AMERICAN UNIVERSITY OF BEIRUT

ANTIMICROBIAL ACTIVITY OF *ORIGANUM EHRENBERGII* EXTRACTS ON RESISTANT BACTERIAL STRAINS

by
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AN ABSTRACT OF THE THESIS OF

Nathalie Joy Malek for Master of Science
Major: Food Technology

Title: Antimicrobial Activity of *Origanum ehrenbergii* Extracts on Resistant Bacterial Strains

The aim of this thesis was to explore the antimicrobial activity of *Origanum ehrenbergii* essential oils and extracts on selected heat resistant bacterial strains of *Escherichia coli*, *Staphylococcus* spp. and *Salmonella* spp., isolated from foods in the Lebanese market.

These bacterial strains were tested at 55, 60, 65, 70 and 75°C, to determine their heat resistance. *E. coli* strains isolated from two different sources of raw meat recorded D_{60} of 7.32 and 28.80 minutes; D_{65} of 0.65 and 1.02 minutes; D_{70} of 0.50 and 0.11 minutes; and D_{75} of 0.38 and 0.11 minutes; respectively. The *Salmonella* strain isolated from Tahini had D_{60}, D_{65}, D_{70} and D_{75} of 9.45, 0.49, 0.19 and 0.19 minutes; respectively. Two other *Salmonella* strains isolated from poultry samples had D_{60} of 3.64 and 3.00 minutes; D_{65} of 0.47 and 0.69 minutes; D_{70} of 0.19 and 0.54 minutes and D_{75} of 0.13 and 0.46 minutes; respectively. The *Staphylococcus* spp. isolated from a chicken sandwich and parmesan cheese had D_{60} of 10.10 and 11.38 minutes, D_{65} of 1.78 and 1.47 minutes, D_{70} of 1.91 and 0.45 minutes and D_{75} of 0.44 and 0.19 minutes. These results, which are higher than what is present in the literature, show that *Salmonella*, *E. coli* and *Staphylococcus* spp. are resistant to thermal processing.

Origanum ehrenbergii was extracted using water and methanol. The extracted compounds were tested against the isolated bacterial strains to determine the inhibition zone diameter, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Essential oils obtained by hydrodistillation were more effective than methanol extracts. MICs of the former were effective at 0.1%, 0.5% and 1%, compared to 1%, 5% and 10% of the latter.

This thesis highlights emerging concerns regarding the safety of certain food products in the Lebanese market. Subsequently, further revision of associated processing conditions is required to ensure compliance with food safety standards and avoid outbreaks.

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ABBREVIATIONS

BHI	Brain Heart Infusion
PCA	Plate Count Agar
TSB	Tryptose Soya Broth
CFU	Colony Forming Unit
spp.	Species
SM1	*Salmonella* isolated from Tahini
SM2	First *Salmonella* isolated from chicken
SM3	Second *Salmonella* isolated from chicken
EC1	First *E. coli* isolated from raw meat
EC2	Second *E. coli* isolated from raw meat
ST1	Staphylococcus spp. isolated from chicken sandwich
ST2	Staphylococcus spp. isolated from parmesan cheese
amu	Atomic mass unit
IU	International unit
GC-MS	Gas Chromatography - Mass Spectrometry
W.D.	Water distillate
M.E.	Methanol extract

CHAPTER 1

INTRODUCTION

1.1. Thesis Objective

Foodborne bacteria are recurrently developing resistance to thermal processing as well as additives. The main aim of this research was to study the heat resistance of various bacterial strains isolated from different foods available in the Lebanese market. Moreover, one goal was also to evaluate the antimicrobial activity of *Origanum ehrenbergii* on these isolated bacterial strains.

1.2. Thesis Organization

This thesis includes three chapters. Chapter 1 is an introduction to the objectives and organization.

Chapter 2 discusses the heat resistance of *Escherichia coli*, *Salmonella* and *Staphylococcus* strains isolated from foods in the Lebanese market.

Chapter 3 highlights the antimicrobial activity of natural extracts from *Origanum ehrenbergii* against the isolated bacteria that have shown resistance to heat. This chapter offers a comparison between water and methanol extracts using disk diffusion method, and determines the Minimum Inhibitory and Minimum Bactericidal Concentrations of both extracts.

CHAPTER 2

EMERGENCE OF HEAT-RESISTANT *SALMONELLA*, *ESCHERICHIA COLI* AND *STAPHYLOCOCCUS* SPP. ISOLATED FROM FOOD PRODUCTS

2.1. Introduction

The Center for Disease Control (CDC) estimated that in the United States 48 million people get sick each year as a result of foodborne illnesses, of which 9.4 million cases are caused by 31 known species of pathogens. Locally as well, the Lebanese Ministry of Public Health (MOPH) data reveal an increasing trend in the number of reported cases of foodborne illnesses over the years; with 57 cases in 2005, 483 in 2010 and 319 in 2012. However, these numbers are underestimated.

In general, more than 90% of foodborne illness cases involve a handful of bacteria like *Staphylococcus aureus, Salmonella, Clostridium perfringens, Campylobacter, Listeria monocytogenes, Vibrio parahaemolyticus, Bacillus cereus,* and Entero-pathogenic *Escherichia coli* (Wagner, 2008). These bacteria are showing increasing resilience to a variety of treatment methods. Of special interest to public health researchers are *Salmonella* and *Escherichia coli*, as they are still strongly associated with foodborne illnesses triggered by the consumption of raw and undercooked meat, or cross-contamination (Jong et al., 2011).

Salmonella, a microorganism that grows at temperatures ranging from 8 to 45°C, at a pH of 4 to 9 and water activity above 0.94, can cause severe infections with only minimal concentrations in food, and is one of the most recurrent causes of foodborne illness. Lianou and Koutsoumanis (2013), showed in their study that even though *Salmonella* is considered a heat sensitive foodborne pathogen, it is exceptionally capable to survive extreme conditions of stress, specifically thermal treatments. The study explains that exposure of *Salmonella* to acidic environments has strengthened its ability to protect itself against heat. Contaminated foods due to handling raw, frozen raw or undercooked animal products can therefore easily spread the microorganism and cause salmonellosis, an infection that can also be transmitted from animals to humans and among humans. Its common symptoms are similar to gastroenteritis such as fever, vomiting, abdominal pain, and watery diarrhea. In more severe cases, infection with *Salmonella* can lead to serious illnesses like typhoid and paratyphoid fevers (Silva & Gibbs, 2012).

In turn, the *Escherichia coli* bacterium is able to grow at temperatures as low as 7-8°C, with an optimum of 35° to 40°C, at a pH range of 4 to 7 and a water activity of 0.935 to 0.995 (Charimba et al., 2010). The Center for Disease Control (CDC) estimates that, in the United States, 73,000 cases of foodborne diseases are caused by *Escherichia coli* infection each year, with 61 cases resulting in death. The Physicians Committee for Responsible Medicine (PCRM) stated that, in spite the high tendency to under-report, *E. coli* infections are likely to increase. The infective doses of *E. coli* range from 2 to 2000 cells. Symptoms vary from abdominal cramps, diarrhea, to severe cases such as

hemorrhagic colitis that can lead to hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (Gabriel, 2012b).

Undercooking or subjecting raw animal products to sub-lethal thermal conditions contributes to the adaptation of viable bacterial cells to higher temperatures, hence to develop resistance. Certain bacteria, especially *E. coli*, adapt to acidic conditions as a result of constant exposure to varied acidic states at sub-lethal concentrations, and develop indirect resistance to heat. In the process, they undergo genetic and physiologic changes in their membrane fatty acid composition (Gabriel, 2012b).

Even though heating has always been a reliable, inexpensive, and effective process in providing safe food for consumption; yet throughout the years, foodborne bacteria are discerning ways to develop resistance to it. Treating with higher levels of heat may not constitute an optimal solution, as it tends to negatively alter the sensory and nutritional quality of food produce and consequently hinder consumer acceptance (Gupta & Abu-Ghannam, 2012). Therefore, this study aims at determining current heat resistance attributes of *Salmonella*, *E. coli* and *Staphylococcus* cultures, by exposing them to five temperatures ranging from 55°C to 75°C, over a time span of 10 minutes. The temperature range was chosen based on the usual thermal processing standards. This intends to identify the heat level and exposure time needed to eliminate those foodborne pathogens. If such levels were found unreasonable, exploring alternative or synergistic methods to end this vicious thermal resistance cycle would prove necessary.

2.2. Materials and Methods

2.2.1. Materials

The medium used to grow the bacteria was the Brain Heart Infusion (BHI) broth[1]. Bacterial colonies and solutions were streaked and plated over Plate Count Agar (PCA) [1]. Tryptose Soya Broth (TSB)[2] was the medium employed during the thermal resistance experiment. Peptone water[1] was utilized for dilution purposes. All media were dissolved in distilled water and sterilized prior to utilization.

Salmonella, *E. coli* and *Staphylococcus* spp. were isolated and stored frozen in 30% glycerol and BHI in the microbiology laboratory of the Agricultural and Food Science Department at the American University of Beirut (AUB). *Salmonella* was isolated from Tahini in July 2012 (also known as sesame paste) (SM1) and chicken in February 2013 (SM2 and SM3), *E. coli* was extracted from raw meat in May 2012 (EC1 and EC2) whereas *Staphylococcus* spp. were isolated from parmesan cheese (ST2) and out of a chicken sandwich (ST1) in May and June 2012; respectively.

[1] BIO-RAD, 3, boulevard Raymond- Pointcare 92430 Marnes-La-Coquette-France
[2] OXOID Ltd., Basingstoke, Hampshire, England

2.2.2. Methods

2.2.2.1. Microbial thermal tolerance

A preliminary study was conducted to determine thermal tolerance. Twenty seven bacterial isolates were tested for thermal tolerance at 73°C for 15 seconds, sixteen were *Salmonella*, six were *E. coli* and five were *Staphylococcus*. Among these bacteria, the ones that survive this process are considered tolerant and will be tested for thermal resistance as explained in the next section. The steps described below are followed to determine thermal tolerance.

Frozen isolates were grown onto PCA and incubated at 37°C for 24 hours. Two bacterial colonies of each of the twenty seven isolates were added to a 5 ml BHI test tube and incubated at 37°C for 24 hours, of which 1ml was taken and added to 99 ml TSB at room temperature; this was the control. 1 ml was transferred from the TSB into a 9 ml peptone water sterile screw cap tube, of which 3 serial dilutions were made in similar tubes to obtain 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} diluted solutions. 0.1ml from the 6^{th} dilution was then spread on PCA and incubated at 37°C for 24-48 hours for counting purpose.

