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The Effect of Lavandula Stoechas on Toxigenesis and the Growth of Vibrio Parahaemolyticus

Omid Maghsoudi[1], Mohsen Zeraatkar[1], Mojtaba Dolatabadi[1], Ahmad Johari[1], Mahdi Barati Karizno[2], Reza Ranjbar[3*]

1. *Doctor of Veterinary Medicine, Faculty of Veterinary Medicine, Islamic Azad University, Karaj Branch, Iran*
2. *Dept. of Food Hygiene, Ferdowsi University of Mashhad, Mashhad, Iran*
3. *Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran*

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ABSTRACT

Background & objective: Outbreak of food-borne diseases has become more and more important these days and using natural food preservers with high durability is under debate. Vegetative essence is a type of food preserver and many studies have been performed on their antimicrobial effects. The purpose of this study wasto investigate antibacterial effects of lavender essence on toxigenesis and the growth of *Vibrio Parahaemolyticus*.

Methods: lavender essence was prepared and its components were identified using GCMS. Determining minimum interceptorgrowth of *Vibrio parahaemolyticus* was assessed in test tubes containing BHI. Thermal resistant hemolysin was measured by Kap-RPLAkit. Growth diagram was prepared after determining toxin formation titration of the bacterium during 0, 2, 3, 4, 6, 8 and 24 hours.

Results: Cineol, Borneol, Camphor, LinaloolL and Alpha-pinen had the highest concentrations in the essence, respectively. Results of minimum intercepter concentration of lavender (0, 0.005, 0.015, 0.03 and 0.045 percent) on *Vibrio parahaemolyticus* showed that 0.03% and higher concentrations had the ability to prevent growth and toxin formation of *Vibrio parahaemolyticus*. In addition, the effect of different concentrations of essence on toxin titration of bacterium showed no toxin at concentrations of 0.030 and 0.045.

Conclusion: lavender essence was able to prevent the growth and toxin formation of Vibrio parahaemolyticus.

Corresponding information: Reza Ranjbar, Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran. E-mail: rranjbar@yahoo.com

Introduction

Modern economic and social changes along with international food trade at universal level have deployed the risk of many food borne diseases. However, producing a healthy food with high durability indicates the necessity of using food preservers. In the recent years, food manufacturers have paid great attention to natural preservers instead of chemical ones due to their unfavorable effects such as being carcinogen, teratogen, acute food poisoning and long-term degradation. Herbal essences have been considered by food health researchers in the recent years and many studies have been focused on their antimicrobial and preservative characteristics. Essences or their components have wide spectrum of antibacterial potency and have anti-parasite, insecticide, antiviral, anti-fungal and antioxidant characteristics (1,2). Essences have the most antibacterial activity if prepared from flowering plants or closely after it. On the other hand, plants of the same species grown in different conditions can have distinctive chemical characteristics and combinations (3).

Approximately, 3000 types of essences have been identified, among which about 300 types are important from commercial aspects. FDA (Food and Drug Administration) has also confirmed essences as food preservative and additive (4). Currently, most common applications of essences in the European Union is related to food industries (as flavors), cosmetics and pharmacy industry (for different applications such as making disinfectants, dental filing material, nutritional supplements, etc.) (5).

Based on chemical structure, active compounds of essences can be classified into three main classes of Terpenes, Terpenoids and Phenyl Propens (6). Terpenoids include thymol, carvacrol, linalool, linalyl acetate, citronellal, piperitone, menthol and geraniol (7,8). Antimicrobial performance of carvacrol, thymol, linalool and menthol against *Listeria monocytogenes, Enterobacter aerogenes, Escherichia coli* and *Pseudomonas aeruginosa* has been studied and the most active antimicrobial compounds were carvacrol and then thymol (9,10,11).

Thymol effect on *Salmonella typhi* form and *Staphylococcus aureus* was studied showing that thymol attached membrane proteins in a hydrophilic way and changed permeability of the membrane of the microorganism (2,12).

