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ACTIVIDAD ANTIMICROBIANA, CITOTÓXICA Y ANTI-INFLAMATORIA DE DIFERENTES ACEITES ESENCIALES, LA DIFUSIÓN DE SUS COMPONENTES MAYORITARIOS APLICADOS EN FASE VAPOR, SU EFECTIVIDAD COMO AGENTES ANTIMICROBIANOS Y SU EFECTO SENSORIAL EN SEMILLAS DE ALFALFA

Tesis presentada en cumplimiento parcial de los requisitos para obtener el Grado de Doctor en Ciencia de Alimentos

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ANTIMICROBIAL, CYTOTOXIC AND ANTI-INFLAMMATORY ACTIVITIES OF DIFFERENT ESSENTIAL OILS, THE DIFFUSION OF THEIR MAIN COMPONENTS WHEN APPLIED IN VAPOR PHASE, AS WELL AS THEIR EFFECTIVENESS AS ANTIMICROBIAL AGENTS AND SENSORY EFFECTS IN ALFALFA SEEDS

In partial fulfilment of the requirements for the Degree of Doctor of Food Science

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ESCUELA DE INGENIERÍA
DOCTORADO EN CIENCIA DE ALIMENTOS

El Dr. Aurelio López Malo Vigil, Profesor del departamento de Ingeniería Química y Alimentos,

Hace constar:

Que la tesis titulada: “EFECTO ANTIBACTERIANO EN FASE VAPOR DIFERENTES ACEITES ESENCIALES, SUS COMPONENTES MAYORITARIOS Y MEZCLAS ENTRE LOS MISMOS EN SISTEMAS MODELO Y GERMINADO DE ALFALFA, Y DEL IMPACTO DE ÉSTOS SOBRE EL SABOR EN GERMINADO DE ALFALFA ” presentada por Ana Cecilia Lorenzo Leal para obtener el grado de Doctora en Ciencia de Alimentos por la Universidad de las Américas Puebla, ha sido realizada en el Departamento de Ingeniería Química, Alimentos y Ambiental bajo su dirección, reuniendo las condiciones necesarias para ser defendida por su autora.

Santa Catarina Mártir, Cholula, Puebla
Junio 2019

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Dr. Aurelio López Malo Vigil
Director de tesis

PROLOGUE

The present doctoral thesis is titled “Vapor phase antibacterial effect of different essential oils, their major components and their mixtures in-vitro and on alfalfa sprouts, and their impact on sprout’s sensory acceptance”, and is divided into four chapters: 1) Evaluation of the efficiency of allspice, thyme and rosemary essential oils on two foodborne pathogens *in vitro* and on alfalfa seeds, and their effect on sensory characteristics of the sprouts, 2) Antimicrobial, cytotoxic, and anti-inflammatory activities of *Pimenta dioica* and *Rosmarinus officinalis* essential oils, 3) Vapor phase antibacterial effect of thyme (*Thymus vulgaris*) and rosemary (*Rosmarinus officinalis*) essential oils and their major components at selected pH’s and temperatures and 4) Vapor phase antibacterial activity, composition and diffusion of allspice, thyme and rosemary essential oils and their major components. The first chapter discusses how the vapors of essential oils could be used as natural preservatives *in vitro* and on alfalfa seeds, inhibiting the growth of two pathogen bacteria (*Listeria monocytogenes* and *Salmonella* Typhimurium) to obtain uncontaminated alfalfa sprouts. The second chapter mentions the antimicrobial effect of two essential oils (allspice and rosemary) against different microbial strains and their cytotoxicity and inflammatory activities. The third chapter discusses the evaluation of the vapor phase antibacterial effect of thyme and rosemary essential oils (EOs) and their major components (thymol and 1,8-cineole, respectively) against three different bacteria (*Salmonella enterica, Listeria monocytogenes* and *Pseudomonas fluorescens*) at selected pH’s and temperatures while the third one explains the importance of the diffusion of the vapors of essential oils major components on the essential oils antimicrobial activity when tested in *in vitro* systems.