Another 1 ml of the initial solution was added to preheated 99 ml TSB at 73°C for 15 seconds. 1 ml was taken from the heated TSB at 15 seconds and added into a 9 ml peptone water sterile screw cap tube of which two serial dilutions were made in similar tubes to reach 10^{-3}, 10^{-4} and 10^{-5} diluted solutions. 0.1 ml of each tube as well as of the 99 ml TSB was then spread on PCA and incubated at 37°C for 24-48 hours. Viable colonies

(30-300 colonies) were enumerated and compared in order to isolate the most tolerant ones. Data were obtained in duplicates and the experiment was done in triplicates.

2.2.2.2. Heat resistance test

Two colonies of each of the tolerant bacterial strains were added to a 5 ml BHI tube and incubated at 37°C for 24 hours. 1ml was taken from this tube and added to a 9 ml peptone water sterile screw cap tube of which six serial dilutions were made in similar tubes to reach 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} diluted solutions. 0.1 ml from the 6^{th} dilution was then spread on PCA and incubated at 37°C for 24-48 hours for counting purpose.

Another 1 ml was transferred into 99 ml TSB held at 55, 60, 65, 70 and 75°C in a water bath which was preheated for half an hour depending on the temperature needed. At each minute for 10 minutes, 1 ml was removed from the heated medium and transferred into 9 ml peptone water sterile screw cap tubes placed on ice. For each temperature, a certain number of dilutions were made to enumerate the bacteria after plating and incubation. In principle, temperatures 55 and 60°C required a 10^{-6} dilution, for 65°C, 10^{-3} was needed, as for 70 and 75°C, 10^{-2} was enough. Data were obtained in duplicates and the experiment was repeated twice.

Thermal resistance to a certain temperature is defined when bacterial strains survive the 10 minutes period. However if their number drops to zero within 10 minutes, they are not considered resistant to this specific temperature.

2.2.2.3. Calculation of D- and z-values and statistical analysis

The CFUs of all bacteria were calculated and their $\log_{10}$ were plotted against time to obtain their decimal reduction time (D-values) for each temperature. It is the time required to kill 90% of the bacterial population at a specific temperature. In addition, the log D-values were plotted against temperature to obtain the thermal death time (z-values), which is the increase in temperature required to reduce to 10^{-1} of its previous value, meaning to kill 90% of the bacterial population. The thermal inactivation reactions of the studied bacteria at different temperatures followed first to several order kinetics with $R^2 \geq 0.8$. Subsequently, the D- and z values were calculated using the following equation: -1/slope (Kennedy et al., 2005). In order to determine whether there was significant difference in bacterial behavior (means of the duplicates) with and without heat treatment, statistical analysis following 2 sample T test were computed using Excel with 95% confidence ($p < 0.05$).

2.3. Results

2.3.1. Thermal tolerance results

The results of the thermal tolerance test conducted in the first phase of the study were as follows. Of the twenty seven bacterial isolates seven were found heat tolerant: three *Salmonella*, two *E. coli*, and two *Staphylococcus*. Thermal tolerance data, under pasteurization conditions (73°C for 15 s), of *Salmonella*, *E. coli* and *Staphylococcus* are presented in Table 2.1.

2.3.2. Heat resistance results of E. coli and Salmonella

In the test for *E. coli* resistance, results of EC1 revealed no significant change in the bacterial behavior at 55 and 60°C ($p>0.05$), unlike the effect of temperatures 65, 70 and 75°C ($p<0.05$; Figure 2.1-a). The thermal inactivation of EC1 at 60°C followed a linear trend with R^2 0.8 and a D-value of 28.80 min. At 65°C it followed 2nd order kinetics with R^2 0.9 and D-value of 1.02 min. While at 70 and 75°C it manifested a 6th order polynomial trajectory with an R^2 of 0.8 and a calculated D-value of 0.11 min. Their z-value recorded 6.06°C (Table 2.2).

Similarly, subjecting EC2 to 55 and 60°C for 10 min did not affect its death rate ($p>0.05$). When the temperature was increased to 65, 70 and 75°C, a clear drop in the log CFU across the 10 min time span was noticed ($p<0.05$; Figure 2.1-b). The thermal inactivation of EC2 at 60, 65, and 70°C followed 3rd order kinetics with an R^2 of 0.8. The same kinetic trend was established at 75°C with an R^2 of 0.9. The respective calculated D-values were 7.32, 0.65, 0.50 and 0.38 min. The z-value was calculated from the obtained D-values and is equal to 4.74°C (Table 2.2).

Regarding the *Salmonella* strains, Figure 2.2-a notes the beginning of a slight decrease in the 60°C curve of SM1, which becomes more pronounced with the increase in time and temperature ($p<0.05$). The thermal inactivation of SM1 at 60°C followed a linear trend with R^2 equals to 0.9 and D-value equals 9.45 min. It continues with a 3rd order polynomial at 65°C with R^2 0.8, and with R^2 0.9 at 70 and 75°C. Their respective D-values were 0.49, 0.19 and 0.19. It was noted that bacterial count was reduced to zero at 70°C

and above, after 2 min of heat treatment (Figure 2.2-a). The z-value obtained from those D-values was 9.14°C (Table 2.2).

While 55°C had still no effect on the death rate of SM2 ($p>0.05$), the decreasing trend at 60°C was more significant following a 2nd order kinetics with R^2 equals 0.9 and D-value recording 3.64 min. The thermal inactivation at 65°C followed a 3rd degree polynomial giving an R^2 0.9 and a D-value of 0.47 min. The 70 and 75°C trajectories represented a 5th degree polynomial with their R^2 0.8 and 0.9 and D-values 0.19 and 0.13 min, respectively. It was noted that its trend persisted till 6 min at 75°C until it completely dropped to zero (Figure 2.2-b). Their calculated z-value was equal to 10.73°C (Table 2.2).

Lastly and again, heating SM3 at 55°C for 10 min did not result in any decrease in its bacterial log CFU ($p>0.05$). However the 60°C trajectory as shown in Figure 2.2-c marked a sharper decline. Thermal inactivation at 55°C followed the 4th order kinetics and continued with 2nd order for 60, 65, 70 and 75°C. All inactivation curves generated a common R^2 of 0.8 and D-values of 3.00, 0.69, 0.54 and 0.46 min respectively of which was derived a z-value of 19.65°C (Table 2.2). It was observed that ultimate death was reached at 70°C for 4 min and at 75°C for 3 min ($p<0.05$; Figure 2.2-c).

2.3.3. Heat resistance results of Staphylococcus

Heating at 55 and 60°C did not seem to affect the performance of ST1 and ST2 ($p>0.05$). The thermal inactivation of ST1 at 60 and 65°C followed a respective 4th and 3rd order polynomial trajectory with an R^2 of 0.8 and D-values recording D_{60} 10.10 min

and D_{65} 1.78 min. While at 70 and 75°C it manifested a respective 2nd and 3rd order kinetics with an R^2 of 0.9 and calculated D-values of 1.91 and 0.44 min respectively. Figure 2.3-a illustrates the resistance of ST1 to all temperatures, its bacterial count did not reach zero even at 75°C for 10 min. Z-value was equal to 12.36°C, which means that the temperature should increase by 12.36°C in order to inactivate 90% of viable cells of this strain (Table 2.2).

ST2 was also resistant, as its bacterial count did not reduce to zero at any combination of time and temperature (Figure 2.3-b). Thermal inactivation of ST2 at 60°C followed a linear trend with a D-value of 11.38 min and continued with 2nd order for 65°C with a D-value of 1.47 min and 3rd order for 70 and 75°C with D_{70} 0.45 min and D_{75} 0.19 min. R^2 was equal to 0.8 for all temperature trajectories. As for z-value, a rise in temperature of 8.57°C is needed to induce a drop of 1 log cycle for this strain (Table 2.2).

2.4. Discussion

The literature shows D-values calculated for the three bacteria analyzed in this research. Gabriel (2012a), and Gabriel and Nakano (2011) who followed the behavior of *E. coli* isolated from apple juice, as well as Gabriel and Arellano (2014), with their study on coconut liquid endosperm, have reported D-values at 55°C that ranged between 0.9 to 23.20 min. This relative sensitivity to heat seems in contrast to the clear resistance behavior found in this study.

For higher temperatures, Charimba and Hugo (2010) and Rajkowski (2012) reported consistently lower D-values in their experiments that treated bacterial samples from traditional South African sausage, catfish and tilapia. Hence, our results have shown higher resistance to heat (D_{60}: 7.32 - 28.80 min, D_{65}: 0.65 - 1.2 min, D_{70}: 0.11 - 0.50 min and D_{75}: 0.11 - 0.38 min), than in the behavior documented in those studies (D_{55}: 7.00 - 9.40 min, D_{60}: 0.93- 4.74 min, D_{65}: 0.06 - 0.17 min and D_{70}: 0.13 min). The z-values obtained in our study (Table 2.2) were also higher than the one mentioned in Rajkowski's (2012) study which was equal to 4.3°C.

Lower D- and z-values of *Salmonella* were reported in Rajkowski (2012)'s study on seafood and meat and Osaili et al. (2013)'s study on chicken (D_{55}: 7.08 - 7.50 min, D_{60}: 0.32 - 0.86 min and D_{65}: 0.03 - 0.06 min; z-values: 3.78 - 4.58°C) as compared to those in our study (D_{60}: 3.00 - 9.45 min, D_{65}: 0.47 - 0.69 min and D_{70}: 0.19 - 0.54 min; z-values: 9.14 - 19.65°C).

Additionally, in their study on thermal resistance of different *Salmonella* species under various conditions and food composition, Doyle and Mazzotta (2000) reported D-values ranging as follows, D_{55}: 36.20 - 8.00 min, D_{60}: 0.20 - 3.06 min, D_{65}: 0.06 - 1.10 min and D_{70}: 0.095 min and his z-values ranged from 3.30 to 10.30°C, which are all slightly lower than our results. The variations noticed in the comparison could be due to experimenting on different species of *Salmonella* as well as to different food composition and media conditions.

While examining the behavior of *Staphylococcus* in ready to eat Indonesian traditional foods, Dewanti-Hariyadi et al. (2011) obtained D_{55} of 6.59-14.3 min and z-values ranging from 3.37 to 6.06°C which are lower than the results of this study. Moreover, Kennedy et al. (2005) reported lower D_{55} (13.00 - 21.70 min), lower D_{60} (4.80 - 6.50 min) and slightly lower z-values (7.7 - 8.0°C) when studying the thermal inactivation of *Staphylococcus* in TSB. Hassani et al. (2006) also recorded a lower D_{65} (0.07 min) and z-value (3.6°C). Therefore, the results of this study have shown higher resistance to heat (D_{60}: 10.10 - 11.38 min and D_{65}: 1.47 - 1.78 min) as well as higher z-values (8.57°C - 12.36°C).