Most commonly studied phenyl propens include eugenol, isoeugenol, vanillin, safrole and cinnamaldehyde. Antibacterial activity of eugenol against 25 lineages of bacteria was studied showing no antimicrobial activity only against one lineage (13).

Isoeugenol and eugenol have considerable higher antimicrobial activity against Gram-negative bacteria, mold, and yeast than Gram-positive ones, while main elements of essences are naturally more active against Gram-positive bacteria (14,15). Eugenol caused partial changes in characteristics of fatty acid in *Pseudomonas fluorescens, Escherichia coli, Brochothrix thermosphacta, Salmonella enterica* and *Staphylococcus aureus* and cellular damages in Escherichia coli and Brochotrix (16). Hydroxyl group of eugenol attached proteins and affected their characteristics.

Although high potential of essences and their compound have been confirmed in experimental studies, their application as food preservers faced some limitations in practice as high concentration of essences is needed to achieve adequate antimicrobial activity. Likewise, hydrophobic components of essences are deactivated with compounds in many food products such as fat, starch and proteins. In addition, antimicrobial potential of essence components depend on factors such as pH, temperature and bacterial infection range (17). Therefore, using food models containing different quantities of fats, proteins or starch can be useful for designing experiments to identify certain essences in different products.

Food borne infections by sea foods are one of the most common reasons of gastrointestinal diseases (18). Vibrio bacteria are Gram-negative and anaerobic bacteria living in sea and fresh waters (19). Fojino introduced *Vibrio parahaemolyticus (V. parahaemolyticus)* for the first time in 1951 as the reason of stomach-intestinal inflammation resulted from consuming affected food. *V. parahaemolyticus*

produces four types of hemolysins including thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH), thermolabile hemolysin (TLH) and £-VPH (22.8 kDa). TDH is thermal resistance and sensitive to trypsin and its thermal stability is in a way that can remain in food after production and is tolerant to 100oC temperature in pH: 6 and for 10 min (20, 21). Fang et al. studied 777 sea foods and reported 54.7% *V. parahaemolyticus* contamination (22). Wong et al. investigated 686 sea foods and 315 samples of Vibrio parahaemolyticus (45.9%) were isolated (23).

Regarding the presence of *V. parahaemolyticus* in sea food products and consequence health risks, this study tried to investigate the effect of lavender essence as a natural preserver on toxin formation and the growth of this pathogen.

Material and Methods

Experimental Plan

First the essence of lavender was prepared and the components were specified by GC MS instrument (Thermoquest Trace GC 2000 FINNIGAN, Eng.). Then, effect of lavender essence was studied on growth and toxin formation of *Vibrio parahaemolyticus* in concentrations of 0, 0.005, 0.015, 0.03, 0.045 percent at 35oC in heart and brain broth over 24 hours.

Preparing Lavender Essence

The essence was prepared using hot vapor distillation using Clevenger instrument, Apparatus model Alright GMB / USA / United State.

Identifying Essence Components

Gas chromatograph instrument connected to mass spectrum (Thermoquest Trace GC 2000 FINNIGAN, Eng) was used to examine and analyze the components of essences. Thermo Quest Finnegan System with capillary column of 30 m long, internal diameter of 250 µm and internal wall thickness of 0.25 µm was used within thermal plan of 50 oC to 26 oC with gradual increase of 2.5 oC/min and maintaining the temperature of 265oC for 30 minutes. Injection module temperature was 250 oC and helium was the carrier gas which passed through the pipe by 1.5 ml/min. Moreover, electrical capacity of the indicator was 70 ev and its ionization source temperature 205 oC.

Studied Bacterium

Studied bacterium was V. parahaemolyticus ATCC 43996. This bacterium was consecutively cultivated twice on heart and brain broth at 37 oC. Bacterium from the second culture was mixed with sterile glycerin with ratio of 1:5 and maintained in Eppendorf micro tubes of 500 µL at -20oC.