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ABSTRACT

Chemically synthesized antimicrobial agents have long been used as preservatives in the food industry. However, the demand for more natural products by consumers has increased over the years, leading to the need of finding sources of natural preservatives, such as herbs and spices, or their extracts or essential oils. Essential oils (EOs) are natural products composed of a mixture of volatile and aromatic compounds extracted from different parts of plants. An alternative source of EOs are the ones extracted from allspice (*Pimenta dioica*), thyme (*Thymus vulgaris*) and rosemary (*Rosmarinus officinalis*), which have inhibited the growth of different microorganism. EOs were tested in vapor phase, and their diffusion was also evaluated. Results showed that thyme EO was the most effective of the tested EOs (allspice, thyme and rosemary) on culture media and on alfalfa seeds, against *Listeria monocytogenes* and *Salmonella* Typhimurium. There also was a significant ($p \leq 0.05$) difference between systems (*in vitro* or on alfalfa seeds) regardless of the microorganism or the evaluated EO. Treatment of alfalfa seed with vapor phase EOs did not affect the seed germination or the sensory acceptability of the sprouts, obtained of treated seeds, were not significant ($p \geq 0.05$) different from sprouts obtained from the non-treated seeds. In addition, the allspice and rosemary EOs showed no inflammatory activity when exposed to human macrophages, but a potent anti-inflammatory activity was measured when the oil from *Rosmarinus officinalis* was exposed to macrophages.

RESUMEN

Los agentes antimicrobianos sintetizados químicamente se han utilizado durante largo tiempo como conservantes en la industria alimentaria. Sin embargo, la demanda de productos más naturales por parte de los consumidores ha aumentado con los años, surgiendo la necesidad de encontrar fuentes de agentes antimicrobianos naturales, como hierbas y especias, o sus extractos o aceites esenciales. Los aceites esenciales son productos naturales compuestos de una mezcla de compuestos volátiles y aromáticos extraídos de diferentes partes de las plantas. Una fuente alternativa de dichos aceites esenciales son aquellos extraídos de pimienta gorda (*Pimenta dioica*), tomillo (*Thymus vulgaris*) y romero (*Rosmarinus officinalis*), que han inhibido el crecimiento de diferentes cepas microbianas. La aplicación de los aceites esenciales fue en fase vapor y evaluándose también a difusión de los mismos. En este trabajo se observó que el aceite esencial de tomillo fue el más efectivo entre los aceites esenciales probados (pimienta gorda, tomillo y romero) en sistemas modelo y en semillas de alfalfa, contra *Listeria monocytogenes* y *Salmonella* Typhimurium. También se observó que hubo una diferencia significativa ($p \leq 0.05$) entre los sistemas (*in vitro* o en semillas de alfalfa) sin importar el microorganismo o el aceite esencial evaluado. El tratamiento de semillas de alfalfa con aceites esenciales en fase de vapor no afectó la germinación de las semillas, ni la aceptabilidad sensorial de los germinados obtenidos. Tampoco se encontró diferencia significativa ($p \geq 0.05$) entre los germinados obtenido a partir de semillas sin tratamiento y los germinados obtenidos de semillas con tratamiento. Además, los aceites esenciales de pimienta gorda y romero no mostraron actividad inflamatoria cuando se expusieron a macrófagos humanos, pero se observó una potente actividad antiinflamatoria cuando el aceite de romero fue expuesto a macrófagos.

GENERAL INTRODUCTION

Chemically synthesized antimicrobial agents have long been used as preservatives in the food industry. However, the demand for more natural products by consumers has increased over the years, leading to the need of finding sources of natural preservatives, such as herbs and spices, or their extracts or essential oils (Rodríguez 2011; Skrinjar and Nemet 2009).

An alternative source of natural preservatives are allspice (*Pimenta dioica*), thyme (*Thymus vulgaris*) and rosemary (*Rosmarinus officinalis*) essential oils, which have inhibited the growth of different microbial strains when applied in liquid phase. The microbial strains included *Listeria monocytogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus feacalis*, *Vibrio alginolyticus*, *Salmonella* Typhimurium, *Rhizoctonia solani*, *Macrophomia phaseolina*, *Salmonella* Senftenberg, and *Salmonella* Give (Du *et al*. 2009; Giarratana *et al*. 2016; Jiang *et al*. 2015; Khaledi, Taheri, and Tarighi 2015; Miladi *et al*. 2013; de Oliveira, Brugnera, and Piccoli 2013; Oussalah *et al*. 2006, 2007; Zabka, Pavela, and Slezakova 2009).

However, when essential oils are applied in liquid phase they generate a significant impact on the sensory attributes of food, as they are usually obtained from spices with a strong aroma and flavor (Başer and Buchbauer 2010). Unlike the liquid phase, the application of essential oils in vapor phase, requires lower concentrations for their use as preservatives. Therefore, the vapor phase application could be a solution to the intense aroma and flavor imparted by essential oils (Olivares-Cruz & Lopez-Malo, 2013).

