken out from incubator and centrifuged at 3000rpm for 10 min at 20 ℃ in a centrifuge machine (Quek and Hu 2008). After centrifugation step, the supernatant in a tube was removed using a pipette gently without disturbing the pellet situated at the bottom of tube. Sterile PBS (Phosphate Saline Buffer) was used for dilution of the bacterial culture across 10-fold dilution.

Using the spectrophotometer, the bacterial solution was standardized at 0.1 McFarland standard at 600nm. The initial pellet in a tube was diluted until the spectrophotometric reading was 0.1. In case of higher dilution (i.e. spectrophotometric reading less than 0.1 OD) the diluent was centrifuged, and the process was repeated. The 10-fold serial dilution for bacterial broth (0.1 OD) was done simultaneously. 1ml from the standard tube was transferred to the next test tube (10^-1) with 9ml of PBS and the solution was mixed using pipette gently and the process was repeated until the last test tube (i.e. 10^8). The bacterial dilution subsequently follows the order as 1, 1:10, 1:10^2, 1:10^3, 1:10^4, 1:10^5, 1:10^6, 1:10^7 to 1:10^8.

The serially diluted bacteria were spread plated in three plates for each dilution. The visible colony for *E. coli* ranging from 30-300 colonies/100µl were obtained after series of dilution at 1:10^5 range containing smooth white, round and raised isolated colonies. Moreover, for 1:10^4 dilution more than thousand colonies were obtained which were too many to count and for 1:10^6 dilution less than 30 colonies were observed from the triplicated petri plates.

3.6 Minimum Inhibitory Concentration (MIC) assay and growth curve

All the MIC plating procedure for designated chemical and extract treatments, broth and bacterial inoculation was performed inside PC2 lab in sterile environment. The freshly optimized volume of 5µl *E. coli* culture in Phosphate Saline Buffer (PBS) at 9.8*10^3 cfu *E. coli*/ml comprising 30-300 colony forming units was preferred for MIC test. 190 µl of nutrient broth is supplied as growth media across all the well to support E. coli growth for 24 hours.

Each disinfectant for the test were sublimated in 2-fold microdilution from its pure form (100%) to zero to observe the lowest inhibitory concentration. An experimental design for 96 well plate invitro assay of minimum inhibitory concentration for 4 treatments viz Pine-o-Cleen®, Bleach, Oregano oil and Oregano extract has been laid out in Appendix 2 and Appendix 3 respectively. The MIC assay was done for bacterial growth assessment using absorbance-based microplate reader (SPECTROstar® Nano) at OD 600nm and growth readings observed in every 30 minutes interval for 24 hours. 4% ZnSo4 was used as the negative control estimating it as the lowest threshold of OD where all the bacterial cells die. 40gm of anhydrous ZnSo4 was dissolved in 100ml of sterile water and a stock solution was prepared. Microcalorimetric evaluation for Zinc sulphate at 250mM (approximately: 40 mg/ml) exhibited the significant bactericidal effect on *Pseudomonas aeruginosa* (Aveledo et al. 2018).

Growth curve analysis using a microplate reader was followed to quantitatively analysed interactive effect between disinfectant and bacteria over a period. Microplate readers were assessed for their high throughput and precise calculation through absorbance-based test (Krishnamurthi et al. 2021).

3.7 Statistical data analysis

All the MIC tests for disinfectants were repeated for threes time and each MIC plates containing triplicates. The mean value and standard deviation error bars were calculated, and graphs designed in MS Excel. version 2403(build 17425.20176). A statistical software IBM SPSS Statistic® (Statistical Package for Social Science) package v. 29.0.1.0(171), Chicago, Illinois, USA was used for the statistical analysis. Data used for the statistical analysis were those examined through the validation control tests. Validation control was performed using reliability analysis in SPSS. The assumption of data normality was carried out using the Shapiro–Wilk test. Similarly, Kruskal-Walli's test was performed for testing significant differences between the disinfectant products considering significant level(α) of 5%.

CHAPTER 4: RESULTS

4.1 E. coli growth curve for positive control.

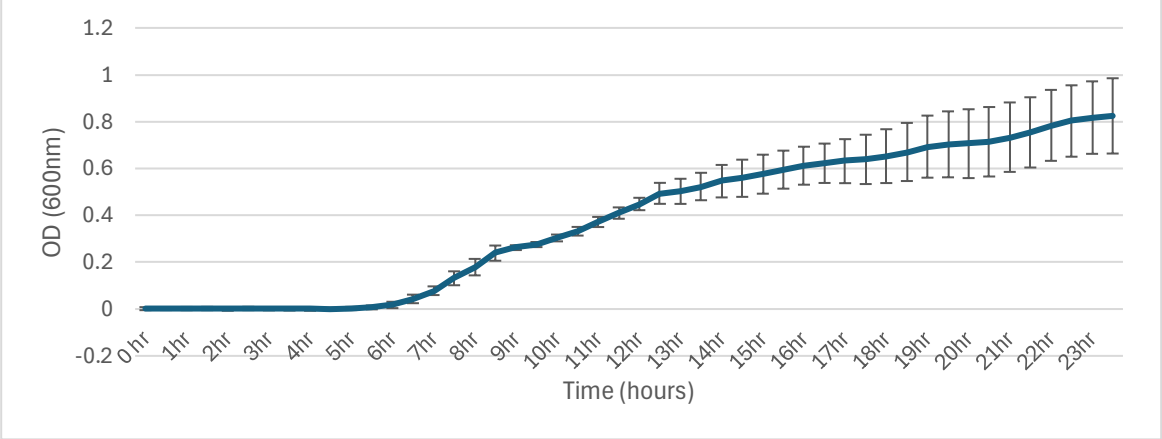


Figure 3: *E. coli ATCC ®15597* growth curve based on absorbance readings for 24 hours. ** n=7 technical replicates, and data expressed in SD ± Mean.**

The assessment of lag, log(exponential) and stationary phase for *E. coli* (9800 cfu/ml) growth in 96 well plate for 24 hours period depicted the viability and growth behaviour in 190ml nutrient broth during MIC assay. The log phase ranged from 6 hours to 12 hours period with a significant difference in OD_{600} readings for every hour as shown in Figure 3. Between 12 hours to 23.5 hours, the bacterial growth rate was slow and no significant increment in absorbance were depicted thereby a stationary phase could be observed. This growth pattern developed in a supplemented nutrient broth for positive control facilitated comparison with all the selected disinfectant for 24 hours. Data was expressed as means of replicates and error bars developed from standard deviation of OD.

4.2 Oregano ethanol extract mediated *E. coli* growth inhibition.

5µl bacterial suspension of *E. coli ATCC®*15597(9800 cfu/ml), nutrient broth(190µl) and Oregano Ethanol Extract (5 µl) across 2-fold dilution from 100% to 0% (positive control) were incubated for

24 hours period at 37 ℃ in a microdilution assay. Absorbance value based on UV/vis spectrum were calculated at 600nm at every 30minutes interval and protocol adjusted for 100rpm shaking in double orbital movement between every cycles. Upon assessment of average OD_{600} reading for bacterial growth across OEE treatment, the highest bacterial inhibition was observed for 0.8% concentration throughout the 24 hours interval as shown in figure 4. A statistically significant difference was observed at 100% concentration for mean absorbance reading in contrast to positive control until 16 hours of incubation as shown in figure 4. The bactericidal effectiveness at this point was similar with the 4% Zinc sulphate (negative control). The error graphs derived from standard deviation values for 0.8% OEE and negative control align in similar fashion representing it as the minimum inhibitory concentration for our indicator ATCC 15597 strain. Deviations in absorbance value was higher in OEE therefore, no significant differences in bacterial OD_{600} were noticed between 0.8%OEE and other OEE concentrations as shown in figure 4.

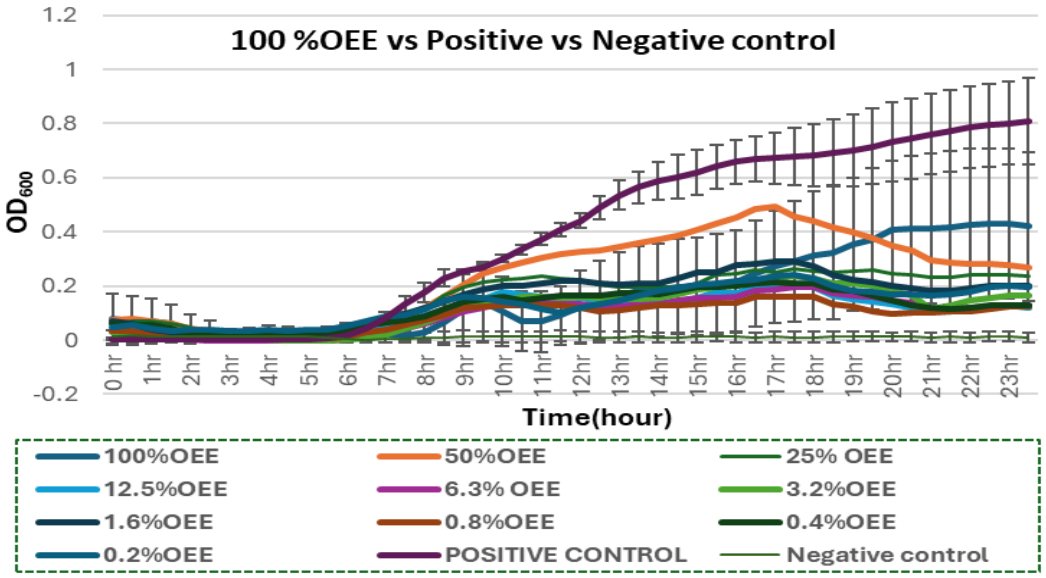


Figure 4: Growth curve analysis for 100% OEE based on mean optical density calculated at every 30 minutes for 24 hours and data expressed in standard error graphs developed for positive, negative control and 100% OEE for ** n=3 technical replicates.**