These variations in D- and z-values could possibly be explained by strains of *E. coli* with higher heat resistance or by the differences in *Staphylococcus* and *Salmonella* species. Further studies would have to be done in order to determine the possible origin of this disagreement.

It seems that the bacterial strains in this study, which were isolated from foods available in the Lebanese market, have shown higher resistance than commonly found in the literature. It may be that bacterial resistance to heat is contributing to contamination and causing foodborne illnesses. High D- and z-values might explain why it is becoming difficult to maintain safe levels of foodborne pathogens in foods by abiding to the standard guidelines. According to the Food and Drug Administration (FDA) and NSW Food Authority (NSWFA) guidelines, Staphylococcus levels in food should not exceed 10^3 CFU per gram to remain “acceptable” for consumption and should not exceed 10^2 CFU per gram to be considered “good” for consumption; whereas *E. coli* levels should be less than 3 CFU

per gram in order to consider the food satisfactory or acceptable for consumption. *Salmonella* however, should not be detected in a 25 g food sample (Silva and Gibbs, 2012). New criteria in food processing may have to be re-established in order to meet the required levels (good, acceptable/satisfactory, unsatisfactory and potentially hazardous) of foodborne pathogens specific to each food category. For instance, longer heat treatment and/or higher temperatures in foods production may be required to insure safe levels of pathogens and at the same time sustain food quality. Meanwhile, a good way in tackling this issue would be through studying other ways of foodborne pathogens inactivation if new guidelines seem to appear unreasonable.

2.5. Conclusion

The studied bacterial strains of *E. coli*, *Salmonella* and *Staphylococcus* have demonstrated resistance to some temperatures as supported by higher D and z-values than what is present in the literature. Hence, the need for exploring and engaging alternative or synergistic methods including natural antimicrobials might seem helpful in reducing cases of foodborne illnesses as well as in preventing post cooking recontamination. This field of research would probably be a plausible solution as it is in accordance with consumers' demand of minimally processed and natural produce.

Table 2.1 Log CFU g^{-1} of *Salmonella, E. coli* and *Staphylococcus* before and after heat treatment at 73°C for 15 s

Bacteria	**Log CFU**	
	Initial*	At 73°C for 15 s*
E. coli (EC1)	8.83 ± 0.362	4.49 ± 1.749
E. coli (EC2)	9.13 ± 0.032	5.66 ± 0.542
***Salmonella* spp.** (SM1)	9.09 ± 0.032	4.35 ± 0.526
***Salmonella* spp.** (SM2)	9.06 ± 0.122	4.66 ± 0.592
***Salmonella* spp.** (SM3)	8.90 ± 0.037	4.50 ± 1.498
***Staphylococcus* spp.** (ST1)	10.36 ± 0.354	6.73 ± 0.852
***Staphylococcus* spp.** (ST2)	10.02 ± 0.688	5.83 ± 0.182

*Results are means ± SD of triplicate measurements

Table 2.2 D-values calculated using the equation D=-1/slope, for the plots of surviving cell numbers (log_{10} CFU) vs. treatment times and z values calculated using the equation z=-1/slope, for the plots of log_{10} D vs. temperature of EC1, EC2, SM1, SM2, SM3, ST1 and ST2 at 60, 65, 70 and 75°C

Isolate	**Origin**	**$D_{(T)}$-values (min)**					**Z values (°C)**
		55°C*	**60°C**	**65°C**	**70°C**	**75°C**	
E. coli (EC1)	Meat	-	28.80	1.02	0.11	0.11	6.06
E. coli (EC2)	Meat	-	7.32	0.65	0.50	0.38	4.74
***Salmonella* spp.** (SM1)	Tahini	-	9.45	0.49	0.19	0.19	9.14
***Salmonella* spp.** (SM2)	Chicken	-	3.64	0.47	0.19	0.13	10.73
***Salmonella* spp.** (SM3)	Chicken	-	3.00	0.69	0.54	0.46	19.65
***Staphylococcus* spp.** (ST1)	Chicken Sandwich	-	10.10	1.78	1.91	0.44	12.36
***Staphylococcus* spp.** (ST2)	Parmesan Cheese	-	11.38	1.47	0.45	0.19	8.57

* The symbol (-) in D_{55} represents an undefined D-value that tends to infinity

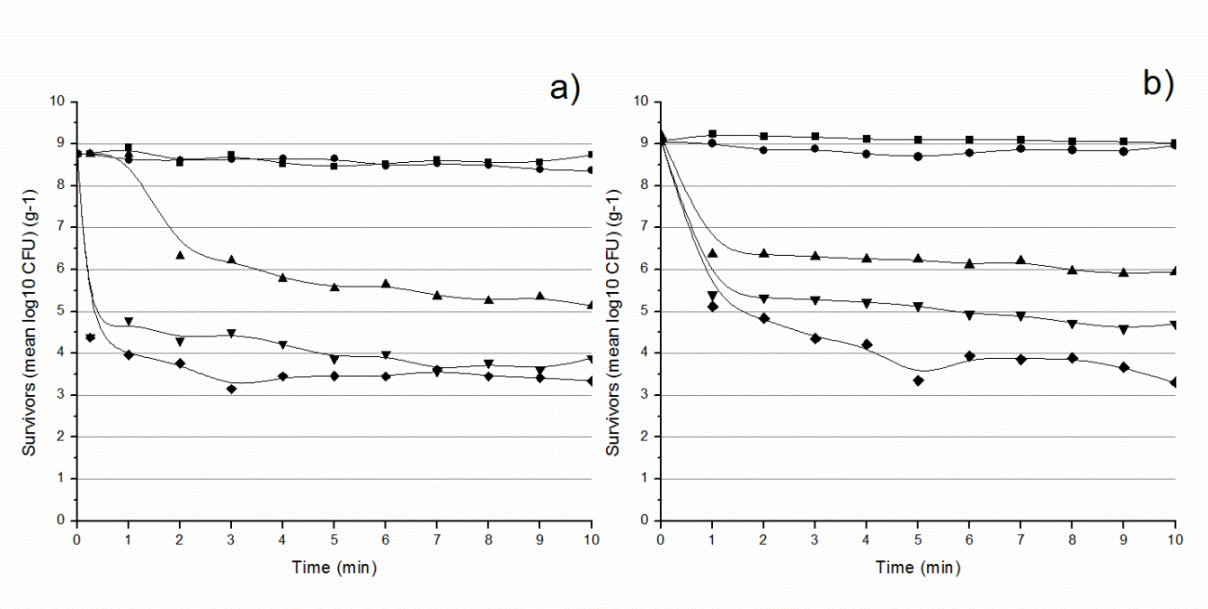

Fig. 2.1 Survivor curves of *E. coli* isolates a) EC1 and b) EC2 at 55 (—■—), 60 (—●—), 65 (—▲—), 70 (—▼—) and 75°C (—◆—)

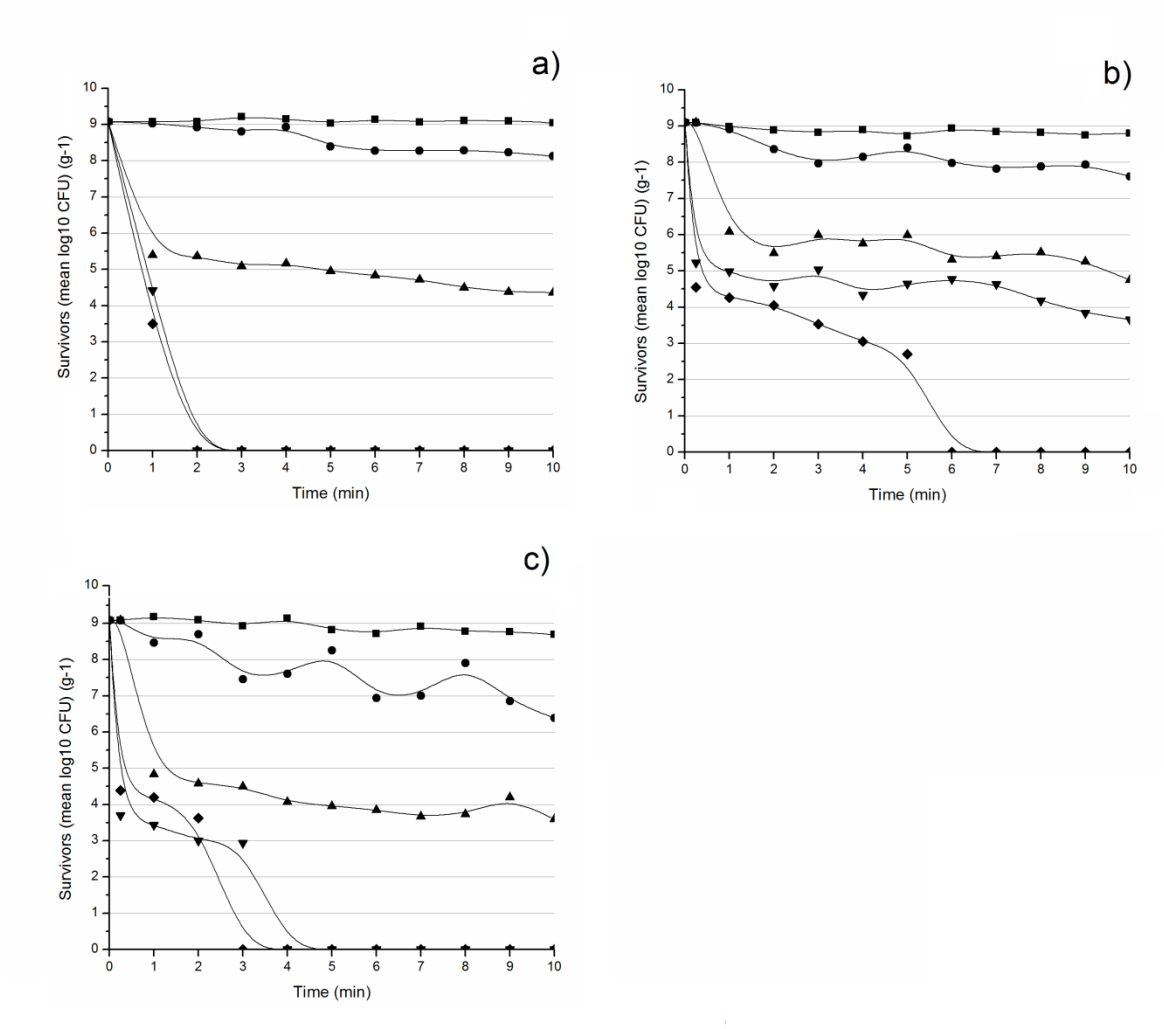

Fig. 2.2 Survivor curves of *Salmonella* isolates a) SM1, b) SM2 and c) SM3 at 55 ( ), 60 ( ), 65 ( ), 70 ( ) and 75°C ( )