Essential oils have been used in traditional medicine to treat different illnesses (Al-Rehaily *et al*. 2002; Braga *et al*. 2007). For example, *Rosmarinus officinalis* (RO) (Lamiaceae) is an herb used worldwide in cuisine, and it can also be used in traditional medicine for its antimicrobial, antiparasitic and anti-nociceptive activities, as well as, it is a strong candidate as an anti-inflammatory and a wound-healing agent (Mangena and Muyima 1999).

The transmission of foodborne pathogens through fresh produce is known as a n increasing problem for the health authorities. For example, sprouts are obtained by soaking the seeds into water from a large variety of seeds (e.g. alfalfa, beans, soybeans, broccoli) and placing them in warm and humid conditions optimal for microbial proliferation (Ding, Fu, and Smith 2013; Galvez *et al*. 2011). Seeds could have up to 6 log of colony-forming units

(CFU)/ g of fresh material, while sprouts could present higher than 3 logs, representing the most common source of contamination for sprout-associated foodborne illnesses. Other indirect contamination sources for sprouts could be related to irrigation water, pre-harvest contamination through fertilizers/manure, or soil quality (Yang *et al*. 2013). Therefore, the aim of this project was to evaluate the antimicrobial, cytotoxic and anti-inflammatory activities of different essential oils, the diffusion of their main components when applied in vapor phase, as well as their effectiveness as antimicrobial agents and sensory effects in alfalfa seeds.

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GENERAL JUSTIFICATION

Essential oils have been studied as an alternative of natural preservatives to inhibit the growth of spoilage or pathogenic microorganisms related to food products. Most studies have been focused on the antimicrobial activity of essential oils when applied in a liquid phase, as a part of a food-product formulation. However, when essential oils are applied in liquid phase they confer a strong aroma and flavor to food products, and the use of a vapor phase is an attractive solution for their applications.

Alfalfa sprouts are a food product highly consumed worldwide that has been related to cases of foodborne illnesses outbreaks; which could be caused by the presence of microorganisms in the seeds used to generate the sprouts. Thus, there is a need to protect the consumer population with a proper disinfection of the seeds, which could be achieved with the use of essential oils as natural preservatives applied in vapor phase.

GENERAL OBJECTIVES

General objective

Evaluate the antimicrobial, cytotoxic and anti-inflammatory activities of different essential oils, the diffusion of their main components when applied in vapor phase, as well as their effectiveness as antimicrobial agents and sensory effects in alfalfa seeds

Specific objectives

- Evaluate the effect of different conditions used to extract allspice essential oil and determine its chemical composition.

- Evaluate the antimicrobial activity *in vitro* of allspice, thyme or rosemary essential oils, and its major components, applied in vapor phase in various concentrations, pH, and temperature against different strains of bacteria, molds, and yeasts.

- Determine the diffusion of the major components of allspice, thyme, and rosemary essential oils in different conditions.

- Determine the cytotoxicity and the inflammatory activity of essential oils of allspice, thyme and rosemary using human-derived macrophages.

- Evaluate the antimicrobial activity of allspice, thyme, and rosemary essential oils applied on alfalfa sprouts in vapor phase at room temperature against *S*. Typhimurium and *L. monocytogenes*.

- Determine flavor's maximum tolerable concentration of allspice, thyme, and rosemary essential oils on alfalfa sprouts.

RESEARCH PAPER I

"Evaluation of the efficiency of allspice, thyme and rosemary essential oils on two foodborne pathogens in *in-vitro* and on alfalfa seeds, and their effect on sensory characteristics of the sprouts"

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Evaluation of the efficiency of allspice, thyme and rosemary essential oils on two foodborne pathogens in *in-vitro* and on alfalfa seeds, and their effect on sensory characteristics of the sprouts

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Abstract

Seeds are usual source of contamination and their sprouts are commonly associated foodborne illness. Therefore, the aim of this study was to evaluate the antibacterial vapor phase efficiency of allspice, thyme and rosemary essential oils on two foodborne pathogens in *in vitro* and on alfalfa seeds, including the chemical profile of the tested EOs and their effect on the sensory characteristics of the sprouts. Antibacterial activity was determined through the minimal inhibitory concentration (MIC) of EOs in vapor phase to inhibit the growth of *Listeria monocytogenes* and *Salmonella* Typhimurium in culture media and on alfalfa seeds. Also, the germination and the effect on sensory characteristics of the sprouts were determined. Thyme EO was the most effective of the tested EOs on culture media and on alfalfa seeds, against both bacteria. When rosemary EO was tested against *L. monocytogenes* in alfalfa seeds, the MIC (4.0 mL/Lair) was higher, compared to the one obtained in culture media (2.7 mL/L_{air}). But when this EO was tested against *S.* Typhimurium, the MIC in alfalfa seeds was lower than in culture media (11.7 vs 13.3 mL/Lair). Allspice EO resulted more effective against both bacteria in alfalfa seeds (6.0 mL/Lair for *L. monocytogenes* and 6.7 mL/L_{air} for *S.* Typhimurium), compared to culture media (12.0 mL/L_{air} for *L. monocytogenes* and 13.3 mL/L_{air} for *S.* Typhimurium). Vapor phase EOs MICs resulted in significant ($p \leq 0.05$) decreases of *L. monocytogenes* and S*.* Typhimurium counts compared to the control. There also was a significant ($p \leq 0.05$) difference between systems (*in vitro* or on alfalfa seeds) despite the microorganism or the evaluated EO. Treatment alfalfa seed with vapor phase EOs, did not affect the seed germination. Sensory acceptability of the sprouts, obtained of treated seeds, did not were significant ($p \geq 0.05$) different of the sprouts obtained from the non-treated seeds.