Preparing Bacterium Inoculation

First the prepared bacterium at -20oC was transferred to culture medium and maintained for 6 hours at 37 oC. Second, culture originated from the first broth and lasted for 6 hours at 37 oC. Then, different proportions of the second culture were transferred to Cuvette tubes containing 4 mL of heart and brain broth until light absorption of the Cuvette tube reached 0.1 at 600 nm using spectrophotometer instrument (Milton Ray Company, USA). Next, another culture was cultivated on heart and brain agar medium using Cuvette tubes so that bacterium quantity per millimeter of heart and brain broth was obtained according to correspondence Cuvette.

Determining Minimum Inhibitory Concentration (MIC) Using Macro Dilution Method

Consecutive concentrations of lavender essence (0, 0.005, 0.015, 0.030 and 0.045 percent) were prepared in two test tubes containing 2 mL of heart and brain broth medium with 5 percent DMSO (Dimethyl sulfoxide). Then, bacterium suspension was inoculated in tubes so that final bacterium concentration of $1*10^5$ cfu/mL was reached. Next, tubes were incubated at 35 oC for 24 hours and opacity in tubes was studied.

Method of Determining Production and Toxin Formation by Kit

At first, 0.1 mL of tubes containing heart and brain broth was taken to determine the amount of toxin and the number of bacteria per ml of heart and brain broth was specified using dilution and cultivation method on BHI Agar medium. Then, Falcon tubes containing the remaining heart and brain broth were centri-

fuged at 3000 rpm for 20 min. In microdilution technique, 25 µL of dilutor was poured in two rows of microplate sinks (no dilutor was added to two first sinks of each row). Then, specified amount of upper liquid resulted from bacterium centrifuge was added to the two first sinks of each row and dilution was performed afterward. Next, 25 µL of sensitize latex solution and 25 µL of control latex added to sinks of the first and second rows, respectively. Afterward, 25 µL of pure toxin and 25 µL of sensitize latex solution were poured in separate sinks as positive control; then microplate was kept in room temperature for 18 hours. Agglutination in each sink shows the presence of toxin and toxin dilution was calculated according to the location of the related sink and the protocol of kit Manufacturer (DENKA Seiken Japan).

Determining Growth Chart

At this stage, the effect of preventive concentrations of the lavender essence on the growth of *V. parahaemolyticus* was assessed over 24 hours. Therefore, 10 mL of prepared solutions containing different concentrations of the essence (0, 0.005 and 0.015) distributed in three tubes. Then, 100 µL of bacterium suspension was added to each three tube (final bacterium concentration was $1*10^5$ cfu/mL). Tubes were incubated at 35 oC and related dilution was prepared from each 3 tubes at 0, 1, 2, 3, 4, 6, 8 and 24 hours and cultivated on a plate containing BHI agar medium. Colony count was performed after 24-hour incubation at 35 oC and the number of bacteria was calculated at each considered hour.

Statistical Analysis

Data analysis was performed by SPSS v.22 (IBM company, USA SPSS Inc,Chicago,IL) using one-way analysis of variance (ANOVA) and Tukey Test.

Results

Analyzing the Components of Lavender Essence

Different components of lavender essence were analyzed by gas chromatograph connected to mass spectrometer. Accordingly, cineol with 49.16 percent had the highest concentration. After that borneol with 10.75 percent, camphor with 8.94 percent, linalool L with 3.95 percent and alpha-pinen with 3.2 had the highest amounts in the essence (Figure 1 and Table 1)

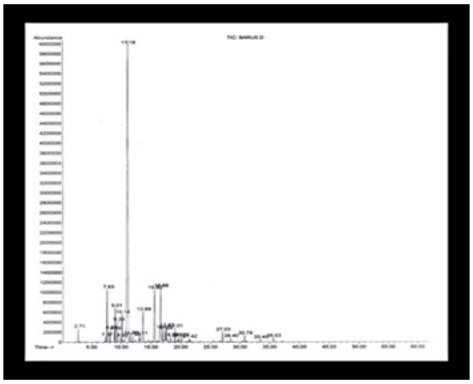

Figure 1. GC/MS Graph of Lavender Essence

Table 1. Chemical Compounds of Lavender Essence

Chemical compound	Percent	Inetcepter index
Alpha-thujene	0.3	7.38
Alpha-pinen	3.2	7.63
Camphene	0.8	8.09
Sabinene	0.9	8.85
Beta-pinen	2.63	9.01
Beta-myrcene	7.87	9.38
Alpha-phellandrene	0.4	9.92
Delta-3-carene	2.73	10.14