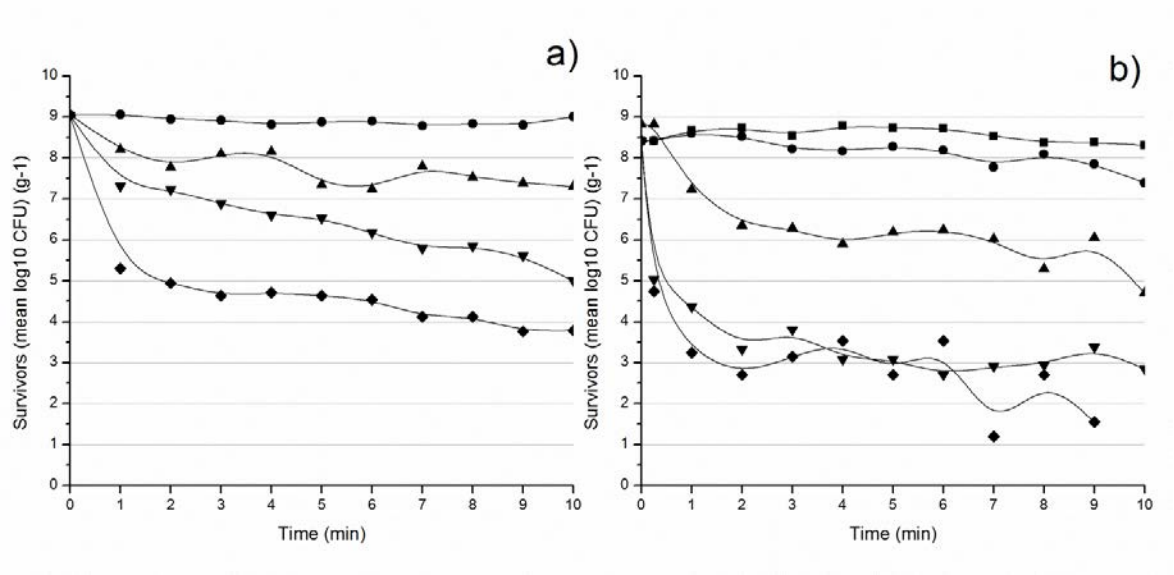

Fig. 2.3 Survivor curves of *Staphylococcus* isolates a) ST1 and b) ST2 at 55 (—■—), 60 (—●—), 65 (—▲—), 70 (—▼—) and 75°C (—◆—)

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CHAPTER 3

ANTIMICROBIAL ACTIVITY OF NATURAL EXTRACTS FROM *ORIGANUM EHRENBERGII*

3.1. Introduction

Bacterial resistance to thermal processing, additives and antibiotics, through various alterations or mutations in their DNA, has become a serious issue faced in the food industry more so due to its continuous amplification. The persistence of this trend will ultimately trap humanity in a vicious cycle, hence the urgent need for alternative or synergistic methods in order to break this cycle and stop the covert behind bacteria resilience.

Consequently, there have been recent advances in the application of non-thermal processing techniques with the hope to treat this issue. Some of these systems include irradiation such as Gamma rays, X-rays and electron beam, UV radiation, high pressure processing (HPP), high pressure carbon dioxide (HPCD) and pulsed electric field (PEF). However these technologies winded up with several limitations summarized in Table 3.1 (Gupta and Abu-Ghannam, 2012). Moreover, it has been reported that many bacteria can survive and subsequently resist such inventions when exposed to their sub-lethal doses (van der Veen and Abee, 2011).

Such hurdles have led researchers to shift their focus towards introducing natural antimicrobials to the food industry and exploring possibilities of incorporating them into food and packages. This is considered a plausible turning point as interest in natural

ingredients is on the rise as well as consumer's demand for minimally processed foods. Due to their natural origins, most of the common plant extracts are GRAS (Generally Recognized As Safe) that can be incorporated into food packages and get in contact with food safely (Negi, 2012). Not only are they considered safe but to our knowledge, bacteria fail to develop resistance against them due to their ultra-complex composition. This leaves bacteria no time to mutate with the presence of hundreds and thousands of chemical constituents within each essential oil which are subsequently capable of easily inhibiting and killing the growth of bacteria.

Researchers have begun their studies on potential applications of essential oils as natural food preservatives. Smith-Palmer, Stewart and Fyfe have studied the possible appliance of bay, clove, cinnamon and thyme essential oils in low-fat and full-fat soft cheese against *Listeria monocytogenes* and *Salmonella enteritidis*. All the oils were active at 1% in the low-fat cheese whereas only clove oil revealed inhibition in the full fat cheese; this explains the importance of the composition of food products (Smith-Palmer et al., 2001). Seydim and Sarikus have tried incorporating essential oils of oregano, rosemary and garlic into whey protein based edible films and tested them against *Escherichia coli O157:H7*, *Staphylococcus aureus*, *Salmonella enteritidis*, *Listeria monocytogenes* and *Lactobacillus plantarum*. Oregano essential oil exhibited the highest level of antibacterial inhibition at 2%, garlic was effective at 3% and 4% whereas rosemary did not show any antimicrobial effect (Seydim and Sarikus, 2006). In another study entitled "Biodegradable gelatin–chitosan films incorporated with essential oils as antimicrobial agents for fish preservation", clove, lavender, fennel, thyme, cypress, pine, herb-of-the-cross and

rosemary essential oils were tested. Mostly clove, followed by thyme, rosemary and lavender were able to reduce bacterial counts (Gomez-Estaca et al., 2010).

The only challenge faced when incorporating essential oils as single components to food products is that most of the time, the needed concentration that is considered enough in inhibiting the growth of bacteria can usually negatively alter the organoleptic properties of foods. However a reasonable solution would be to discover and develop synergies between several compounds, aiming to end up with lower essential oil concentrations without deteriorating the organoleptic quality of foods.

The focus of this study revolves around oregano oil as it is classified among the strongest antimicrobial agent. Oregano primarily serve as spices with remarkable flavor worldwide, and their oils, extracted from the plant *Origanum,* are typically utilized for their antimicrobial, antifungal, antiviral, nematicidal, cytotoxic and antioxidant characteristics. Specifically, their antimicrobial activity stems from their abundance in carvacrol and thymol (Halawi, 2005). Oregano oils were found more successful in inhibiting *E. coli* than lemongrass oil and cinnamon oil in apple puree edible films, (Rojas-Graü et al., 2006) and more effective than rosemary and garlic on some other bacteria in whey protein based films (Seydim and Sarikus, 2006) or in chitosan films (Zivanovic et al., 2005). The Mediterranean environment favors the growth of oregano and enhances its essential oils' yield which is generally moderate.

Origanum ehrenbergii, specie of Oregano belonging to the Prolaticorolla section, was selected for this study in particular for several reasons. As a start, the common

availability of this plant in the Lebanese mountains motivates the subject of this research; it grows rapidly on dry granite based lands and in pinewood of acidic soils at an altitude of up to 1500 m. To the best of our knowledge, few studies were conducted on this plant in particular. Regarding its chemical composition, it was found that the dominant essential oils composition of *Origanum ehrenbergii* were thymol (28.5 - 59.2%), carvacrol (21.2 - 78.5%), p-cymene (5.5 - 10.6%) and γ-terpinene (2.6 - 14.7%) (Figuérédo et al., 2005). Along with their precursors monoterpene hydrocarbons p-cymene and γ-terpinene, carvacol and thymol belong to the phenolic group of antimicrobial compounds in plants (Halawi, 2005). Chemical analyses have shown that thymol and p-cymene are compounds with major antioxidant, anti-inflammatory and anticholinesterase activities (Loizzo et al., 2009). In his thesis, Halawi (2005) has tested *Origanum ehrenbergii*'s essential oils on specific bacteria, and showed that they were effective against *Staphylococcus aureus* in vitro. There is not much research conducted on its possible inhibition properties against a wide variety of bacteria specifically the ones that are acquiring resistance to food processing.

In the course of this study, the substance of *Origanum ehrenbergii* was extracted using water and methanol. The chemical composition of their active components was determined by Gas Chromatography - Mass Spectrometry (GC-MS) and their concentrations were calculated. These extracts were used against the *E coli*, *Salmonella* and *Staphylococcus* isolates that were previously tested for heat resistance. At the end of this research we would be able to detect the antimicrobial activity of *Origanum ehrenbergii* oil and extracts on these isolates, compare their effectiveness with thermal

treatment and antibiotics application and finally suggest some food application interventions.

3.2. Materials and Methods

3.2.1. Materials

Wild *Origanum ehrenbergii* was collected at an altitude of 1650 m during the month of June. The samples were dried in the shade over a one week period, ground and vacuum packed (Appendix 1).

Five bacterial isolates used in the heat resistance test were selected for this study. They are the following, SM1 and SM2 which are the *Salmonella* isolated from Tahini and chicken, respectively; EC1 and EC2 which are the *E coli* isolated from raw meat, respectively and ST1 and ST2 which are the *Staphylococcus* isolated from a chicken sandwich and Parmesan cheese, respectively.

3.2.2. Methods

3.2.2.1. Water distillation

Each 100 g at a time was subjected to conventional hydrodistillation for 3 to 5 hours. The essential oil was then separated using a separatory funnel or syringe, centrifuged (Megafuge 1.0R Heraeus)[3], dried from water through the rotary evaporator

[3] Kendro Laboratory Products D-37520 Osterode

(LABCONCO) then dissolved in a solution of 40% dimethylsulfoxide (DMSO)[4] and water in order to attain the following concentrations, 10%, 5%, 1%, 0.5% and 0.1% then exposed to ultrasonic waves to insure homogenization.

3.2.2.2. Methanol extraction

The remaining plant material was soaked in methanol (34860)[5] at a ratio of 1:10 for 16 hours, suction-filtered through Whatman No 1 filter paper using a Buchner funnel and washed with methanol. The filtrate was subjected to centrifugation for clarification and the methanol fraction was completely evaporated through the rotary evaporator. The extract was then dissolved in 40% DMSO and water to attain the following concentrations, 10%, 5%, 1%, 0.5% and 0.1% then exposed to ultrasonic waves to insure homogenization.