Keywords *Salmonella* Typhimurium, *Listeria monocytogenes,* Allspice (*Pimenta dioica*) essential oil, Thyme (*Thymus vulgaris*) essential oil, Rosemary (*Rosmarinus officinalis*), Alfalfa sprouts, Sensory evaluation, Chemical composition.

1. Introduction

Bacteria are challenging microorganisms to control in the food industry and are the main cause of foodborne illnesses (USDA, 2012). Sprouts are grown from a great variety of seeds, including alfalfa, which are usually consumed raw in sandwiches or salads, and are known to be a common vehicle for bacterial foodborne illnesses (FDA, 2017). Generally, seeds could contain a microbial load of 6.0 log CFU/g, while sprouts could have counts > 3 logs. In addition, seeds are the most common source of contamination for sprout-associated foodborne illness. Other indirect contamination sources for sprouts could be irrigation water, pre-harvest contamination through fertilizers/manure, or soil quality (Yang *et al*., 2013). Some contamination sources for seeds, could be irrigation water, pre-harvest contamination through fertilizers/manure, and soil quality (Yang *et al*., 2013).

The Food and Drug Administration (FDA) mentions that there are 46 reported outbreaks of foodborne illnesses related to sprouts in the United States over 20 years (between 1996 and 2016); detecting microorganisms such as *Salmonella, Listeria monocytogenes*, and *Escherichia coli* O157:H7. The prevalence of *Salmonella* in seeds is reported to be higher than in the final product (sprouts) while the prevalence of *L. monocytogenes* in seeds and in sprouts was similar (FDA, 2017).

There has been a worldwide increase in consumer demand for freshly produced fruit and vegetables mainly because they are associated with health benefits. Similarly, the demand for more “natural” products has increased over the years; thus, there is a need to find natural sources of food preservatives, such as herbs and spices, and their extracts and/or essential oils (Hyldgaard *et al.,* 2012; Kim *et al.,* 2012) that may reduce the risk of foodborne illnesses. Essential oils (EOs) are complex mixtures of volatile and aromatic compounds, extracted from different parts of plants (Burt, 2004), including EOs extracted from allspice (*Pimenta dioica*), thyme (*Thymus vulgaris*), and rosemary (*Rosmarinus officinalis*). These EOs have shown antimicrobial activity (in liquid or vapor phase) against different microbial strains, such as *L. monocytogenes, Staphylococcus aureus*, *St. epidermidis*, *Micrococcus luteus*, *Bacillus cereus, E. coli, Pseudomonas aeruginosa, Enterococcus feacalis, Vibrio alginolyticus, Salmonella* Typhimurium*, Rhizoctonia solani, Macrophomia phaseolina, Salmonella Senftenberg, S. Give, Aspergillus ochraceus, A. parasiticus,* and *A. niger* (Boskovic *et al.,* 2016; Du *et al*., 2009; Han *et al.,* 2014; Khaledi *et al*., 2015; Kim *et al*., 2016; Mattos De Oliveira *et al.,* 2013; Miladi *et al*., 2013). However, when EOs are applied in liquid phase (directly), they produce a significant impact on the

sensory attributes of the food because of their strong aroma and flavor. Unlike liquid phase, vapor phase application (indirectly) of EOs, requires lower concentrations for their use as antimicrobials. Therefore, vapor phase application could be a solution to the adverse effects of the intense aroma and flavor of EOs in foods (Lee *et al*., 2018).

There are few studies that mention the use of allspice, thyme, and rosemary EOs as antimicrobials against *Salmonella* Typhimurium and *L. monocytogenes* in vapor phase, and even fewer assessing their antibacterial activity in alfalfa seeds. Thus, the aim of this research was to evaluate the antibacterial vapor phase efficiency of allspice, thyme and rosemary essential oils on two foodborne pathogens *in vitro* and on alfalfa seeds, including a chemical profile of the tested EOs and their effect on the sensory characteristics of the sprouts.