Chemical compound	Percent	Inetcepter index
1,8-cineole	49.16	11.18
Ocimene <(E)-B->	0.49	11.51
Alpha-terpinolene	0.5	13.11
Linalool L	3.95	13.69
Camphor	8.94	15.63
Borneol	10.75	16.66
Terpinen-4-ol	1.04	16.97
Cryptone	1.3	17.34
Alpha-terpineol	1.16	17.56
Isophorone<4-methylene>	0.49	18.27
Unknown	0.99	19.01
Cumin aldehyde	0.38	19.56
Linalool acetate	0.5	20.09
Thuganol acetate <iso-3>	0.23	21.42
Trans-caryophyllene	0.93	27.03
Trans-beta-farnesene	0.3	28.40
Gamma-cadinene	0.58	30.79
Caryophyllene oxide	0.33	33.45
Cubenol<1,10-di-epi>	0.53	35.63
Sum	95.7	

MIC Results of Lavender Essence on Growth and Toxin Formation of *Vibrio Parahaemolyticus*

In this research, the effect of five different concentrations of lavender essence (0, 0.005, 0.015, 0.030 and 0.045 percent) was assessed on growth and toxin formation of *V. parahaemolyticus*. Results showed that 0.03 percent and higher concentrations had the ability to prevent the growth of *V. parahaemolyticus.*

The Effect of Lavender on TDH Titration

Different concentrations of lavender essence had significant effect on the titration of produced toxin of *V. parahaemolyticus*, so that titration of produced toxin in concentrations of 0, 0.005 and 0.015 percent of lavender essence at 35oC were 1.256, 1.128 and 1.8, respectively (Table 2 and Figure 2).

V.parahaemolyticus Growth Chart Affected by Lavender Essence

As shown in table 2, concentrations of 0.005 and 0.015 percent of lavender essence (under-interceptor concentrations) reduced bacterium growth significantly compared with the control group ($P < 0.05$), so that logarithm of bacterium number in these two concentrations at the fourth hour of incubation were 3.04 and 2.69, respectively; however, logarithm of bacterium growth in the mentioned hour for the control group was 7.74. Bacterium growth reduced from zero to sixth hour and increased from eighth hour. The difference of bacterium growth between the two concentrations of 0.005 and 0.015 percent of essence was not significant, but was significantly different between the two mentioned concentrations and the control group (Figure 3 and Table 3).

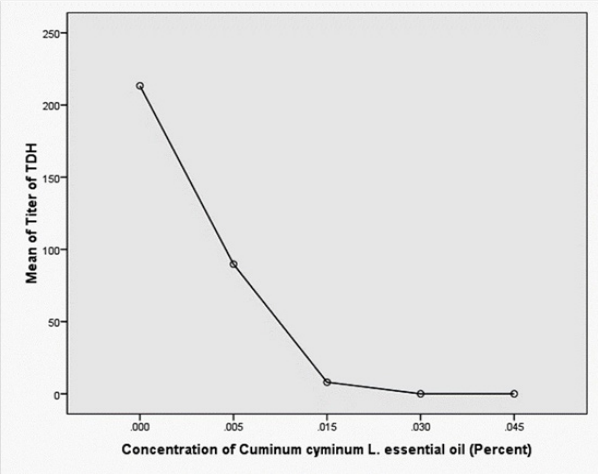

Figure 2. The Effect of Different Concentrations of Lavender Essence on TDH Titration