4.2 Oregano essential oil antibacterial activity assessment.

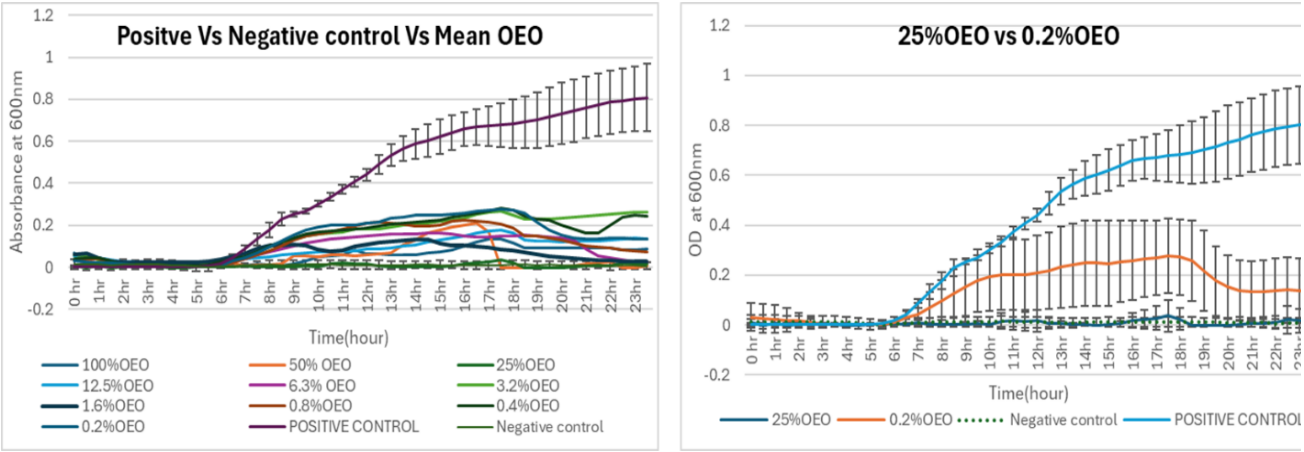


Figure 5: Comparative analysis of 25% OEO, 0.2% OEO and Positive control. Data expressed in SD ± Mean based on ** n=3 technical replicates.**

5µl bacterial suspension of (9800 cfu/ml), nutrient broth(190µl) and Oregano essential oil (5 µl) across 2-fold dilution from 100% (active ingredient: Carvacrol 89.08%) to 0% (positive control) were incubated for 24 hours period at 37 ℃ in a microdilution assay. Absorbance values were assessed at 600nm at every 30minutes interval and protocol was adjusted for 100rpm shaking in double orbital movement between every cycles. Oregano essential oil (OEO) at 25% concentration represented a lowest mean absorbance exhibiting the minimum bacterial growth across each 30 minutes interval. The highest mean absorbance was observed for 0.2%OEO but still a significant bacterial control was observed across stationary phase than positive control. The highest inhibitory concentration of 25% OEO revealed an outstanding biocidal effect similar as negative control throughout incubation period as shown in figure 5. Therefore, the minimum inhibitory concentration for Oregano essential oil was its 25% dilution.

4.3 Effect of bleach on the bacterial growth.

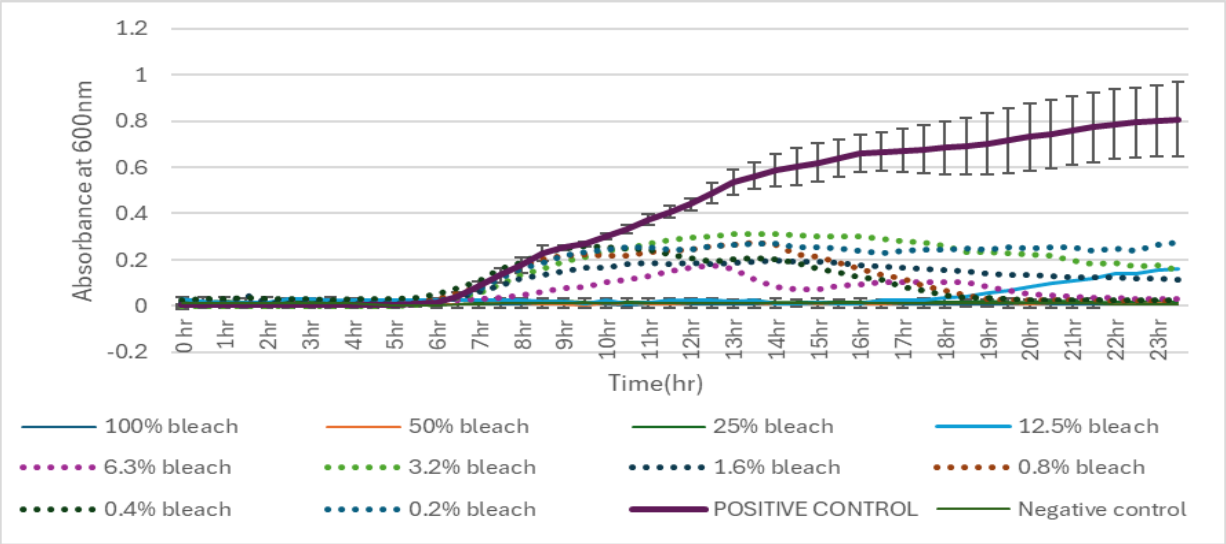


Figure 6: Disinfection status of bleach at different concentration accessed in optical density growth curve at every 30 minutes within 24 hours. Data expressed as Mean absorbance and error bars given for positive control and negative control from standard deviation, ** n=3 technical replicates.**

5µl bacterial suspension (9800 cfu/ml), nutrient broth(190µl) and bleach (5 µl) across 2-fold dilution from 100% (Sodium hypochlorite 42gm/L with available chlorine of 4% w/v and sodium hydroxide 9gm/L) to 0% (positive control) were incubated for 24 hours period at 37 ℃. Absorbance values were assessed at 600nm at every 30minutes interval and protocol was adjusted for 100rpm shaking in double orbital movement between every cycles. The solid lines represented the significant effect of bleach across 100%, 50% and 25% dilutions. Upon assessment of absorbance for these three respective concentrations using a standard deviation error bars against the growth control, highly significant difference for average OD_{600} values were observed as shown in Figure 6. The mean value for these three dilutions exhibited similarity with negative control depicted by error bars. The microplate (SPECTROstar® Nano) reading at 600nm for all of

three dilutions is approximately “0” throughout 24 hours therefore, the minimum inhibitory concentrations for bleach was found to be at 25% dilution level.

4.4. Pine-o-Cleen® associated inhibitory effect.

A 5µl bacterial suspension (9800 cfu/ml), nutrient broth(190µl) and Pine-o-Cleen® (5 µl) across 2-fold dilution from 100% (1.6% w/w Benzalkonium chloride active ingredient in 1.5L) to 0% (positive control) were incubated for 24 hours period at 37 ℃ in a 96 well plate. Absorbance values were determined by microplate reader (SPECTROstar® Nano) at 600nm at every 30minutes interval and protocol was adjusted for 100rpm shaking in double orbital movement between every cycles. Mean data for absorbance exhibited the lowest *E. coli ATCC ®15597* OD at 3.2% POC concentration. The highest mean bacterial absorbance was obtained for 100% POC followed by 50% POC for 24 hours as shown in the figure with dot lines. The average absorbance reading for all POC treatment ranges from 0 to approximately 0.2 OD. Error bars for 25% POC express the similarity in bacterial OD of 25%POC, 12.5% POC, 6.3% POC, 3.2% POC and 1.6% POC respectively with the negative control for 24 hours observation. Therefore, the minimum inhibitory concentration for POC was its 1.6% dilution. Optical density for 100% and 50% POC treatment did not follow the similar trend of bacterial growth for lag phase and log phase as seen for positive control.

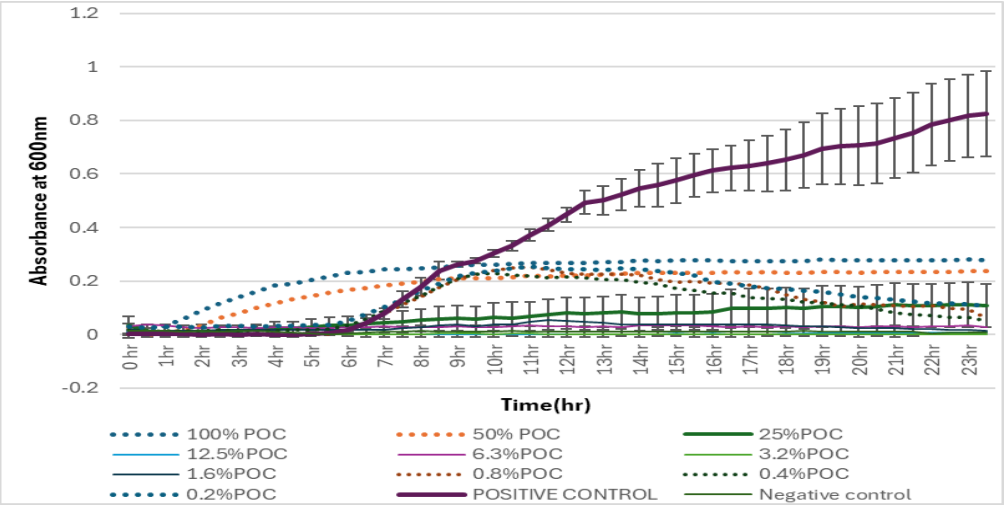


Figure 7: Optical density graph for growth curve analysis of Pine-o-Cleen® disinfected *E. coli* ATTC® 15597 well plates. Data expressed in SD ± Mean. Error bars for positive control, 25% and negative control, ** n=3 technical replicates.**

4.5 Data Analysis

Cronbach's alpha test value of 0.017 for disinfectant concentration, time and optical density upon reliability analysis was obtained. From Shapiro-wilk test, the significance level(p-value) of all four disinfectants was less than expected p value ($p<0.05$) indicating that the absorbance data did not follow a normal distribution as shown in Table 1. Therefore, to prevent the inaccuracy from parametric test, a non-parametric method for statistical analysis using Kruskal-Walli's test was performed.