2. Material and methods

2.1 Bacterial strains

Bacterial strains (*Salmonella enterica* serovar Typhimurium ATCC14028 and *Listeria monocytogenes* Scott A) were obtained from the Food Microbiology Laboratory strain collection of Universidad de las Americas Puebla (UDLAP, Mexico, Puebla), and were maintained on Tryptic Soy Agar (TSA; Difco, BD, Sparks, MD) slants at 5 °C.

Cultures were prepared by inoculating the bacteria (*S.* Typhimurium and *L. monocytogenes*) into 10 mL of Tryptic Soy Broth (TSB; Difco, BD, Sparks, MD) and incubated at 35 °C for 24 h. Inoculum cell concentration was adjusted to 10^8 or 10^5 CFU/mL for subsequent use in the culture mediums and seeds respectively (Reyes-Jurado *et al*., 2016).

2.2. Essential oils, components and plant materials

Allspice (*Pimenta dioica*) EO was obtained from Liquid Gold® (Evansville, IN) while thyme and rosemary EOs were purchased from Hersol® laboratories (San Mateo Atenco, Estado de México, Mexico). Alfalfa seeds were purchased from Hortaflor® (Rancho Los Molinos, Tepoztlán, Morelos, Mexico).

2.3. Gas chromatography/mass spectrometry (GC/MS) analysis

The studied EOs were analyzed by a gas chromatographer with a 6850 Series Network (Agilent Technologies, Santa Clara, CA), equipped with a mass selective detector (5975C VL) and with a triple-axis detector (Agilent Technologies). Separation of the components was achieved by an HP-5MS (5% phenyl – 95% polydimethylsiloxane) capillary column (30m by 0.35 mm, 0.25 µm film thickness). The carrier gas was helium at a constant flow mode of 1.5 mL/min. The temperature of the column was initially around 60 °C for 10 min, increasing every 5 min until reaching 240 °C, and maintained at 240 °C for 50 min. The injector temperature was 240 °C. Retention indices were calculated by a homologous series of n-alkanes C8 to C18 (Sigma, St. Louis, MO). Compounds were identified by comparing mass spectra obtained with the reported in the US NIST (National Institute of Standard Technology) Library, and Shimadzu retention index (RI) isothermal equation (Reyes-Jurado *et al*., 2016).

2.4. Vapor phase antibacterial activity *in vitro*

2.4.1. Inverted Petri dish method

Plates prepared with TSA were platted with 50 µL of inoculum of each bacteria, using a spiral plater Autoplate 4000 (Spiral Biotech, Norwood, MA).

Minimum inhibitory concentration (MIC) refers to the minimum concentration necessary to inhibit the visible growth of the studied microorganism (López-Malo *et al.*, 2005.). This value was considered to evaluate the antibacterial activity using the inverted Petri dish technique. This method consists in placing a sterile paper disc (Whatman No. 1, diameter 55 mm) on the lid of the Petri dish and impregnating it with a known volume of EOs. The volumes tested varied from 5 to 1900 µL, depending on the tested EO and the studied bacteria. The previously inoculated culture medium with the paper disc on the lid was immediately inverted, sealed with Parafilm® and incubated at 35 °C for 24 h (Miladi e*t al.*, 2013). Quantification of colony forming units (CFU/mL) was performed when growth was observed, using a Q-Count counter and corresponding software (Spiral Biotech, Norwood, MA). The obtained MICs were expressed as mL of EO per L of air. Tests were performed by triplicate.

2.5. Vapor phase antibacterial activity *in vivo*

2.5.1. Seed disinfection

Alfalfa seeds were disinfected by submersing them in a 70% ethanol solution for 1 min, followed by dipping in 0.75% NaClO solution for 3 min, and then rinsed four times with sterilized distilled water. Seeds were dried overnight using Whatman filter paper sheets at 35 °C in a laminar flow hood (Kotana *et al*., 2013).

2.5.2. Seed inoculation

For seed inoculation, 1 g of disinfected seeds (approximately 420 seeds) and 1 mL of the adjusted inoculum were transferred to a tube with 8 mL of TSB, and incubated with shaking (BT25, Yamato Scientific Co., Japan) at 35 °C for 1 h. Inoculated seeds were then drained and dried overnight using Whatman No, 1 filter paper sheets at 35 °C in a laminar flow hood (Kotana *et al*., 2013).