3.2.2.3. GC-MS

Depending on the mass of the oil and extract, a certain 99.9% dichloromethane[5] volume was added to obtain the concentration of 100 ppm. Standard solutions of thymol (T0501)[5], carvacrol (282197)[5], para-cymene (C121452)[5] and gamma-terpinene (223190)[5] at several concentrations (2, 4, 6, 8, 10 and 15 ppm) were prepared in order to draw their calibration curves (Area vs. Concentration) and to identify them in the samples tested. All the calculations performed in order to obtain these solutions are listed in Appendix 2. 1 ml of the prepared solution was analyzed on a Trace GC Ultra coupled to a DSQ II MS equipped with a TG-5MS column (30 m x 0.25 mm, df: 0.25 µm) with a flow rate of

[4] MERCK-Schuchardt, 328, Germany
[5] Sigma-Aldrich, St. Louis, USA

1ml/min, helium was the carrier gas. Injection volume was 1µl. The temperature programming was set for 5 min at 50°C up to 300°C at 5°C/min with a delay starting time of 6 min. The injector and detector temperatures were 250°C and 230°C, respectively. The ion source was set at 230°C and the mass range was from 33 to 450 amu (atomic mass unit). Identification of the compounds of interest was carried out by comparing their mass spectra and structure to those in the library and to the standards.

3.2.2.4. Antimicrobial resistance testing

3.2.2.4.1. Disk Diffusion

Two colonies of each of the *E coli*, *Staphylococcus spp.* and *Salmonella* isolates that were screened for heat resistance were tested against their specific antimicrobial agents[6] (Penicillin (6µg), Tetracycline (30µg or 10IU), Streptomycin (500µg), Chloramphenicol (30µg), Amoxycillin (25µg), Erythromycin (15µg), Gentamicin (500µg) and Neomycin (30UI or 18µg)) by the Kirby-Bauer Single Disc Diffusion method with standard antibiotics disks. Three disks were placed on agar plates containing 0.1ml of inoculate (colonies in 5ml BHI) from the 4^{th} dilution, which is equivalent to10^5 CFU/ml, and incubated at 37°C for 24 hours. Strains were classified as sensitive or resistant based on standardized inhibition zones which were measured with a metric ruler then compared to standard cut-offs according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute guidelines (CLSI).

[6] BIO-RAD, 3, boulevard Raymond- Pointcare 92430 Marnes-La-Coquette-France

The solutions of essential oils prepared followed the same procedure on the most resistant bacteria in order to detect microbial inhibition.

3.2.2.4.2. Minimum Inhibitory (MIC) and Minimum Bactericidal Concentrations (MBC)

The MIC is defined as the lowest antibacterial concentration needed to stop the growth of bacteria all throughout the incubation period. To know the minimum inhibitory concentration of the oil and extract, 1ml of each concentration of oil and extract was added to 1ml bacterial inoculum from the 4^{th} dilution that has a concentration of 10^5 CFU/ml and incubated at 37°C for 18 hours. The turbidity of the mixture was compared visually and spectrophotometrically, the clear tubes indicate no bacterial growth (the wavelength chosen for the methanol extract solution ranged from 600 to 540 nm whereas that of water distillation varied between 540 and 420 nm depending on the color and turbidity of the solution). Consequently, in order to discern whether the oil or extract is not only inhibitory but also bactericidal, 0.1 ml from each tube was plated on PCA and incubated at 37°C for 24 hours, counted and compared. The MBC is the lowest concentration of antibacterial substance inducing negative growth (less than 30 colonies).

3.2.2.5. Data analysis

Duncan's test was performed to determine if the means of inhibition zones diameters at different concentrations were significantly different from each other at 95% confidence interval ($\alpha = 0.05$). The analyses were conducted using SPSS v. 21.0 (SPSS Inc., Chicago, IL, USA).

3.3. Results

3.3.1 Oil yield and chemical analysis

The yields obtained from 100 g plant material subjected to water distillation and methanol extraction were around 1 ml (0.9 g) and 12.9 g respectively. In reference to their calibration curves (Appendix 3), concentrations of thymol (17.96 ppm) and carvacrol (3.96 ppm) in the essential oils obtained through water distillation were higher than those in the methanol extract (thymol: 2.82 ppm and carvacrol: 0.90 ppm). This is also manifested by their greater areas illustrated in Figures 1 & 2 in Appendix 3. The GC-MS could not detect peaks for para-cymene and gamma-terpinene even when running the oil and extract at a concentration of 500 ppm (Appendix 2).

3.3.2 Antimicrobial resistance tests

The inhibition zone diameters formed by the application of the water distillate and methanol extract on the bacterial plates are stated in Tables 3.2 and 3.3 along with their MICs and MBCs. The MICs were determined based on the percent transmittance of the bacterial mixtures (with water distillate and methanol extract) as compared to the control (antibacterial solution without bacteria) which is set at 100% transmittance (Appendix 4). Any level below 100% indicates bacterial growth. SM1 was inhibited at 1.0% water distillate (W.D.) versus 10.0% methanol extract (M.E.), SM2 at 0.1% W.D. versus 5.0% M.E, ST1 at 0.5% W.D. versus 1.0% M.E., ST2 and EC1 at 1.0% W.D. versus 5.0% M.E.

and EC2 at 0.1% W.D. versus 1.0% M.E.. Methanol or DMSO and water without oil added do not acquire any antimicrobial properties as they did not show an inhibitory effect against the tested isolates. There was no inhibition of growth with the vehicle control (40% DMSO/water or methanol).

3.3.3 Antibiotic susceptibility tests

According to the antimicrobial susceptibility cut-offs set by the EUCAST (2014) and CLSI (2011) guidelines (Table 3.5), it is inferred that the *Salmonella* isolates (100%) showed resistance to neomycin, gentamicin and streptomycin and were susceptible to penicillin, amoxycillin, tetracycline and chloramphenicol. The *E coli* isolates (100%) were found resistant to penicillin, neomycin, gentamicin and streptomycin and susceptible to amoxycillin, tetracycline and chloramphenicol. Concerning *Staphylococcus* isolates, ST1 showed multidrug resistance to almost all antibiotics tested (penicillin, neomycin, chloramphenicol, erythromycin and gentamicin), while ST2 was only resistant to gentamicin (Table 3.4). It has been observed that five out of six isolates showed resistance to at least three antibiotics.

3.4. Discussion

The abundance of thymol and carvacrol in the plant material of this study is in accordance with a previous study conducted in 2005 on two populations of *Origanum ehrenbergii* (Figuérédo et al., 2005). On the other hand, its flowering aerial parts are mainly abundant in thymol and *p*-cymene (Loizzo et al., 2009). The essential oils obtained

from water distillation proved to be more effective than those in the methanol extract, as the former's MICs (Table 3.2) were significantly lower than the latter's (Table 3.3) for all tested bacteria. In addition, due to the fact that inhibition zones of the water distillate were significantly greater than those of the methanol extract for most concentrations (Tables 3.2, 3.3). Moreover, at a concentration of 10%, the water distillate was able to kill SM1, ST2, EC1 and EC2 while the methanol extract was only able to kill SM1 at the same concentration. This stronger antibacterial activity could be correlated to the higher concentration of thymol and carvacrol in the water distillate as compared to the methanol extract. It can therefore be concluded that water distillation is more effective and successful than methanol extraction. It is interesting to point out that bacteria with larger zones of inhibition are not always the ones resulting in lower MICs and MBCs values because disk diffusion is mainly influenced by the solubility and volatility of the oil (Bouhdid et al., 2008).

Antibacterial activity of essential oils could be used to outweigh the inefficiency of usual thermal processing and antibiotic cure. Part of this inefficiency is confirmed in the second chapter of this work, where bacterial behavior was analyzed under heat treatments at different temperatures ranging from 55 to 75°C; it was proven that *Salmonella*, *E. coli* and *Staphylococcus* spp. are resistant to most temperatures for a period of 10 minutes. While the results of this study also show that these same bacteria are resistant to some antibiotics, the essential oils of *Origanum ehrenbergii* on the other hand were effective and inhibitory at low concentrations. Antibiotic resistance is expected to increase if bacteria frequently get exposed to them in a disorganized manner. Hence the

importance of investing in incorporating *Origanum ehrenbergii*'s essential oils into food and food packages. This intervention could imply substitution or supplementation of the oils to pasteurization as well as their addition to foods, which require cooking at high temperatures; this would ultimately preserve nutritional quality while insuring food safety. They could also be added solitarily to food products that are preferably eaten raw.

When applying such interventions, it should be taken into account that the appropriate concentration of essential oil needed to inhibit bacteria does not negatively alter the taste of food. Fortunately in this study, the inhibitory concentrations of essential oils obtained from water distillation (0.1%, 0.5% and 1%), are considered organoleptically acceptable according to a study in which it was concluded that the addition of 0.6% or 0.9% essential oil was found to be organoleptically acceptable (Gupta & Abu-Ghannam, 2012). However, when used in food systems, it was noticed that the effective essential oils levels needed often become greater than would normally be organoleptically acceptable (1-3%). This is due to the presence of fat, carbohydrate, protein, salt and pH differences that influence the activity of these antimicrobials in foods (Holley and Patel, 2005). Therefore testing *Origanum ehrenbergii*'s essential oils on contaminated foods with different composition is necessary to see how the effectiveness of the oils varies.

In this case, if the needed inhibitory concentration of *Origanum ehrenbergii* oil exceeds the organoleptically acceptable level, synergy with other kinds of essential oils at lower concentrations could be a solution. A different way to minimize their taste effects would be to encapsulate the oils into nanoemulsions and enclose them within the food product. This technique enhances the stability of volatile compounds, protects them as

much as possible from interacting with the macronutrients that constitute food products and it improves their antimicrobial efficacy due to their slow release into the cells (Donsì et al., 2011). Another innovative idea would be to keep the original inhibitory level and market new food products with oregano flavour. This suggestion would mostly be possible for dairy products and other processed foods notably spicy foods where the acceptable sensory threshold is relatively high.

Nevertheless, this technique is not always feasible. As a matter of fact, introducing the essential oil as an ingredient in certain food products poses a concern. In order to avoid this, applying active packaging by encapsulating the essential oils into polymers of edible and biodegradable coatings or films induce slow release to the food or headspace of packages without being part of the food itself.

3.5. Conclusion

The resulting *Origanum ehrenbergii*'s water distillate proved more effective than its methanol extract on the selected foodborne bacteria. Moreover, it turned out to be inhibitory at organoleptically acceptable concentrations as defined for essential oils in general.

The crude essential oils of Oregano are classified as GRAS by the FDA (Hyldgaard, Mygind, & Meyer, 2012); however, more research is needed to specify the acceptable daily intake of *Origanum ehrenbergii*'s oils in specific before they can be used in food products. It might also be important to run a complete chemical analysis on *Origanum ehrenbergii*'s components and make sure they are all safe for consumption.

After which complementary analysis should be done by testing the oils on contaminated foods with different composition to see how the effectiveness of the oils varies. Lastly, sensory evaluation of the end products could be performed in order to double check that consumers would show a favorable reception towards *Origanum ehrenbergii* supplemented foodstuff.