Table 2. TDH Titration Affected by Different Concentration of Lavender (0, 0.005, 0.015, 0.030 and 0.045 percent) at 35 oC

Bacterium per ml	OD	Titration of produced toxin	Lavender essence concentration
$8.7*10^8$	0.86	1.256	0
$8.7*10^8$	0.86	1.128	0.005
$8.7*10^8$	0.86	1.8	0.015
-	-	No growth	0.03
-	-	No growth	0.045

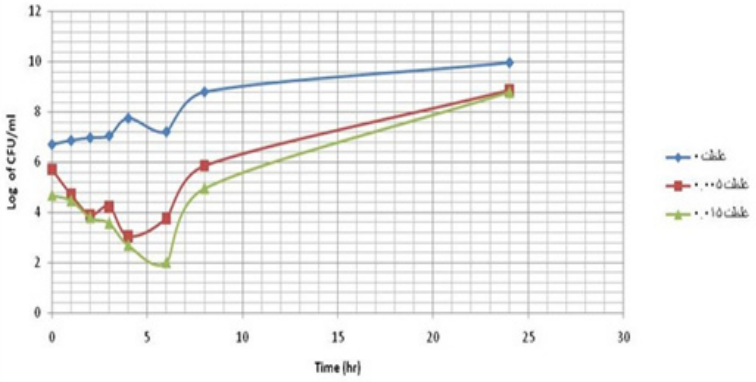

Figure 3. *Vibrio Parahaemolyticus* Growth Chart Affected by Different Concentrations of Lavender Essence (0, 0.005 and 0.015) During 24 Hours

Table 3. *Vibrio Parahaemolyticus* Growth Chart Affected by Different Concentrations of Lavender Essence (0, 0.005 and 0.015) During 24 Hours

Concentration Time	Zero	1	2	3	4	6	8	24
Zero	$4.9*10^6$	$7.2*10^6$	$9.2*10^6$	$1.1*10^7$	$5.5*10^7$	$1.6*10^7$	$6.2*10^8$	$9.3*10^9$
0.005	$5.2*10^5$	$5.1*10^3$	$8*10^3$	$1.7*10^3$	$1.1*10^3$	$6*10^3$	$7.3*10^5$	$7.7*10^8$
0.015	$4.8*10^3$	$3*10^3$	$6.3*10^3$	$3.8*10^3$	$5*10^2$	$1*10^2$	$9.3*10^3$	

Discussion

One of the most important food borne pathogens is *V. parahaemolyticus*, which leads to food poisoning by consuming fish and its products and other sea foods infected by the bacterium. This bacterium leads to gastroenteritis. As some food products are consumed half-baked or even raw, there is always the risk of infection. There have been many reports of isolating this bacterium from sea foods around the world. In a study by American Food and Drug Association performed on 635 samples of sea foods, 86% contained *V. parahaemolyticus* (18). Increase of microbial growth in food harms people health and national economy, which necessitates maintaining the quality and surveillance strategies. In this study, the effect of different concentrations of lavender essence was assessed on the growth and toxin formation of *V. parahaemolyticus* with incubation of 105 in vitro. This study methodology is similar to Osawa et al. who used ELISA method, Kap-Rpla Kit (24).

In this study, titration of TDH (toxin formation) in concentrations of zero, 0.005 and 0.015 percent were 1:8, 1:128 and 1:256, respectively, but no toxin formation was observed in 0.030 and 0.045 percent. Bacterium growth diagram also showed considerable growth decrease in concentrations of 0.005 and 0.015 percent in comparison with control group during 24 hours, especially at sixth hour.

Many studies have been performed to investigate the effect of different compounds on the growth and toxin formation of *V. parahaemolyticus* (25).

Antibacterial effects against *V. parahaemolyticus* have been reported using different oily essences such as rosemary, oregano, clove, horse radish and Garlic (26).