Table 1: Shapiro-Wilk Test for assessment of normality for Bleach, Oregano Ethanol Extract (OEE), Oregano Essential Oil (OEO) and Pine-o-Cleen® (POC) at p-value (<0.05).

OD	DISINFECTANT	Statistic	Df	Sig.
	Bleach	0.730	4320	<0.01
	OEE	0.848	3360	<0.01
	OEO	0.713	3360	<0.01
	POC	0.769	4320	<0.01

a.Lilliefors Significance correction.

The independent sample test Kruskal-Walli's test for disinfectants expressed a t-static value of 498.177 at p<0.01(df=3, n=15360) referring to a statistically significant differences in the OD distribution among disinfectant groups.

Table 2: Pairwise Comparisons of disinfectant based on OD_{600} at significance level, (p<0.05).

Sample 1- Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig	Adj.Sig.[a]
OEO-Bleach	445.407	100.578	4.428	<0.001	0.000
OEO-POC	-675.409	100.578	-6.715	<0.001	0.000
OEO-OEE	2218.841	106.679	20.799	<0.001	0.000
Bleach-POC	-230.002	94.082	-2.445	0.014	0.087
Bleach-OEE	-1773.434	100.578	-17.632	<0.001	0.000
POC-OEE	1543.432	100.578	15.346	<0.001	0.000

a. Bonferroni correction.

The aim of the Kruskal-Walli's test was to compare the statistically significant differences in the distributions of OD values between multiple groups of disinfectants used. A post-hoc pairwise comparison using Kruskal-Walli's test depicts that at p<0.05 significance level, distribution of

absorbance (OD_{600}) between all the disinfectants was statistically significant except for bleach-POC. An adjusted p-value (p=0.087) for a test statistic of -230.002 between Bleach and POC was higher than the expected ($p<0.05$), therefore the null hypothesis that both disinfectants had similar antimicrobial effect was accepted.

CHAPTER 5: DISCUSSION

5.1. Growth control response in MIC test.

MIC test has been considered as an effective tool to address antimicrobial resistance, invitro assay of new bactericidal compounds and mostly used as gold standard in susceptibility testing (Andrews 2001). *E. coli ATTC®15597* strain had been selected before for anti-bacterial response at varying temperature and their effect was studied for different chemical disinfectant on the surface of strawberry fruits (Yu et al. 2001). The non-pathogenic strain was found to be nutrient responsive and significant reduction in bacterial volume was realized while comparing between the surface inoculation and inoculum injected conditions in strawberry fruit. An excess supply of nutrient through broth prior to incubation of bacteria in MIC test reduced the chances of outliers from bacterial death in nutrient deficit conditions. Moreover, OD_{600} reading is also correlated with the average cell mass present in the growth media and depend on the media composition itself for microbial growth response.(Schaechter et al. 1958). In the Luria broth the estimated absorbance at 600nm for the end of steady-state growth was observed to be 0.6 to1 OD for *E. coli*.(Sezonov et al. 2007). This absorbance reading was approximately equal to the initiation of stationary phase observed for nutrient broth cultured *E. coli ATTC®15597* strain in our case for positive control as shown in figure 3. The lag phase observed for *E. coli* was approximately 6 hours which was considered as normal incubation time for bacterial cell kinetic growth. This period had been effective to ensure disinfection and sterilization in a surgical space prior to robust growth afterwards.(Percin et al. 2015).

5.2. Oregano ethanol extracts and Oregano essential oil related bacterial response.

Biocidal activity found against the tested strain from use of ethanolic extract of oregano show efficacy at different concentration with an approximate OD_{600} of 0.2 inhibiting logarithmic growth compared to positive control. Even though the complete stagnation from dead cells were not depicted at any level of dilutions for 24 hours, still bacterial arrest was visible until 14 hours for most of the laboratory prepared OEE as shown in Appendix 4. Oregano extracts at 100% and 50% concentration were found effective for Klebsiella pneumonia and 100%, 50%, 25% and 13% concentrations were effective for Staphylococcus aureus in 18-24 hours tube dilution test. The actual concentration of bioactive of Oregano extracts was not discussed in this paper (Amri et al. 2023).

Similarly, bacteriostatic role of Oregano essential oil was scrutinized for its 25% attenuations resulting a long-term suppression in 24 hours surveillance. Our findings were similar with OEO effectiveness against Staphylococcus aureus and klebsiella pneumonia where inhibition was obtained from 100% to 25% dilutions in DMSO (Amri et al. 2023). Carvacrol acts by compromising cell membranes causing a leakage of cytoplasmic fluid in *E. coli.* Protein concentration from 400 to 480 µg/ml was detected to be continuously dispersed from the ruptured cells by Bradford method and membrane lysis was confirmed by protein molecular profile analysis in SDS-PAGE. (Khan et al. 2017). This activity of cell membrane disruption by non-polar monoterpenes of Carvacrol was also reported in antibacterial test for Carvacrol combined with erythromycin against erythromycin resistant Group-A Streptococci(GAS).(Magi et al. 2015). Carvacrol (450 µg/ml) exhibited a complete death of 10^7 cfu/ml *E. coli* within two hours of incubation in a time kill assay and created higher oxidative stress across *E. coli* bacterial cells which was examined through H2DCFDA [5(6) -Carboxy-2',7'-dichlorofluorescein diacetate] and rhodamine 123 fluorescent staining and microscopy (Khan et al. 2017).

5.3. Pine-o-Cleen® responsive effect on *E. coli ATCC®15597.*

Four proportions of POC at 25%, 12.5%, 6.3% and 3.2% exhibited optimal lethality towards the indicator ATCC 15597 strain. Lag phase in any bacteria is a preparative period and there is no significant increment in bacterial cells therefore, higher OD for 100%POC and 50%POC observed in dotted lines during 1 to 8 hours period in figure-8 was uncommon. The higher initial OD might be influenced by inoculum size or bacterial clumps during bacterial inoculation step for microdilution (Bertrand 2019). Optical density for BAC inhibited bacteria consequently decreased in 0.2% POC, 0.8%POC, and 0.4%POC after 10 hours of incubation which is unusual response for dead cells in turbidimetric test. BAC is a broad-spectrum disinfectant that kills food borne pathogens including gram negative and gram-positive bacteria, fungus, virus, and their spores. Its amphiphilic surfactants allow easy interaction of cationic charges with lipid and water fractions of bacterial cell membrane thereby induced lysis and leakage of cytoplasmic content (Fazlara and Ekhtelat 2012). The sudden rise in OD at 0.2%, 0.8% and 0.4%POC during active growth period might be influenced by the cytolysis and leakage from bacterial cells into the broth. The permanent loss of cell membrane integrity in *Escherichia coli* strains *C9490, H1071, and NCTC 8003* was also higher during exponential growth phase during osmosis and pressure treatment whereas cells at stationary phase were more resistant to same pressure level. (Pagán and Mackey 2000).

5.4. Bleach responsive effect on *E. coli ATCC®15597* strain.

Bleach containing 0.5 to 2 mg/L free Chlorine residue is commonly used disinfectant for water treatment and gram-negative Legionella growth control in water distribution system 0.2mg/ml or below this concentration, recolonization of bacteria was expected to occur in the water across the sink. (Queensland Health, 2017). Attenuations at 6.5% level (equivalent to 0.25 mg/ml free chlorine) and below for our treatment also bleach did not show significant reduction in gram negative *E. coli ATCC®15597* strain. Since belonging to same proteobacteria in gamma taxonomic

group, the cell membrane lipids for Legionella were characterized by the presence of phosphatidyl glycerol, cardiolipin, phosphatidylethanolamine and monomethyl phosphatidylethanolamine whereas similar lipid profile were found for *E. coli* species comprising phosphatidyl glycerol, cardiolipin and phosphatidylethanolamine (Sohlenkamp and Geiger 2016). Bleach might have similar inhibitory effect for both bacteria at this concentration because of same membrane lipid compositions. Hypochlorite ions at neutral ph (7) was observed nullifying the bacterial cells through disruption of cellular membrane in *E. coli* ascribed by EGFP (Enhanced Green Fluorescent Protein) detection test for leaked protein (Mizozoe et al. 2019). Higher efficiency of bleach from oregano oil and extracts might be influenced by the complementary factors like sodium hydroxide. Corrosive effect of NaOH on the membrane layer by reverse osmosis and deionization at 0.4% concentration on bacillus ATCC 9372 strain cells had also been found (Mazzola et al. 2006). Moreover, NaOH is alkaline in nature thus, it increases the ph value of the solution and helps in liberation of hypochlorite ions from sodium hypochlorite into the solutions (Mizozoe et al. 2019).

5.5. Interpretation of results from data analysis

The Cronbach's alpha measure indicated value of 0.017 is less than the acceptable value for research purpose (0.7 to 0.8). The consistency of data for our research did not meet the acceptable threshold for performing the parametric test. Antimicrobial testing for plant derived polyphenols had been limiting because of the inherent complex compound structure in them which are prone to be inconsistent with susceptibility test (Bubonja-Šonje et al. 2020). The Kruskal-Wallis test for 4 disinfectants exhibit a statistical difference in OD between them. The statistical difference was examined based on variation of OD data from central tendency, so the variation could be from any of the groups of concentrations among disinfectants. In our test, the level of bacterial inoculum used was 9.8*10^3 colony/ml for each MIC well plates that corresponds to 49 colony/5µl. This bacterial volume was incubated into 190 µl of nutrient broth

therefore the cells count at the initial period might not reach the threshold required for susceptibility analysis. The inconsistency in OD reading may be influenced by the inoculum size with lower cell counts than 5*10^5 cfu/ml rendering the false susceptibility values in microdilution test (Bubonja-Šonje et al. 2020).