2.5.3. Seed treatment with essential oils

The antibacterial effect of the essential oils against the studied bacteria was quantified by minimum inhibitory concentration (MIC) using an airtight container (ca. 1.5 L, 21 cm long X 11.5 cm wide X 10.5 cm high). One gram of inoculated seeds with *L. monocytogenes* or *S.* Typhimurium was placed inside of an airtight container over a plastic mesh attached to a circular glass container (7 cm diameter, 3 cm high), and exposed to different concentrations of EO vapors (Lee *et al.,* 2018) at room temperature for 24 h.

2.5.4. Antibacterial activities of essential oils in alfalfa seeds

After the treatment with EO vapors, the seeds were crushed in a sterile mortar and pestle with 9 mL of sterile peptone water (1.0 g/L) until a seed slurry was obtained. One milliliter of the slurry was serially diluted in 9 mL of sterile peptone water and poured plated (1 mL) in TSA. Petri dishes were incubated at 35 °C for 24 h and colony forming units (CFU/mL) were quantified (Lee *et al*., 2018; Singh *et al*., 2003). Tests were performed by triplicate.

2.5.5. Seed germination on Petri dish

Seed germination percentage was determined by incubating (at room temperature) 10 dry, inoculated seeds treated with EOs in a Petri dish (60×15 mm) over sterile Whatman No. 1 filter paper and wetted with 4 mL sterilized distilled water. Germinated seeds were considered germinated if a 2-mm radicle had emerged; they were observed under a microscope magnified 20× (Forty, American Optical Corporation, USA) and counted after 24

h of incubation. Germination was calculated by percentage (Kotana *et al*., 2013). Tests were performed by triplicate.

2.6. Sensory evaluation

Triangle tests were utilized to compare treated alfalfa sprouts (with each of the three tested EOs, allspice, thyme and rosemary) with nontreated alfalfa sprouts. The amount of EO used on treated seeds for this evaluation was the MIC obtained from the seed treatment with EOs previously explained. The sensory panel group consisted of 51 untrained panelists, and regular consumers of alfalfa sprouts; for each test, three samples were simultaneously presented, one different from the other two, all were randomly selected from treated or untreated alfalfa sprouts.

For sample preparation, the sprouting method reported by Landry *et al*. (2014) was utilized with some modifications. Treated and untreated alfalfa seeds were placed in a 750 mL flask and soaked with 112.5 mL (15%) of drinking water at 25 °C for 24 h. The water was then removed, sprouting was continued for 3 days at the same temperature and then alfalfa sprouts were stored at 5 °C until used, but for no longer than 1 day.

With the purpose of having samples at room temperature, they were taken out of the fridge 1 h before the test; then placed in a white plate and presented to panelists. Samples were accompanied by a neutral flavored cookie and a glass of water to clean panelists' palates. Tests were performed at UDLAP's Sensory Evaluation Laboratory (Stone *et al*., 2012).

Complementary to the triangle test, panelists were asked to mark the degree of difference that they detected (slight, moderate, strong, or extreme); and their preference among the samples (equal samples or the different one). Finally, panelists were encouraged to write additional comments.

2.7. Statistical analyses

MICs obtained data were analyzed using a general linear model with Minitab statistical package (ver. 17, Minitab Inc., State College, PA). Statistical analysis of the germination data was performed by ANOVA and Tukey's mean comparison tests ($p \leq 0.05$), using Minitab and the results of the triangle tests were analyzed with minimum number of correct

judgments to establish significance ($p \leq 0.05$) for triangle tests presented by Stone *et al.* (2012).

Results and discussion

3.1. Chemical composition of the essential oils

Allspice, thyme, and rosemary EOs main components were identified by GC–MS, and their calculated retention indices are reported in Table 1. The main component of allspice EO was eugenol (89.55%); Mcymene (36.77%) and thymol (16.98%) of thyme EO; α-pinene (27.39%), camphor (20.64%) and 1,8 – cineole (20.89%) of rosemary EO. Burt (2004) mentioned that rosemary EO may contain 6–14% of 1,8–cineole and 2–10% of β–pinene; thyme EO, 10–64% of thymol, 2–10% of carvacrol, and 2–31% γ–terpinene; this chemical composition is similar to that obtained in our analysis. Miladi *et al.* (2013) found in rosemary EO, α–pinene, 1,8–cineole, β–pinene, and camphene as the main components; again, similar to the reported results in this study except for α–pinene. For thyme EO, these authors reported that thymol, p-cymene, and γ-terpinene were its main components. On the other hand, Attokaran (2011) stated that allspice EO contained 80–87% of eugenol, 4–8% β-caryophyllene, and 0.2–0.5% of β-phyllandrine; which coincide with our findings, especially in the case of eugenol.