Yutaka et al. studied antibacterial effects of 18 vegetative species on *V. parahaemolyticus*. Consequently, basil, clove, garlic, horse radish, marjoram, oregano, rosemary and thyme showed antibacterial effects at 30 oC. The lowest MIC was 0.125% related to clove and marjoram in an environment rich in nutrients. Decreasing maintenance temperature has little effect on minimum intercepter concentration except for turmeric. Sensitivity to different essences was the same between different clinical serotypes (27).

Antimicrobial activity of fresh garlic and lemon extracts on lineage of *V. parahaemolyticus* pandemics was studied using disc diffusion technique indicating that the two extracts prevented growth of *V. parahaemolyticus* (28).

Al-Jedah et al. studied fish sauce inoculation ($1 * 10^4$ cfu/mL) with *E. coli, S. typhi* form, *Staphylococcus aureus* and *V. parahaemolyticus* showing that spices and other compounds (wheat and lemon) had preventive effects on corresponding pathogens and only *V. parahaemolyticus* remained alive after 7 hours at 250 oC and was identified after 21 days. However, all of these pathogens in the control group except Staphylococcus aureus remained alive until 28 days (29).

Antibacterial effects of combined extracts of cranberry and oregano on *V. parahaemolyticus* were studied showing that antibacterial activity of the combination of these two extracts was more than each one alone, also the efficiency of these two extracts with lactic acid was higher (30).

Rattanachaikunsopon et al. studied antimicrobial activity of an oily garlic essence against *Vibrio cholerae* in vitro and in food model. Fatal effect on different lineages of *V. cholerae* was confirmed in vitro with MIC of 3.13 to 25 μg/mL, also the essence reduced the number of bacteria in food model (31).

According to Badiee, cumin essence in concentrations of 0.005 and 0.015 percent reduced toxin formation of *V. parahaemolyticus* at 37 oC (32).

Effect of different concentrations of Shirazian thyme essence on *V. parahaemolyticus* growth was studied in BHI medium indicating that the essence could prevent bacterium growth (33).

Imelouane et al. studied antibacterial effect of lavender essence using propagation method in solid medium and it was found that the essence prevented bacterial growth of *Listeria monocytogenes*, Haemophilus influenza, Neisseria meningitidis and different species of Streptococcus (34).

In Rasooli and Rezaee investigation, antibacterial activity of lavender essence on the growth of *S. au-*

reus and *E. coli* was studied using MIC and MBC methods; MIC was 0.030 (35).

According to the report of Food Health Codex Committee, even small amount of *V. parahaemolyticus* in raw sea food makes its unusable (30).

In a study by Dixon, infectious dosage of *V. parahaemolyticus* was reported as 107 to 105 in raw and cooked sea food (36).

Kaneko showed that ingestion of $2*10^5$ to $3*10^7$ number of positive *V. parahaemolyticus* Kanagawa bacteria led to quick symptoms of gastroenteritis, while volunteers eating 1.6*1010 numbers of *V. parahaemolyticus* bacteria only showed diarrhea (37).

Most common method of extracting essences in industry is distillation. In the present study, components of lavender essence were identified using GC mass instrument and the resulted decomposed composition included 49.16% 1,8-cineol, 10.75% borneol, 8.94% camphor, 9.53% linalool L and 3.2% alpha-pinen. Lavender essence resulted from distillation was a yellow or greenish yellow liquid.

Gorena et al. analyzed components of lavender using gas mass spectroscopy and most common parts were menthol, beta-pinon and menthon (38).

Imelouane et al. identified 29 constituents in lavender oily essence in which gama-cineol with 41.28% had the highest amount and p-chlorophenyl was the least one (34).

In conclusion we found that lavender essence was able to prevent the growth and toxin formation of *Vibrio parahaemolyticus* in vitro. This finding encourages us to evaluate inhibitory effects of such natural substances on this organism in vivo.

Other pathogenic species of *Vibrio* including *V. cholerae* are among most common food and water borne bacterial pathogens in our country (39-43). Similar studies are suggested to determine antibacterial effects of lavender essence on endemic pathogenic *V. cholera* in the future.

Conflict of interest

the authors declare that there is no conflict of interests.

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