5.6. Relevance of research for general purpose.

Bleach is a cost-effective commercial disinfectant for cleaning household space, hospital areas and food supply chains. 0.1% sodium hypochlorite solution had been proposed for hospital grade disinfectant according to the standard disinfection procedure. (Australian Nursing and Midwifery Federation, 2020). Our findings were relevant for sterilizing water-soluble *E. coli* strains using the lowest concentration of bleach(Mazzola et al. 2006). Many studies evaluated unique property of Carvacrol as antimicrobial agent against multiple bacterial strain, fungi and yeast by changing cell membrane potential, homeostasis, reduced ATP level or biofilm disruption. Despite of efficacy, their utilization in domestic cleaning required comprehensive study of the degradation effect on environment (Mączka et al. 2023). Oregano essential oil efficacy estimation from our research could be used with modification with surfactants and complementary antibacterial agents to boost their efficacy only for research purpose so far. Similarly, the ethanol extracts from laboratory preparations also required further analytical test and toxicity evaluation and safety certification prior to use for general purpose.

5.7. Limitations of study

Extraction: Rotary evaporation could not confirm if the ethanol in the final extract is fully dried off and free from ethanol. The antibacterial assessment using concentrated Oregano ethanol extracts may also require 70% ethanol as a control to further check if bacterial inhibition is from the extracts or the ethanol itself. The concentration step of extracts at higher temperature was

feasible because of high boiling point of Carvacrol but it might compromised an underlying phenolic compounds and antioxidants that was lost by heating (Alagawany et al. 2015). Furthermore, the concentrated extracts are in semi-liquid state therefore actual calibration of oregano extracts on weight basis was not possible therefore complete drying of extracts by freeze drying procedure should be necessary.

MIC test: Age of bacterial broth in serial dilution highly effect the bacterial growth in MIC assay, outliers may prevail from presence of higher number of dead cells. Susceptibility assessment by spectrophotometric analysis cannot fully explain the cause of antibacterial activity of plant derived ethanol extracts thus other confirmatory assay is important to relate its efficacy from antioxidant present in sample. Moreover, microdilution of Oregano essential oil with sterile water did not allow proper mixing of hydrophobic oil components thus, it severely hindered the feasible interaction of Carvacrol and oil constituents to bacterial cell. The proper diluent including DMSO, or acetone or Tween 80 in broth could facilitate for homogeneous mixing and dilution of the oil (Donadu et al. 2021, Hood et al. 2003).

5.8. Conclusion and Future directions

Turbidimetric method is the simplest way to evaluate the bacterial growth trend by assessment of their standard growth curves in antibacterial susceptibility testing for disinfectants. The research findings from data analysis and interpretation exhibited biocidal role of the Oregano and chlorine-based disinfectants. Our analysis could only confirm the presence of antibacterial nature in laboratory formulated product which activity level was different from the chemical disinfectants. Microdilution assay for confirmation of the absorbance activity fails to provide consistent data for the tested products. The experiment demands for more than 3 MIC tests with 3 replicates for each disinfectant to limit the inconsistency in data.

This research will guide for the assessment of antimicrobial compounds in fresh Australia grown oregano products. Further evaluation of the Oregano extract content will be necessary to estimate the underlying antimicrobial compounds through Gas Chromatography will be essential to derive proper understanding. The choice of growth media also could influence the MIC to some extent therefore, MIC test in multiple media will be helpful to generalize the MIC values for essential oils. Similarly, susceptibility test including MBC (Minimum Bactericidal Concentration), inhibition assay through disk diffusion and comparative assessment with commercially available extracts should be followed to ensure the susceptibility test output. The research will provide insights to prepare the ethanol extracts and allows wise selection of diluent and proper spectrophotometric evaluation for disinfectants in days to come. In conclusion, Oregano essential oil and ethanol extract both were found to be promising solutions for their use as an active ingredient in development of novel bactericide.

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APPENDICES

APPENDIX 1: BIOSAFETY COMPLETION CERTIFICATE

Flinders University

Flinders University Institutional Biosafety Committee

Certificate of Completion

This is to certify that

Prakash Neupane

satisfied the

Biosafety Training requirements of Flinders University

The following topics have been successfully completed:

Introduction to Biosafety
Physical Containment Facilities

October 2023

Professor Melissa Brown
Chairperson
Institutional Biosafety Committee
Flinders University

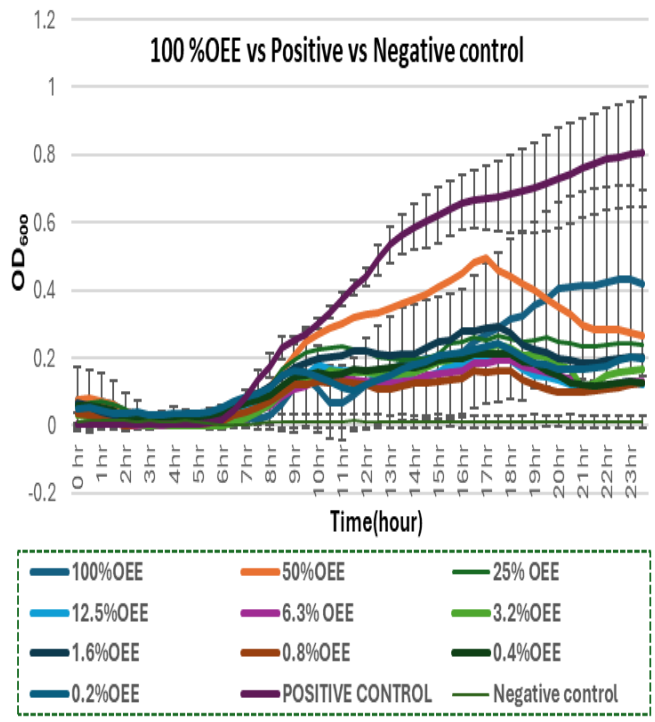
APPENDIX 2:_MIC PLATE LAYOUT FOR 96 WELL PLATE FOR BLEACH AND PINE-O-CLEAN DISINFECTANTS TESTING.

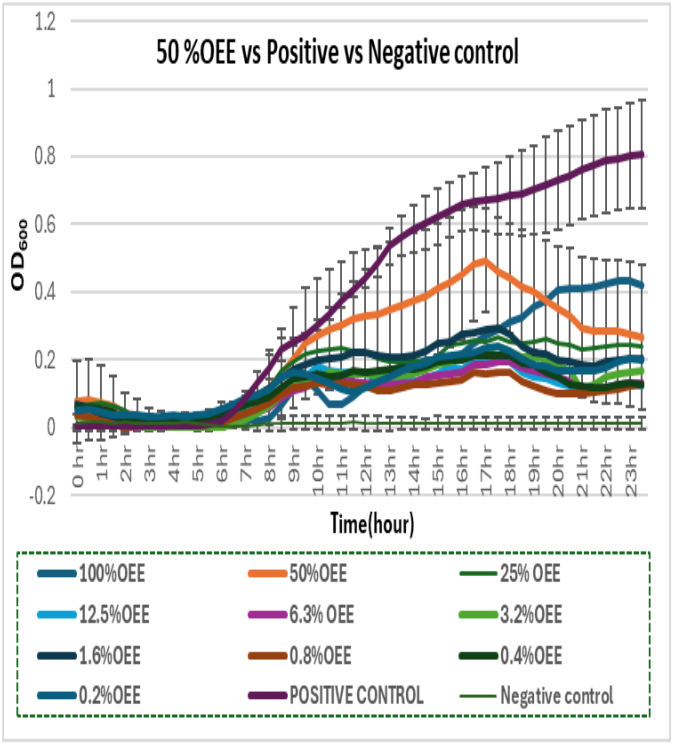
Concentration Name	1 100%	2 50%	3 25%	4 12.5%	5 6.3%	6 3.2%	7 1.6%	8 0.8%	9 0.4%	10 0.2%	11 0%- Positive control (bacteria+ water+broth)	12 Negative Control (4% ZnSo4 + Bacteria+ broth)
A Bleach	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	Broth=190μL +*E. coli*=5μL+water=5 μL	Broth=190μL +*E. coli*=5μL+ZnSo4=5 μL
B Bleach	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	Broth =190μL +*E. coli*=5μL+water=5 μL	Broth=190μL +*E. coli*=5μL+ZnSo4=5 μL
C Bleach	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	BROTH=190μL Bleach =5μ L *E. coli* =5μL	Broth =190μL +*E. coli*=5μL+water=5 μL	Broth=190μL +*E. coli*=5μL+ZnSo4=5 μL
D Pine-o-Cleen®	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	Broth =190μL +*E. coli*=5μL+water=5 μL	Broth=190μL +*E. coli*=5μL+ZnSo4=5 μL
E Pine-o-Cleen®	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	Broth =190μL +*E. coli*=5μL+water=5 μL	Broth=190μL +*E. coli*=5μL+ZnSo4=5 μL
F Pine-o-Cleen®	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	BROTH=190μL POC =5μ L *E. coli*=5μL	Broth =190μL +*E. coli*=5μL+water=5 μL	Broth=190μL +*E. coli*=5μL+ZnSo4=5 μL
G. Blank (Bleach)	Broth=190 μL Bleach=5 μL PBS=5 μL	Broth=190 μL Bleach=5 μL PBS= 5 μL	Broth=190 μL Bleach=5 μL PBS=5 μL	Broth=190 μL Bleach=5 μL PBS= 5 μL	Broth=190 μL Bleach=5 μL PBS=5 μL	Broth=190 μL Bleach=5 μL PBS= 5 μL	Broth=190 μL Bleach=5 μL PBS=5 μL	Broth=190 μL Bleach=5 μL PBS= 5 μL	Broth=190 μL Bleach=5 μL PBS=5 μL	Broth=190 μL Bleach=5 μL PBS= 5 μL		
H. Blank (Pine-o-Cleen®)	Broth=190 μL POC=5 μL PBS=5 μL	Broth=190 μL POC=5 μL PBS=5 μL	Broth=190 μL POC=5 μL PBS=5 μL	Broth=190 μL POC=5 μL PBS=5 μL	Broth=190 μL POC=5 μL PBS=5 μL	Broth=190 μL POC=5 μL PBS=5 μL	Broth=190 μL POC=5 μL PBS=5 μL	Broth=190 μL POC=5 μL PBS=5 μL	Broth=190 μL POC=5 μL PBS=5 μL	Broth=190 μL POC=5 μL PBS=5 μL		