Table 1. Main components of allspice, thyme, or rosemary essential oils (EOs) determined by gas chromatography-mass spectrometry.

Compound	Percentage in allspice EO	Percentage in thyme EO	Percentage in rosemary EO	Retention Index
Eugenol	89.55	-	-	1356
α-Terpineol	2.04	-	4.25	1457
Caryophyllene oxide	1.48	3.52	-	1582
1,8 – cineole	1.06	-	20.89	1026
α-Cadinol	0.86	-	-	1652
M-Cymene	-	36.77	-	1082
Thymol	-	16.98	-	1288
Caryophyllene	-	9.20	-	1417
γ-Terpinene	-	7.83	-	1055
α-Pinene	-	5.38	27.39	0930
Camphor	-	-	20.64	1141
Camphene	-	-	7.16	0946
Borneol	-	-	3.69	1165

3.2. Vapor phase antibacterial activity

The antibacterial activity of allspice, thyme, or rosemary EOs against *L. monocytogenes* and *S.* Typhimurium was tested by the inverted Petri dish method and the obtained results are shown in Fig. 1; where it can be observed that the three tested EOs exhibited antibacterial effects (at different concentrations) against the two studied bacteria.

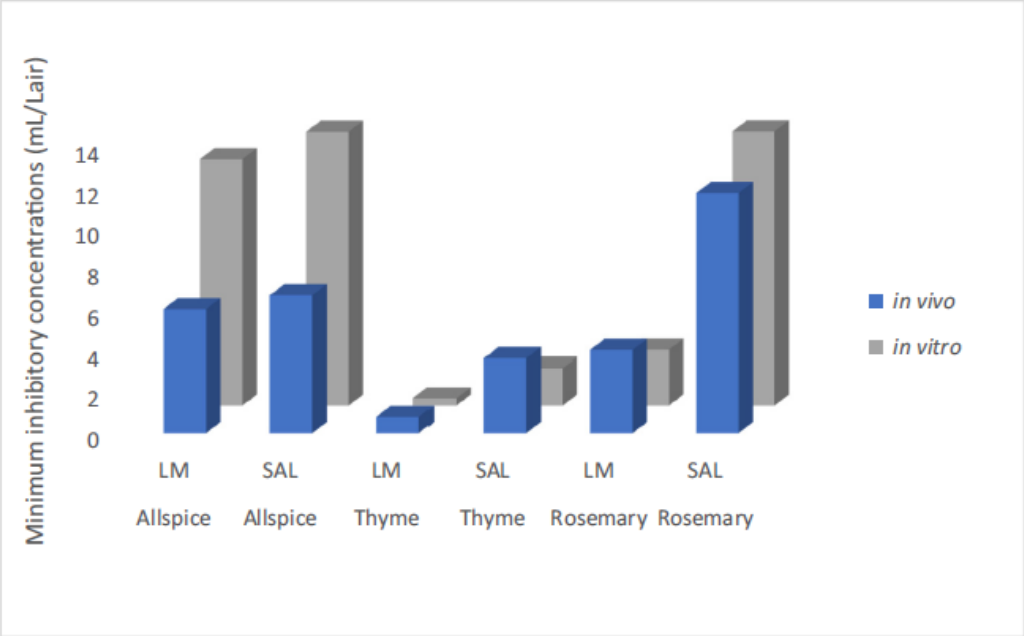

Figure 1. Minimum inhibitory concentrations (MICs, mL/Lair) of allspice, thyme, or rosemary essential oils against *L. monocytogenes* (LM) and *S.* Typhimurium (SAL).

Thyme EO displayed the strongest antibacterial activity against both bacteria (0.4 mL/Lair for *L. monocytogenes* and 1.8 mL/Lair for *S.* Typhimurium) followed by rosemary EO (2.7 mL/Lair for *L. monocytogenes* and 13.3 mL/Lair for *S.* Typhimurium), and allspice EO (12.0 mL/Lair for *L. monocytogenes* and 13.3 mL/Lair for *S.* Typhimurium). Obtained MICs for thyme EO were higher than those found in the literature, but MICs for allspice and rosemary were similar to those reported. Nodorostova *et al.* (2009) evaluated the antibacterial effect of thyme EO against *L. monocytogenes* by the disc volatilization method, and determined a MIC of 0.26 µL/cm^3, lower than that reported in this study. Using the same method, Mattos De Oliveira *et al.* (2013) tested the vapor phase antimicrobial activity of rosemary EO against *L. monocytogenes* and reported that with 0.72 µL/cm^3 there was an insignificant reduction of bacterial growth. Likewise, Du *et al.* (2009) studied the vapor-phase diffusion of allspice EO from tomato films against *S. enterica* and *L. monocytogenes*, where 3% (w/w) of the EO, slightly reduced bacterial growth.