Table 3.1 Non thermal processing: advantages vs. limitations

Non Thermal Processing Technique	Advantage	Limitation
Irradiation *(Gamma rays, X-rays, electron beam)*	Effective for several foods Many different sources available	Limited public acceptance Lipid oxidation & texture degradation
UV radiation	No chemicals are used Non-heat related method	Long term exposure can be harmful to workers
HPP *(High pressure processing)*	Can be used for both solid and liquid samples.	Changes in quality of food has been observed
HPCD *(High pressure carbon dioxide)*	CO2 is GRAS, nonflammable and non-toxic	Not very successful for solid foods & Commercial application is still not a success
PEF *(Pulsed electric field)*	Pulse applied for a short period so no generation of heat	Cannot be applied to foods which cannot withstand high fields or form bubbles

Table 3.2 Antibacterial activity of *Origanum ehrenbergii*'s essential oils extracted by water distillation (W.D.) in solid and liquid media

Bacteria*	Source	Inhibition zone diameter† W.D. concentration					MIC‡	MBC§
		0.1%	0.5%	1%	5%	10%		
***Salmonella* spp.** (SM1)	Tahini	0.0 ± 0.0^{a}	11.3 ± 1.5^{b}	10.0 ± 0.0^{b}	15.3 ± 0.6^{c}	15.0 ± 2.0^{c}	1.0	10.0
***Salmonella* spp.** (SM2)	Chicken	0.0 ± 0.0^{a}	10.7 ± 0.6^{b}	10.7 ± 1.2^{b}	12.3 ± 2.3^{bc}	14.0 ± 2.0^{c}	0.1	-
***Staphylococcus* spp.** (ST1)	Chicken Sandwich	10.3 ± 0.6^{a}	10.7 ± 6.4^{a}	14.0 ± 6.9^{ab}	14.3 ± 5.8^{ab}	24.7 ± 1.2^{b}	0.5	-
***Staphylococcus* spp.** (ST2)	Parmesan Cheese	0.0 ± 0.0^{a}	$<10.0\pm1.7^{a}$	11.0 ± 1.2^{b}	12.7 ± 3.1^{bc}	18.0 ± 1.0^{c}	1.0	10.0
E. coli (EC1)	Meat	0.0 ± 0.0^{a}	11.0 ± 1.0^{b}	10.3 ± 0.6^{b}	15.7 ± 2.1^{c}	18.3 ± 1.5^{d}	1.0	10.0
E. coli (EC2)	Meat	0.0 ± 0.0^{a}	$<10.0\pm0.0^{b}$	10.0 ± 0.0^{bc}	13.7 ± 0.6^{c}	13.0 ± 0.0^{c}	0.1	10.0

Variables with different superscripts are significantly different ($p < 0.05$)

*Final bacterial density was 10^5 CFU/ml

†Inhibition zone diameters (mm) produced around the discs by adding 20 µl of essential oil. Values are means of three measurements

‡ MIC: minimum inhibitory concentration (as % v/v)

§ MBC: minimum bactericidal concentration (as % v/v)

Table 3.3 Antibacterial activity of *Origanum ehrenbergii*'s methanol extract (M.E.) in solid and liquid media

Bacteria*	**Source**	**Inhibition zone diameter**[†] **M.E. concentration**					**MIC**[‡]	**MBC**[§]
		0.1%	**0.5%**	**1%**	**5%**	**10%**		
***Salmonella* spp.** (SM1)	Tahini	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	10.0 ± 0.0^{b}	11.0 ± 1.0^{c}	10.0	10.0
***Salmonella* spp.** (SM2)	Chicken	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	$< 10.0\pm0.0^{b}$	11.0 ± 1.0^{c}	12.0 ± 10.8^{c}	5.0	-
***Staphylococcus* spp.** (ST1)	Chicken Sandwich	8.7 ± 15.0^{a}	13.3 ± 15.3^{a}	13.0 ± 5.2^{a}	15.0 ± 8.7^{a}	16.0 ± 5.6^{a}	1.0	-
***Staphylococcus* spp.** (ST2)	Parmesan Cheese	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	$< 10.0\pm0.0^{b}$	11.7 ± 0.6^{c}	15.3 ± 1.2^{d}	5.0	-
E. coli (EC1)	Meat	$< 10.0\pm0.0^{a}$	$< 10.0\pm0.0^{a}$	$< 10.0\pm0.0^{a}$	10.0 ± 0.0^{b}	14.0 ± 1.0^{c}	5.0	-
E. coli (EC2)	Meat	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	$< 10.0\pm0.0^{b}$	11.0 ± 1.0^{c}	1.0	-

Variables with different superscripts are significantly different ($p < 0.05$)

*Final bacterial density was 10^5 CFU/ml

[†]Inhibition zone diameters (mm) produced around the discs by adding 20 µl of methanol extract. Values are means of three measurements

[‡] MIC: minimum inhibitory concentration (as % v/v)

[§] MBC: minimum bactericidal concentration (as % v/v)

Table 3.4 Antibiotics susceptibility of *Salmonella, Staphylococcus spp.* and *E coli* (Disc Diffusion)

Bacteria*	**Source**	**Inhibition zone diameters†**							
		Pen	**Amo**	**Tetra**	**Neo**	**Chlora**	**Ery**	**Gen**	**Strep**
***Salmonella* spp.** (SM1)	Tahini	14.3 ±1.1^{S}	40.0 ±0.0^{S}	32.7 ±0.6^{S}	15.7 ±0.6^{R}	30.0 ±0.0^{S}	-	25.7 ±0.6^{R}	27.0 ±0.0^{R}
***Salmonella* spp.** (SM2)	Chicken	22.7 ±0.6^{S}	40.0 ±0.0^{S}	33.7 ±0.6^{S}	17.0 ±0.0^{R}	31.3 ±0.6^{S}	-	26.7 ±0.6^{R}	27.0 ±1.0^{R}
Staphylococcus spp. (ST1)	Chicken Sandwich	0.0 ±0.0^{R}	34.3 ±0.6^{S}	31.0 ±1.0^{S}	14.3 ±0.6^{R}	30.7 ±0.6^{S}	0.0 ±0.0^{R}	23.0 ±0.0^{R}	-
Staphylococcus spp. (ST2)	Parmesan Cheese	33.7 ±0.6^{S}	40.0 ±0.0^{S}	40.0 ±0.0^{S}	22.0 ±0.0^{S}	30.3 ±0.6^{S}	29.3 ±2.1^{S}	32.3 ±0.6^{R}	-
E. coli (EC1)	Meat	0.0 ±0.0^{R}	36.3 ±1.5^{S}	31.7 ±0.6^{S}	15.3 ±0.6^{R}	31.3 ±1.2^{S}	-	25.0 ±0.0^{R}	26.3 ±0.6^{R}
E. coli (EC2)	Meat	0.0 ±0.0^{R}	35.0 ±1.0^{S}	31.0 ±1.0^{S}	14.0 ±0.0^{R}	32.3 ±0.6^{S}	-	25.3 ±0.6^{R}	25.3 ±0.6^{R}

*Final bacterial density was 10^5 CFU/ml

†Inhibition zone diameters (mm) produced around the antibiotic discs. Values are means of three measurements

^{R} Resistant

^{S} Susceptible

Table 3.5 Guidelines for antimicrobial susceptibility cut-offs as set by EUCAST* (2014) and CLSI† (2011)

Antibiotics	Resistant (mm) ‡	Intermediate resistance (mm)	Susceptible (mm)
Penicillin 10 µg* (Enterobacteriaceae)	<14	-	≥14
Penicillin 5 µg† (*Staphylococcus spp.)*	≤9	10-13	≥14
Amoxicillin 10 µg* (Enterobacteriaceae)	<14	-	≥14
Amoxicillin 20/10 µg† (*Staphylococcus spp.)*	≤19	-	≥20
Tetracycline 30 µg† (Enterobacteriaceae)	≤11	12-14	≥15
Tetracycline 30 µg* (*Staphylococcus spp.)*	<19	19-21	≥22
Neomycin 10 µg * (Enterobacteriaceae)	<12	12-14	≥15
Neomycin 10 µg * (*Staphylococcus* spp.)	<22	-	≥22
Chloramphenicol 30 µg* (Enterobacteriaceae)	<17	-	≥17
Chloramphenicol 30 µg* (*Staphylococcus spp.)*	<18	-	≥18
Erythromycin 15 µg* (*Staphylococcus spp.)*	<18	18-20	≥21
Gentamicin 10 µg* (Enterobacteriaceae)	<14	14-16	≥17
Gentamicin 10 µg* (*Staphylococcus* spp.)	<22	-	≥22
Streptomycin 10 µg† (Enterobacteriaceae)	≤11	12-14	≥15

‡ mm = zone of inhibition diameter in millimeters

3.6. References

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APPENDIX 1. *ORIGANUM EHRENBERGII* DATA SHEET

Plant Data Sheet*

Plant Name	*Origanum ehrenbergii*		
Date of Collection	June 6, 2012	Plant Life Stage:	Flowering
Origin	Wild ☑	Cultivated	
Location	Region: Sannine	Altitude: 1650 m	GPS 33°54'14.52"N 35°51'8.12"E
Weather Conditions	Temperature around 25°C	Rain or Sun ☑	Wind
Habitat	Soil clay	Hidden	Open ☑
Population	Density medium	Spreading	
Harvesting	Flowers	Stems	Whole ☑
Drying Info	Dried in shade	Duration: one week	Location: AREC
For Cultivated Only			
Seeds	Origin	Treatment	Location
Condition	Water treatment	Fertilizer	
Cultivation Year			

*Provided by Mr. Khaled Sleem, plant production specialist at the Nature Conservation Center (AUB) and field coordinator at IBSAR (AUB)

APPENDIX 2. GC-MS SOLUTIONS PREPARATION

- Preparation of the standard stock solutions (2000 ppm):

Thymol: 99.5%

2000 ppm → 2 mg/ml

Weigh 2 mg (0.002 g) thymol powder in 1 ml DCM to obtain 2000 ppm.

Carvacrol: 98 %

98 % → 980000 ppm

C1V1 = C2V2

980000 ppm x V1 = 2000 ppm x 1000 µl

V1 = 2.04 µl + 997.9 µl DCM to obtain 2000 ppm.

Gamma-Terpinene: 97 %

97 % → 970000 ppm

C1V1 = C2V2

970000 ppm x V1 = 2000 ppm x 1000 µl

V1 = 2.06 µl + 997.9 µl DCM to obtain 2000 ppm.

Para-Cymene: 99 %

99 % → 990000 ppm

C1V1 = C2V2

990000 ppm x V1 = 2000 ppm x 1000 µl

V1 = 2.02 µl + 997.9 µl DCM to obtain 2000 ppm.