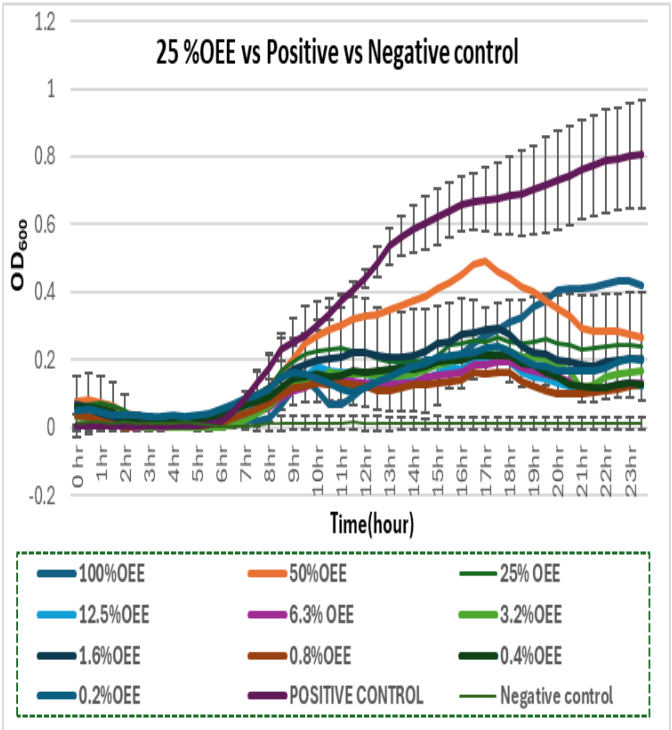
APPENDIX 3: MIC PLATE LAYOUT FOR 96 WELL PLATE FOR OREGANO ETHANOL EXTRACT AND OREGANO ESSENTIAL OIL TESTING.

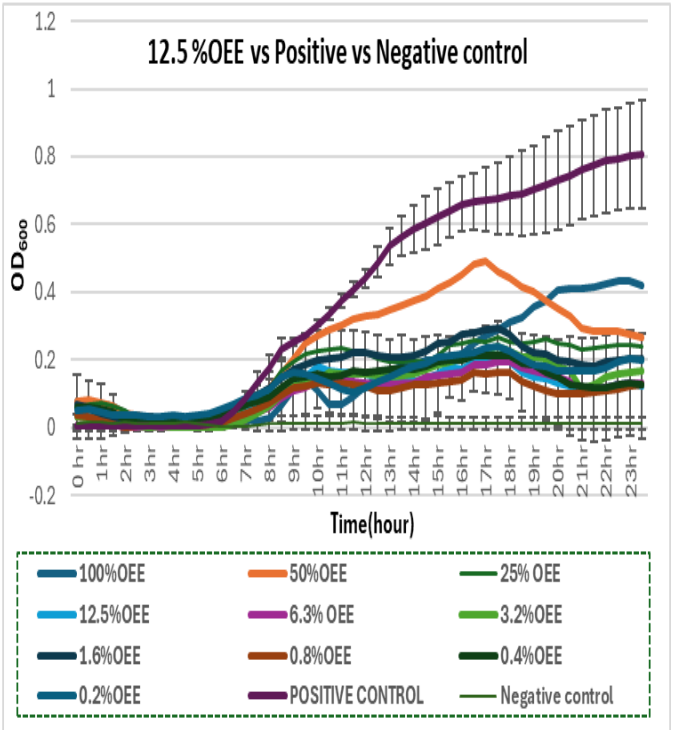
Concentration / Name	1 100%	2 50%	3 25%	4 12.5%	5 6.3%	6 3.2%	7 1.6%	8 0.8%	9 0.4%	10 0.2%	11 0% Positive control (bacteria +water+ broth)	12 Negative Control (4% ZnSo4 + Bacteria+ broth)
A Oregano ethanol extract (OEE)	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	Broth=190 µL +*E. coli*=5µL+water=5 µL	Broth=190µL +*E. coli*=5µL+ ZnSo4=5 µL
B Oregano ethanol extract (OEE)	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	Broth =190µL +*E. coli*=5µL+water=5 µL	Broth=190µL +*E. coli*=5µL+ ZnSo4=5 µL
C Oregano ethanol extract (OEE)	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µ L *E. coli* =5µL	BROTH=190µL OEE =5µL *E. coli*=5µL	Broth =190µL +*E. coli*=5µL+water=5 µL	Broth=190µL +*E. coli*=5µL+ ZnSo4=5 µL
D Oregano essential oil (OEO)	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	Broth =190µL +*E. coli*=5µL+water=5 µL	Broth=190µL +*E. coli*=5µL+ ZnSo4=5 µL
E Oregano essential oil (OEO)	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	Broth =190µL +*E. coli*=5µL+water=5 µL	Broth=190µL +*E. coli*=5µL+ ZnSo4=5 µL
F Oregano essential oil (OEO)	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	BROTH=190µL OEO =5µ L *E. coli*=5µL	Broth =190µL +*E. coli*=5µL+water=5 µL	Broth=190µL +*E. coli*=5µL+ ZnSo4=5 µL
G. Blank (Oregano Essential Oil)	Broth=190 µL OEO=5 µL PBS=5 µL	BROTH=190µL OEO =5µ L PBS=5µL	BROTH=190µL OEO =5µ L PBS=5µL	BROTH=190µL OEO =5µ L PBS=5µL	Broth=190 µL OEO=5 µL PBS=5 µL	BROTH=190µL OEO =5µ L PBS=5µL	BROTH=190µL OEO =5µ L PBS=5µL	BROTH=190µL OEO =5µ L PBS=5µL	Broth=190 µL OEO=5 µL PBS=5 µL	BROTH=190µL OEO =5µ L PBS=5µL		
	Broth=190 µL OEE=5 µL PBS=5 µL	Broth=190 µL OEE=5 µL PBS=5 µL	Broth=190 µL OEE=5 µL PBS=5 µL	Broth=190 µL OEE=5 µL PBS=5 µL	Broth=190 µL OEE=5 µL PBS=5 µL	Broth=190 µL OEE=5 µL PBS=5 µL	Broth=190 µL OEE=5 µL PBS=5 µL	Broth=190 µL OEE=5 µL PBS=5 µL	Broth=190 µL OEE=5 µL PBS=5 µL	Broth=190 µL OEE=5 µL PBS=5 µL		

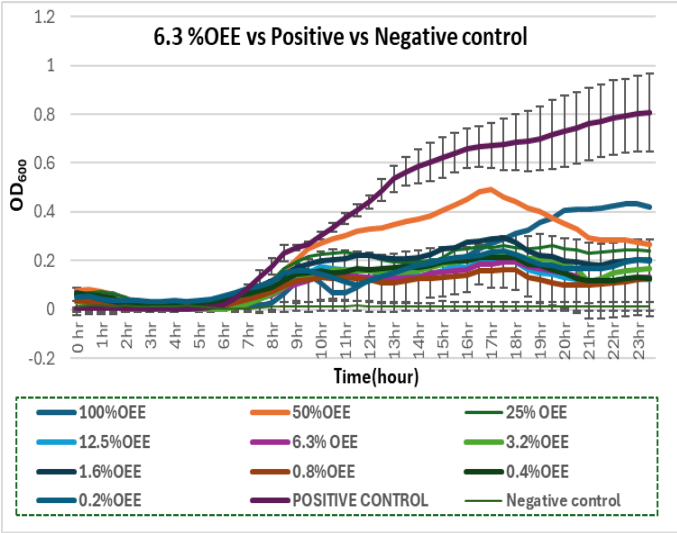
APPENDIX 4: OREGANO ESSENTIAL EXTRACT MEDIATED BACTERIAL OD COMPARISION AT 100%, 50%,25%,12.5%,6.3%,3.2%,1.6%,0.4% AND 0.2% WITH POSITIVE AND NEGATIVE CONTROL.