- Preparation of 20 ppm from the 2000 ppm standard stock solutions:

C1V1 = C2V2

2000 x V1 = 20 x 1000 µl

Volume needed for all standards to reach 20 ppm:

V1 = 10 µl + 990 µl DCM

- The chosen standards concentrations to draw their calibration curves:

(1^{st} trial)

0.01 ppm: V1 = (0.01 x 1000 µl) / 20 ppm = 0.5 µl + 999.5 µl DCM

0.4 ppm: V1 = (0.4 x 1000) / 20 = 20 µl + 980 µl DCM

1.5 ppm: V1 = (1.5 x 1000) / 20 = 75 µl + 925 µl DCM

5 ppm: V1 = (5 x 1000) / 20 = 250 µl + 750 µl DCM

Updated concentrations to obtain clearer peaks: (2^{nd} trial)

2 ppm: V = (2 x 1000) / 20 = 100 µl + 900 µl DCM

4 ppm: V = 200 µl + 800 µl DCM

6 ppm: V = 300 µl + 700 µl DCM

8 ppm: V = 400 µl + 600 µl DCM

10 ppm: V = 500 µl + 500 µl DCM

15 ppm: V = 750 µl + 250 µl DCM

- Preparation of 100 ppm from the oil subjected to water distillation (WD):

Mass of the oil = 0.15 g

C= 150 mg / 10 ml = 15 000 ppm

Volume needed to reach 100 ppm:

V1 = (100 x 2000 µl) / 15 000 = 13.33 µl + 1986.66 µl DCM

- Preparation of 100 ppm from methanol extracts (ME):

Mass of the extract = 0.01 g

C = 10 mg / 10 ml = 1 mg/ml = 1000 ppm

V1 = (100 x 1000) / 1000 = 100 µl + 900 µl DCM

Given that the concentration of carvacrol in the methanol extract was less than 2 ppm, and the concentration of thymol in the water distillate was higher than 15 ppm; 0.5 and 1 ppm of the standard carvacrol and 25 ppm of the standard thymol were added to their calibration curves.

- Preparation of the above concentrations from the 20 ppm initial solutions:

1 ppm (carvacrol): V = (1 x 1000) / 20 = 50 µl + 950 µl DCM

0.5 ppm (carvacrol): V (0.5 x 1000) / 20 = 25 µl + 975 µl DCM

25 ppm (thymol): V (25 x 1000) / 2000 = 12.5 µl + 987.5 µl DCM (Prepared from the 2000 ppm initial standard solution because the final volume would be greater than 1000 µl if prepared from the 20 ppm solution).

Para-cymene and gamma-terpinene's peaks did not show in 100 ppm, therefore 500 ppm WD and ME were prepared to check if they would appear:

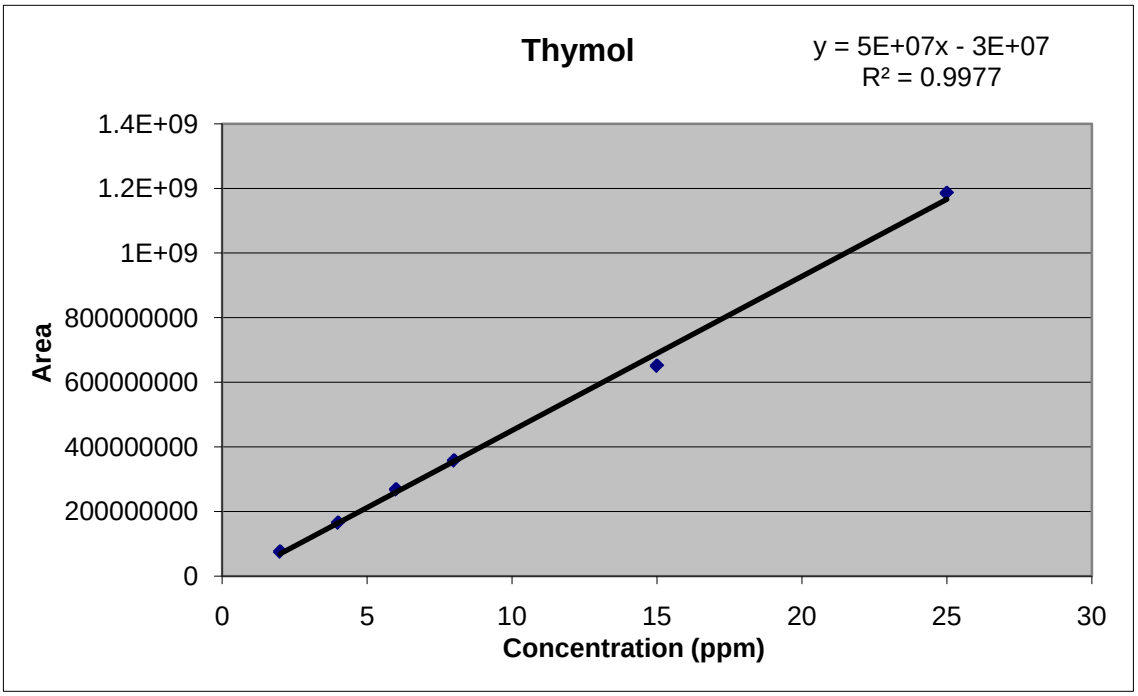
Volume needed to reach 500 ppm WD from its 15 000 ppm original solution:

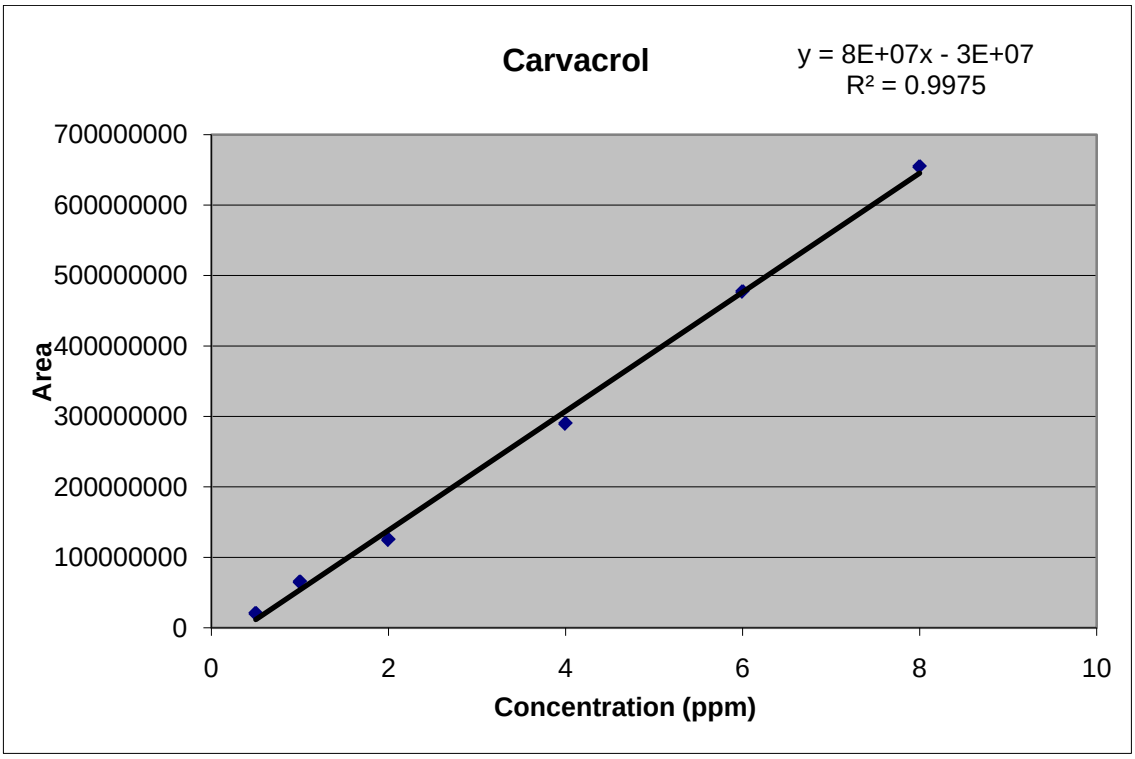
V1 = (500 x 1000 µl) / 15 000 = 33.33 µl + 966.66 µl DCM

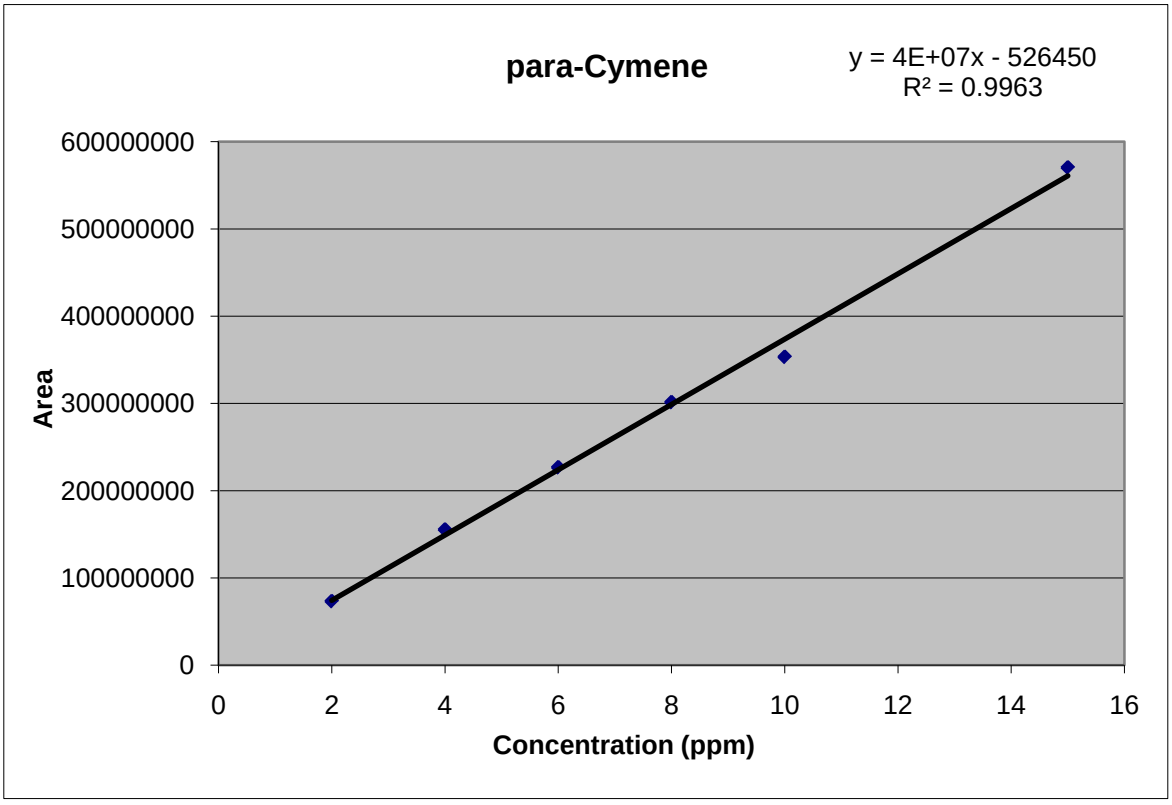
Volume needed to reach 500 ppm ME from its 1000 ppm original solution:

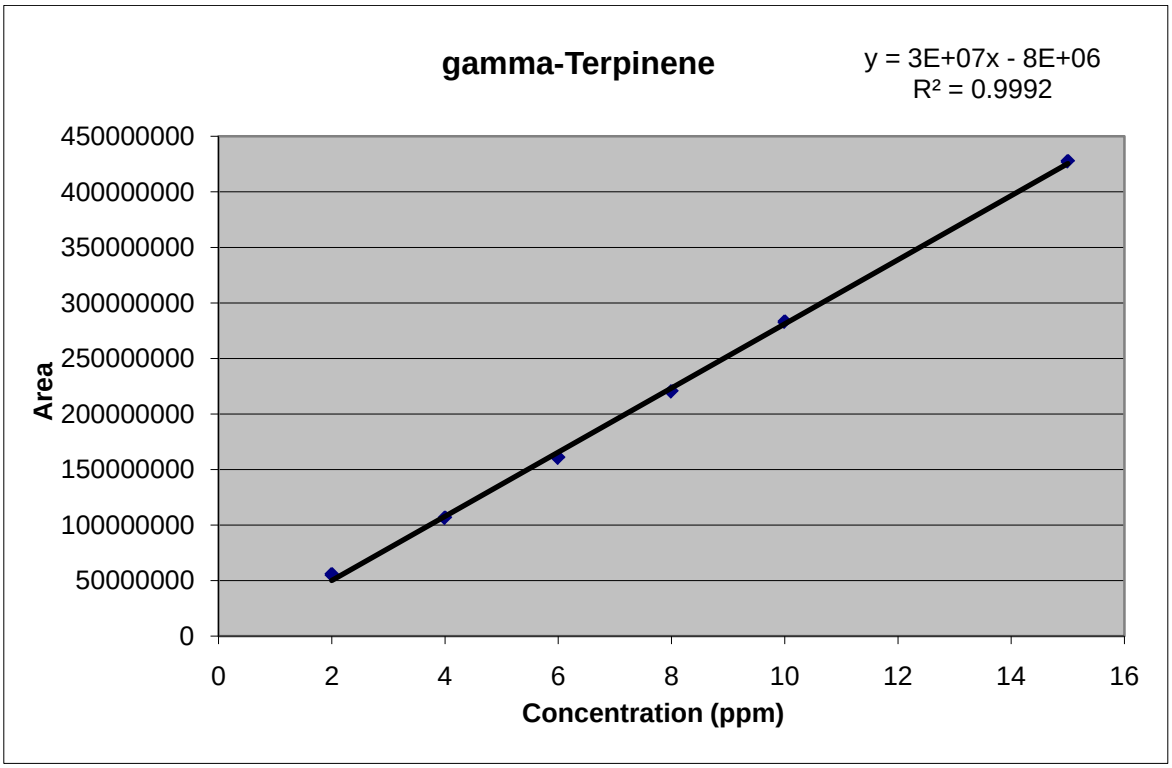
V1 = (500 x 1000 µl) / 1000 = 500 µl + 500 µl DCM

APPENDIX 3. CALIBRATION CURVES, GC-MS GRAPHS AND STRUCTURES OF *ORIGANUM EHRENBERGII*'S ACTIVE COMPOUNDS




Carvacrol
y = 8E+07x - 3E+07
R² = 0.9975
700000000
600000000
500000000
400000000
300000000
200000000
100000000
0
Area
0
2
4
6
8
10
Concentration (ppm)


para-Cymene
y = 4E+07x - 526450
R² = 0.9963
600000000
500000000
400000000
300000000
200000000
100000000
0
Area
0
2
4
6
8
10
12
14
16
Concentration (ppm)



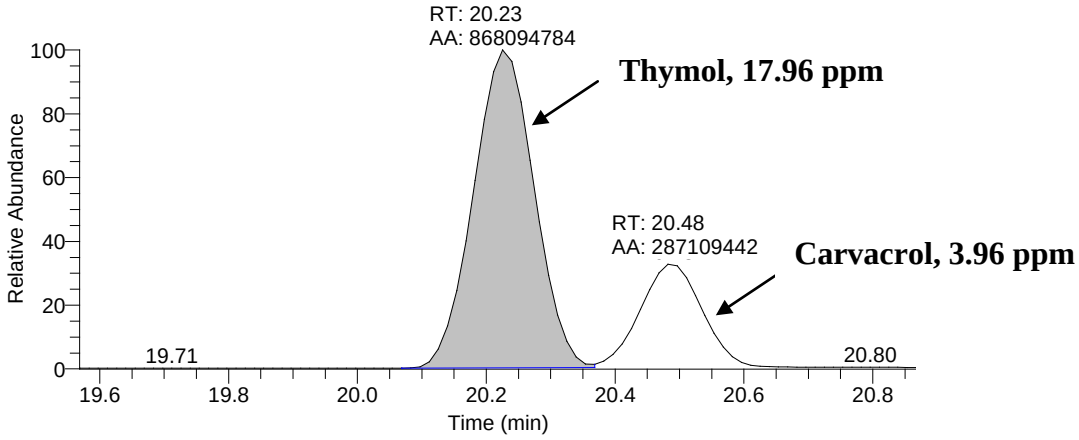


Figure 1: Relative abundance of thymol and carvacrol in 100 ppm water distillate (W.D.)

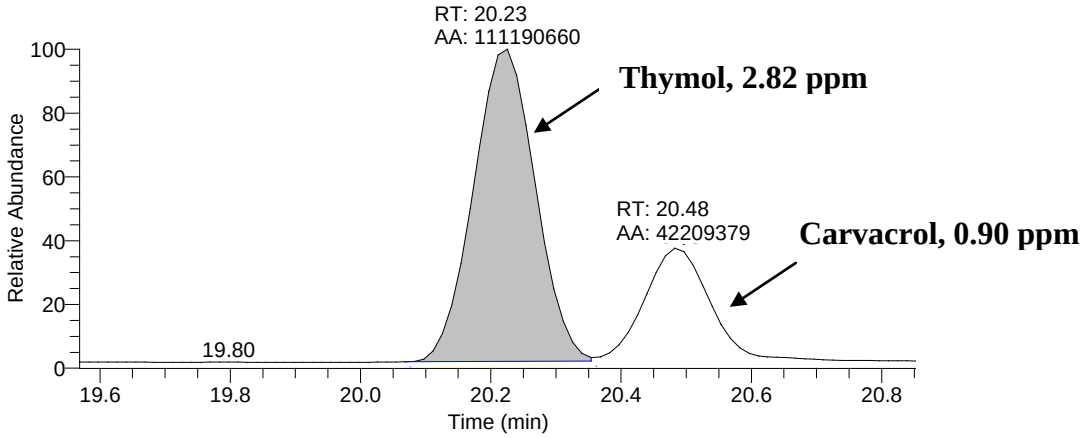
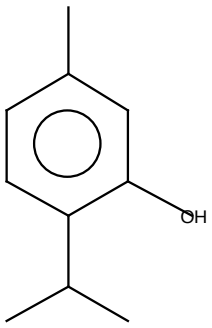


Figure 2: Relative abundance of thymol and carvacrol in 100 ppm methanol extract (M.E.)

Compound structure

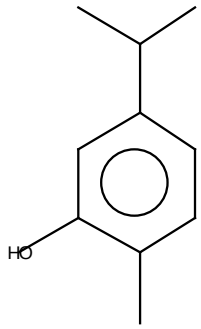
Thymol

Phenol, 5-methyl-2-(1-methylethyl)-



Phenol, 2-methyl-5-(1-methylethyl)-

Carvacrol



APPENDIX 4. SPECTROPHOTOMETER RESULTS

% transmittance of bacterial mixture with essential oil (B+W) and methanol extract (B+M) as compared to the control* (100% transmittance)

Antibacterial concentration (%)	Transmittance (%)†	
	B+W	B+M
	SM1	
10	120.10 (120.00, 120.20)	116.43 (120.00, 112.87)
5	119.29 120.00, 118.59)	84.47 (85.72, 83.22)
1	117.55 (120.00, 115.10)	80.03 (79.47, 80.60)
0.5	79.50 (76.76, 82.25)	59.19 (58.36, 60.02)
0.1	74.04 (73.71, 74.38)	51.25 (52.32, 50.19)
	SM2	
10	120.00 (120.00, 120.00)	120.00 (120.00, 120.00)
5	119.66 (120.00, 119.32)	100.80 (95.60, 106.00)
1	109.49 (108.42, 110.56)	89.44 (91.18, 87.71)
0.5	114.44 (113.73, 115.15)	58.49 (56.07, 60.91)
0.1	100.94 (99.35, 102.54)	52.39 (54.91, 49.88)
	ST1	
10	120.00 (120.00, 120.00)	120.00 (120.00, 120.00)
5	119.91 (120.00, 119.83)	120.00 (120.00, 120.00)
1	112.91 (113.09, 112.74)	116.04 (120.00, 112.08)
0.5	100.23 (101.33, 99.13)	93.57 (91.27, 95.88)
0.1	77.98 (78.08, 77.89)	91.66 (93.06, 90.27)
	ST2	
10	120.00 (120.00, 120.00)	120.00 (120.00, 120.00)
5	119.80 (120.00, 119.61)	119.71 (119.88, 119.54)
1	116.12 (117.25, 114.99)	90.04 (91.33, 88.76)
0.5	60.44 (59.10, 61.78)	79.53 (77.93, 81.14)
0.1	57.47 (64.74, 50.20)	79.04 (82.16, 75.93)
	EC1	
10	119.35 (118.70, 120.00)	120.00 (120.00, 120.00)
5	120.00 (120.00, 120.00)	118.66 (120.00, 117.33)
1	112.71 (113.54, 111.88)	89.02 (88.04, 90.01)
0.5	82.25 (82.32, 82.19)	82.36 (81.31, 83.42)
0.1	71.11 (70.33, 71.90)	82.00 (84.89, 79.11)
	EC2	
10	120.00 (120.00, 120.00)	120.00 (120.00, 120.00)
5	119.77 (120.00, 119.54)	119.48 (120.00, 118.97)
1	112.22 (112.15, 112.30)	101.59 (101.13, 102.06)
0.5	103.29 (101.98, 104.61)	95.94 (96.76, 95.12)
0.1	111.39 (111.70, 111.09)	93.32 (93.57, 93.08)

*Antibacterial solution without bacteria

†Results are means (range) of duplicate measurements