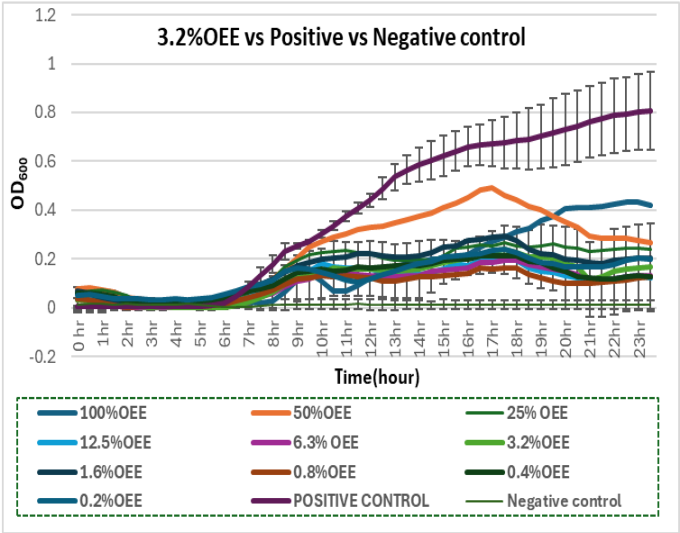


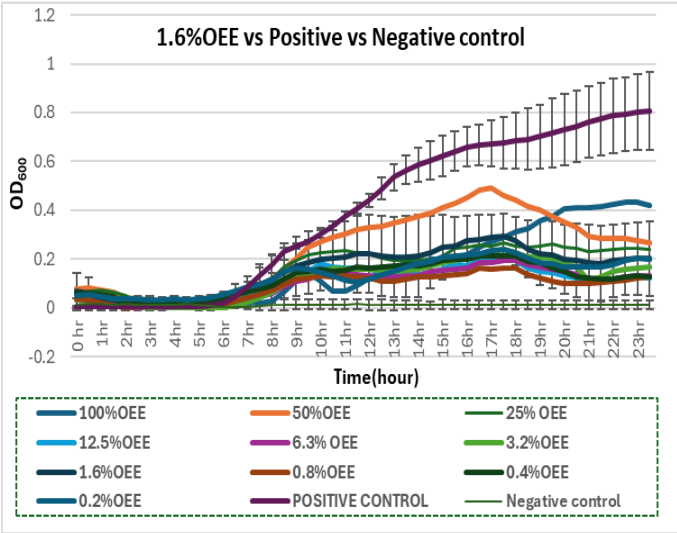


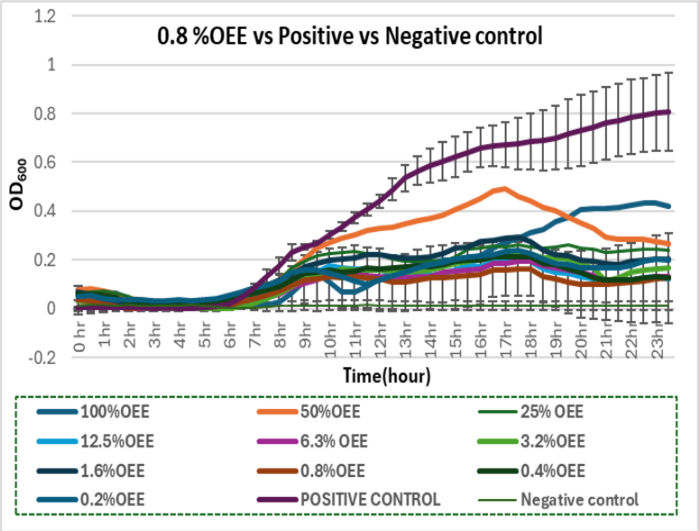


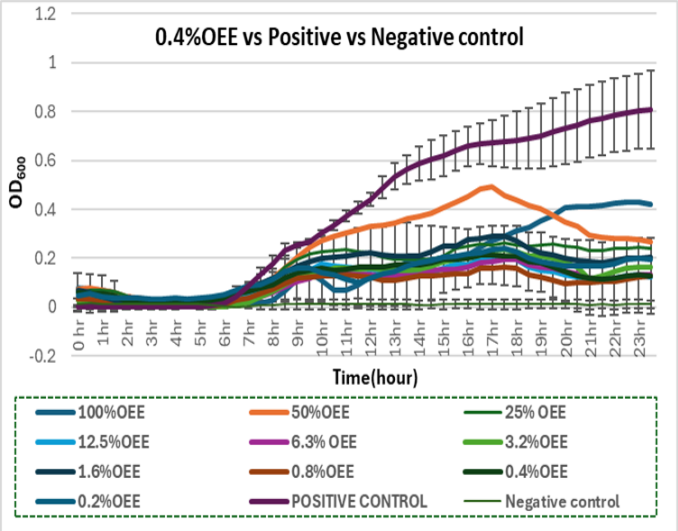


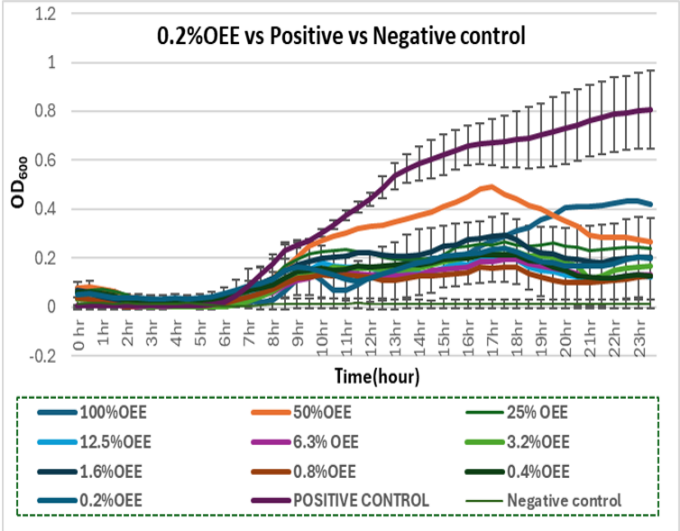

6.3 %OEE vs Positive vs Negative control
OD600
Time(hour)
100%OEE
50%OEE
25% OEE
12.5%OEE
6.3% OEE
3.2%OEE
1.6%OEE
0.8%OEE
0.4%OEE
0.2%OEE
POSITIVE CONTROL
Negative control


3.2%OEE vs Positive vs Negative control
OD600
Time(hour)
100%OEE
50%OEE
25% OEE
12.5%OEE
6.3% OEE
3.2%OEE
1.6%OEE
0.8%OEE
0.4%OEE
0.2%OEE
POSITIVE CONTROL
Negative control

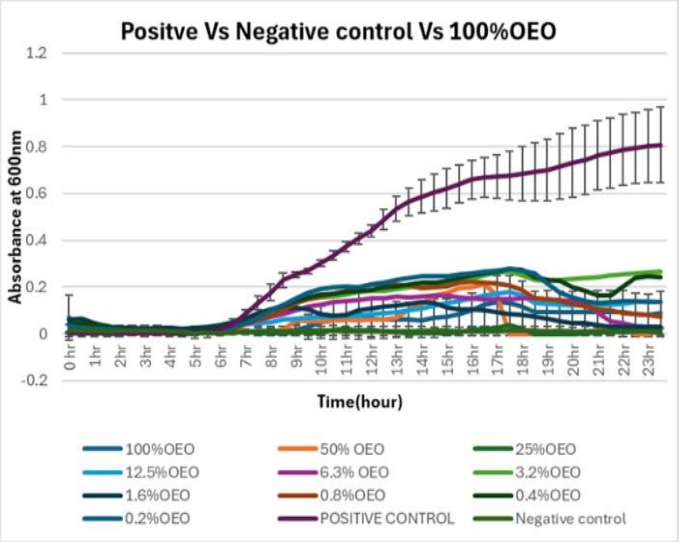

1.6%OEE vs Positive vs Negative control
OD600
Time(hour)
100%OEE
50%OEE
25% OEE
12.5%OEE
6.3% OEE
3.2%OEE
1.6%OEE
0.8%OEE
0.4%OEE
0.2%OEE
POSITIVE CONTROL
Negative control

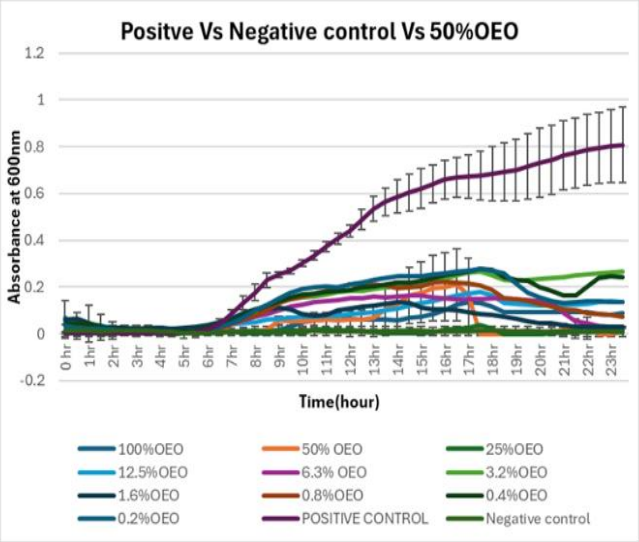

0.8 %OEE vs Positive vs Negative control
OD600
Time(hour)
100%OEE
50%OEE
25% OEE
12.5%OEE
6.3% OEE
3.2%OEE
1.6%OEE
0.8%OEE
0.4%OEE
0.2%OEE
POSITIVE CONTROL
Negative control

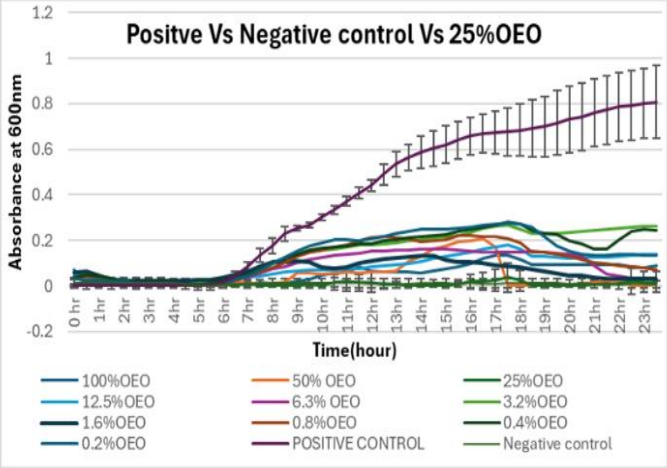

0.4%OEE vs Positive vs Negative control
OD600
Time(hour)
100%OEE
50%OEE
25% OEE
12.5%OEE
6.3% OEE
3.2%OEE
1.6%OEE
0.8%OEE
0.4%OEE
0.2%OEE
POSITIVE CONTROL
Negative control

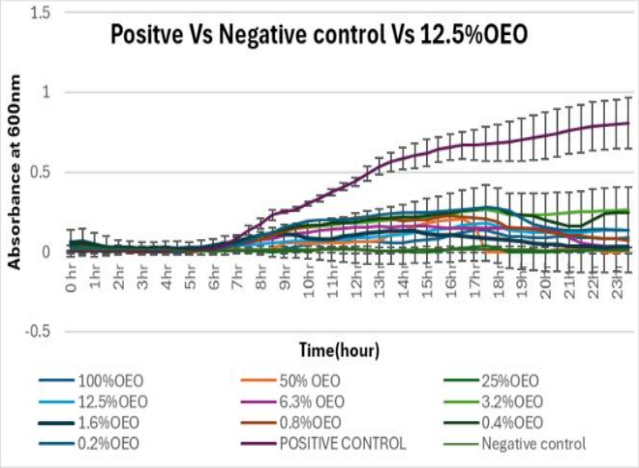

0.2%OEE vs Positive vs Negative control
OD600
Time(hour)
100%OEE
50%OEE
25% OEE
12.5%OEE
6.3% OEE
3.2%OEE
1.6%OEE
0.8%OEE
0.4%OEE
0.2%OEE
POSITIVE CONTROL
Negative control

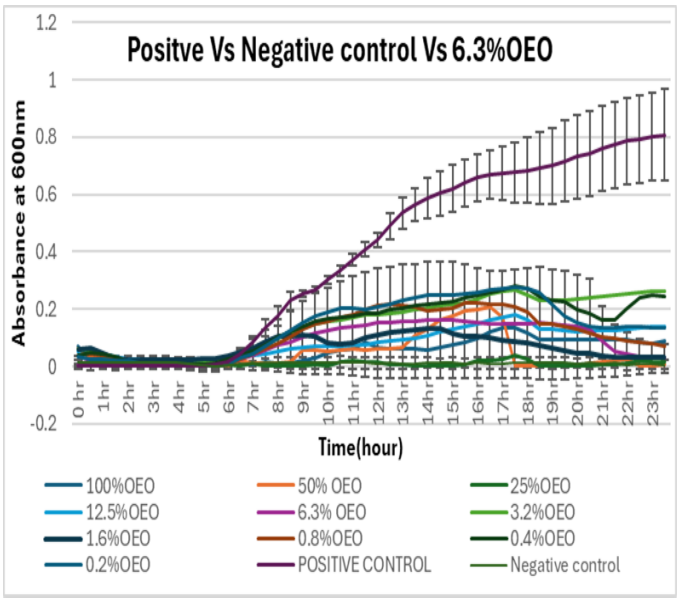
APPENDIX 5: OREGANO ESSENTIAL OIL MEDIATED BACTERIAL OD COMPARISION AT 100%, 50%, 25%, 12.5%, 6.3%, 3.2%, 1.6%, 0.4% AND 0.2% WITH POSITIVE AND NEGATIVE CONTROL.

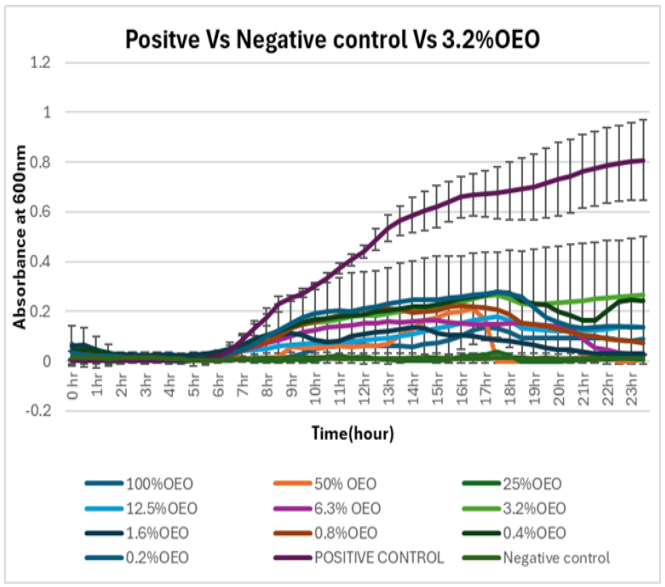


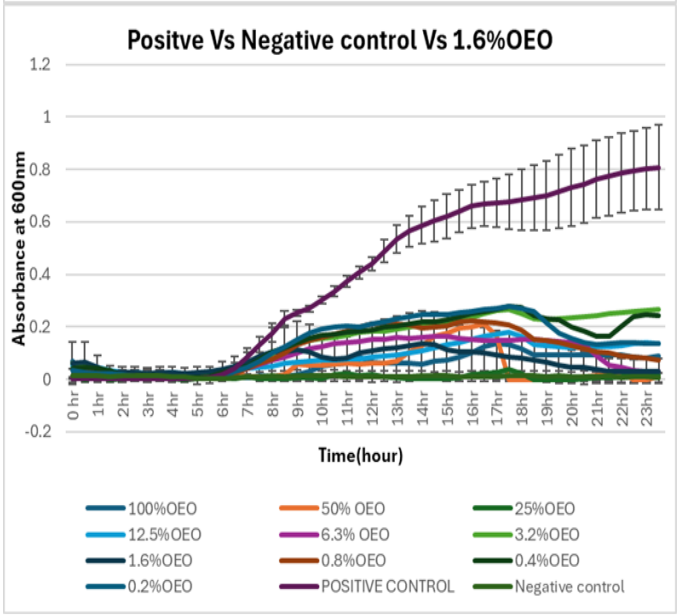


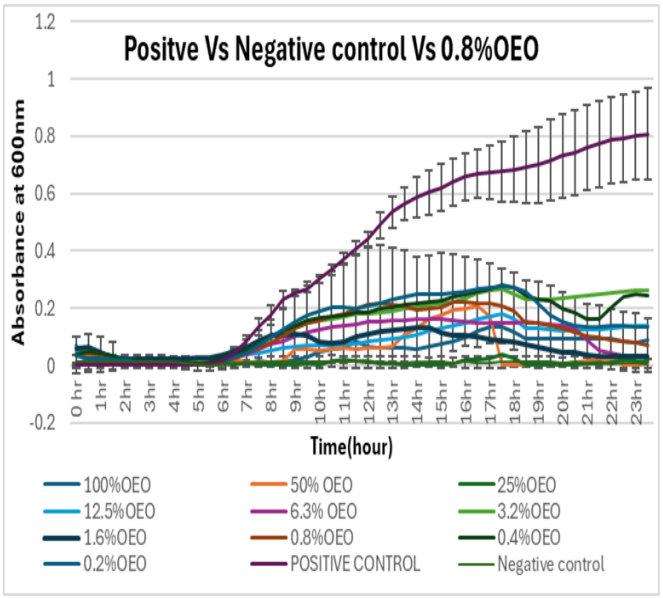


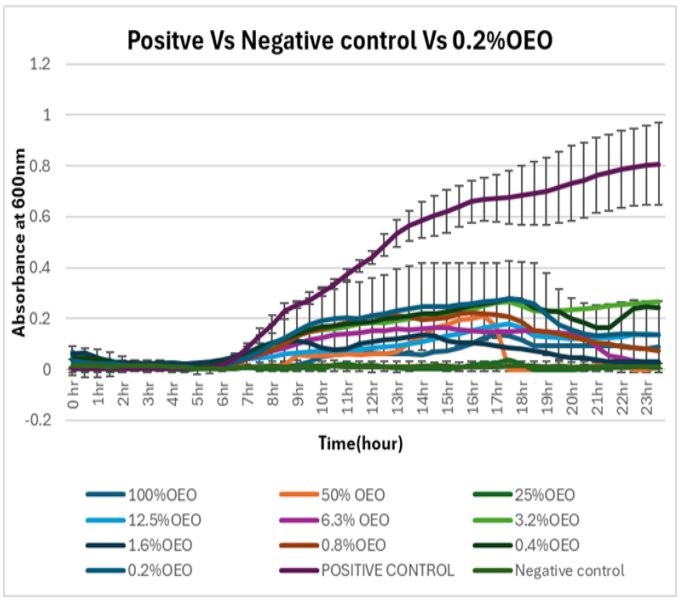


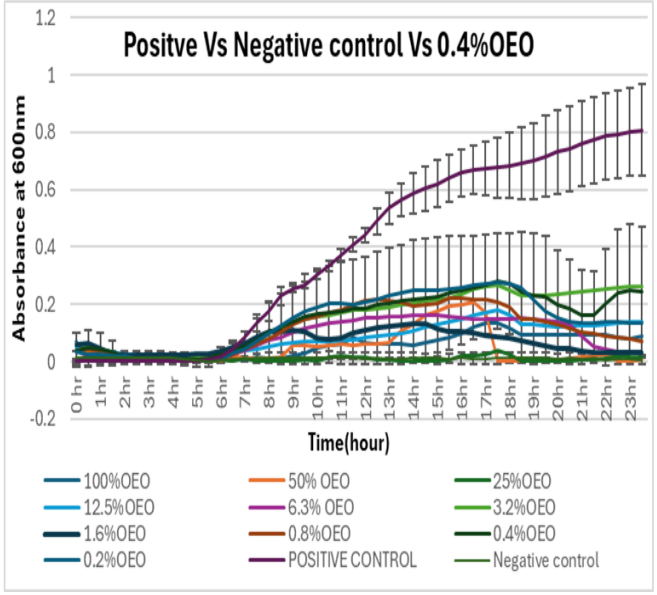

Positve Vs Negative control Vs 6.3%OEO
Absorbance at 600nm
Time(hour)
100%OEO
50% OEO
25%OEO
12.5%OEO
6.3% OEO
3.2%OEO
1.6%OEO
0.8%OEO
0.4%OEO
0.2%OEO
POSITIVE CONTROL
Negative control


Positve Vs Negative control Vs 3.2%OEO
Absorbance at 600nm
Time(hour)
100%OEO
50% OEO
25%OEO
12.5%OEO
6.3% OEO
3.2%OEO
1.6%OEO
0.8%OEO
0.4%OEO
0.2%OEO
POSITIVE CONTROL
Negative control

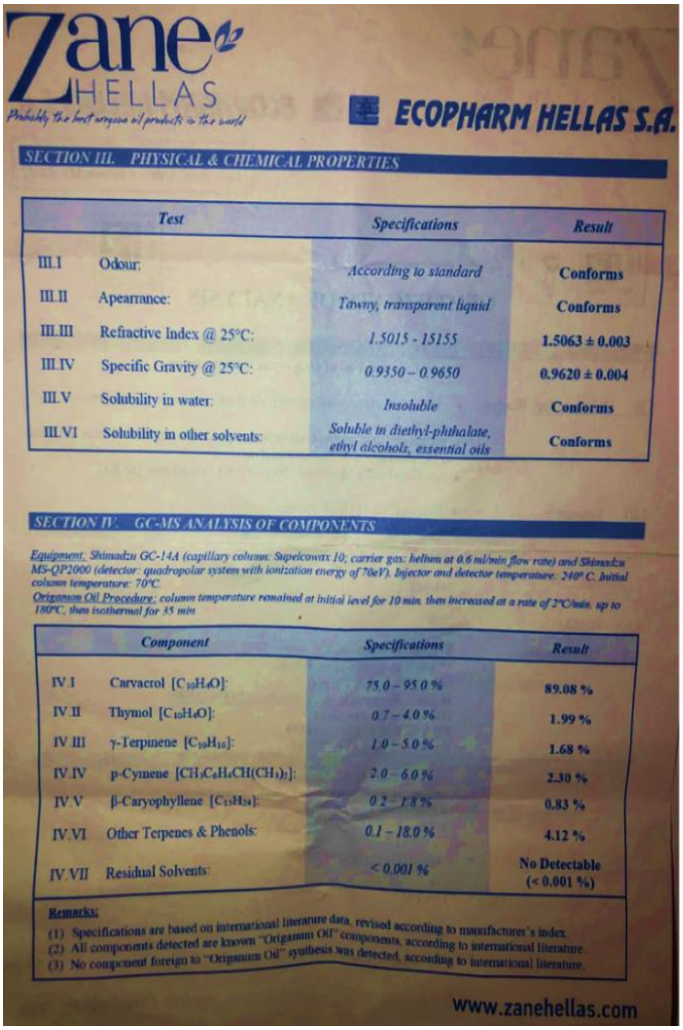

Positve Vs Negative control Vs 1.6%OEO
Absorbance at 600nm
Time(hour)
100%OEO
50% OEO
25%OEO
12.5%OEO
6.3% OEO
3.2%OEO
1.6%OEO
0.8%OEO
0.4%OEO
0.2%OEO
POSITIVE CONTROL
Negative control


Positve Vs Negative control Vs 0.8%OEO
Absorbance at 600nm
Time(hour)
100%OEO
50% OEO
25%OEO
12.5%OEO
6.3% OEO
3.2%OEO
1.6%OEO
0.8%OEO
0.4%OEO
0.2%OEO
POSITIVE CONTROL
Negative control


Positve Vs Negative control Vs 0.2%OEO
Absorbance at 600nm
Time(hour)
100%OEO
50% OEO
25%OEO
12.5%OEO
6.3% OEO
3.2%OEO
1.6%OEO
0.8%OEO
0.4%OEO
0.2%OEO
POSITIVE CONTROL
Negative control


Positve Vs Negative control Vs 0.4%OEO
Absorbance at 600nm
Time(hour)
100%OEO
50% OEO
25%OEO
12.5%OEO
6.3% OEO
3.2%OEO
1.6%OEO
0.8%OEO
0.4%OEO
0.2%OEO
POSITIVE CONTROL
Negative control

APPENDIX 6: OREGANO ESSENTIAL OIL CHEMICAL COMPOSITION PROFILE.



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SECTION III. PHYSICAL & CHEMICAL PROPERTIES

	Test	Specifications	Result
III.I	Odour:	According to standard	Conforms
III.II	Apearrance:	Tawny, transparent liquid	Conforms
III.III	Refractive Index @ 25°C:	1.5015 - 15155	1.5063 ± 0.003
III.IV	Specific Gravity @ 25°C:	0.9350 – 0.9650	0.9620 ± 0.004
III.V	Solubility in water:	Insoluble	Conforms
III.VI	Solubility in other solvents:	Soluble in diethyl-phthalate, ethyl alcohols, essential oils	Conforms

SECTION IV. GC-MS ANALYSIS OF COMPONENTS

Equipment: Shimadzu GC-14A (capillary column: Supelcowax 10; carrier gas: helium at 0.6 ml/min flow rate) and Shimadzu MS-QP2000 (detector: quadropolar system with ionization energy of 70eV). Injector and detector temperature: 240° C. Initial column temperature: 70°C.
Origanum Oil Procedure: column temperature remained at initial level for 10 min. then increased at a rate of 2°C/min. up to 180°C, then isothermal for 35 min

	Component	Specifications	Result
IV.I	Carvacrol [$C_{10}H_{14}O$]:	75.0 – 95.0 %	89.08 %
IV.II	Thymol [$C_{10}H_{14}O$]:	0.7 – 4.0 %	1.99 %
IV.III	γ-Terpinene [$C_{10}H_{16}$]:	1.0 – 5.0 %	1.68 %
IV.IV	p-Cymene [$CH_3C_6H_4CH(CH_3)_2$]:	2.0 – 6.0 %	2.30 %
IV.V	β-Caryophyllene [$C_{15}H_{24}$]:	0.2 – 1.8 %	0.83 %
IV.VI	Other Terpenes & Phenols:	0.1 – 18.0 %	4.12 %
IV.VII	Residual Solvents:	< 0.001 %	No Detectable (< 0.001 %)

Remarks:
(1) Specifications are based on international literature data, revised according to manufacturer's index.
(2) All components detected are known "Origanum Oil" components, according to international literature.
(3) No component foreign to "Origanum Oil" synthesis was detected, according to international literature.

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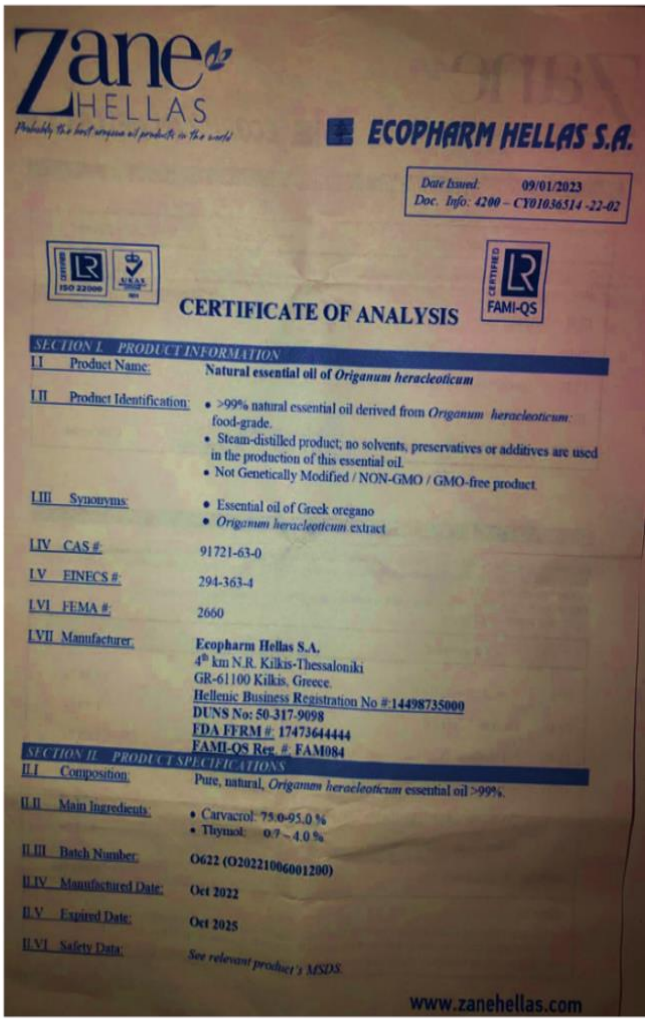

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ISO 22000 AUTH. CERTIFIED FAMI-QS

CERTIFICATE OF ANALYSIS

SECTION I. PRODUCT INFORMATION

I.I Product Name: **Natural essential oil of *Origanum heracleoticum***

I.II Product Identification:
- >99% natural essential oil derived from *Origanum heracleoticum* food-grade.
- Steam-distilled product; no solvents, preservatives or additives are used in the production of this essential oil.
- Not Genetically Modified / NON-GMO / GMO-free product.

I.III Synonyms:
- Essential oil of Greek oregano
- *Origanum heracleoticum* extract

I.IV CAS #: 91721-63-0

I.V EINECS #: 294-363-4

I.VI FEMA #: 2660

I.VII Manufacturer:
Ecopharm Hellas S.A.
4th km N.R. Kilkis-Thessaloniki
GR-61100 Kilkis, Greece.
Hellenic Business Registration No #:14498735000
DUNS No: 50-317-9098
FDA FFRM #: 17473644444
FAMI-QS Reg. #: FAM084

SECTION II. PRODUCT SPECIFICATIONS

II.I Composition: Pure, natural, *Origanum heracleoticum* essential oil >99%.

II.II Main Ingredients:
- Carvacrol: 75.0-95.0 %
- Thymol: 0.7 – 4.0 %

II.III Batch Number: O622 (O20221006001200)

II.IV Manufactured Date: Oct 2022

II.V Expired Date: Oct 2025

II.VI Safety Data: *See relevant product's MSDS.*

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