Furthermore, in this study we observed that *L. monocytogenes* was more susceptible to EOs than *S.* Typhimurium, as reported by various authors (Han *et al.,* 2014; Soković *et al.*, 2010; Techathuvanana *et al.,* 2014). Resistance of Gram-negative bacteria, could be related to their hydrophilic cell wall, which helps them against penetration of hydrophobic

compounds found in EOs (Rivera Calo *et al.,* 2015). We observed that different factors affect MICs, such as tested EO, microorganism, medium and method of study, which makes it difficult to compare results from different authors.

MICs of allspice, thyme, or rosemary EOs against *L. monocytogenes* or *S.* Typhimurium in inoculated alfalfa seeds are displayed in Fig. 1. As in the culture media, the most effective tested EO against studied bacteria was thyme (0.8 mL/Lair for *L. monocytogenes* and 3.4 mL/Lair for *S.* Typhimurium); both MICs were higher than those in culture media. When rosemary EO was tested against *L. monocytogenes* in alfalfa seeds, the MIC (4.0 mL/Lair) was higher compared to the one obtained in culture media (2.7 mL/Lair). But when this EO was probed against *S.* Typhimurium, the MIC in alfalfa seeds was lower (11.7 mL/Lair vs 13.3 mL/Lair). Allspice EO was more effective against both studied bacteria in alfalfa seeds (6.0 mL/Lair for *L. monocytogenes* and 6.7 mL/Lair for *S.* Typhimurium) compared to the values obtained in culture media (12.0 mL/Lair for L. monocytogenes and 13.3 mL/Lair for *S.* Typhimurium) as can be seen in Fig. 1. Vapor phase EOs MICs resulted in significant ($p \leq 0.05$) decreases of *L. monocytogenes* and *S.* Typhimurium viable cells compared to the control. Between treated alfalfa seeds and the control seeds, there was a decrease of 5 log CFU/g. between treated culture medium and the control medium, the decrease was of 7 log CFU/g. There also was a significant ($p \leq 0.05$) difference between systems (*in vitro* or on alfalfa seeds) regardless of the tested microorganism or the EO.

Our findings demonstrate an effective vapor phase antibacterial activity of allspice, thyme and rosemary EOs in alfalfa seeds, against *S.* Typhimurium and *L. monocytogenes*. The antibacterial effect of thyme EO (against both bacteria) and rosemary EO against *L. monocytogenes* tended to be lower when they were utilized in alfalfa seeds than on laboratory media, while the vapors of allspice EO were more effective to inhibit bacterial growth bacteria in alfalfa seeds. An explanation for these results would be that it is harder to remove or inactivate pathogens when they penetrate vegetable surfaces (Seo and Frank, 1999). Nuñez and D'aquino (2012) reported that the presence of organic matter reduced the antibacterial effect of clove EO and that this decrease varied from one microorganism to another. Consequently, it is important to consider the influence of surface structures of alfalfa seeds. The antibacterial efficacy of vapors of the EOs, could also be affected by different factors (Lee *et al*., 2018) such as relative humidity, pH, water activity, among others, between the studied systems.

Lee *et al*. (2018) evaluated the antimicrobial activities of gaseous thyme EO against *L. monocytogenes* on laboratory media and on radish sprouts through the MIC. These authors found that there was a significant ($p \leq 0.05$) decrease of *L. monocytogenes* counts, which agree with our findings. There are a few studies related to alfalfa seeds and sprouts treated with EOs, but the comparison was difficult since different methods and EOs were used against diverse microorganisms.

It has been known that the major components of allspice, thyme, or rosemary EOs (Table 1) can be related with the observed antibacterial effect; the main component of tested allspice EO was eugenol (representing 89.55% of the total EO), which has been used to protect foods from different microorganisms during storage, and it has been reported as an effective antibacterial against *B. cereus, B. subtilis, St. aureus, E. coli, P. aeruginosa* and *Salmonella typhi* trough the inhibition of amino acid decarboxylase, the interruption of amylase and protease production, and by the deterioration of the cell wall (Lee *et al*., 2018). The tested thyme EO major component was thymol, which also has demonstrated antibacterial effects against *B. subtilis, E. coli, Klebsiella pneumoniae* and *St. aureus*; this compound causes lipid perturbation in the microbial plasma membrane and penetrates the microorganism's cell in order to exert antimicrobial effects (Kumar Trivedi *et al.,* 2015; Lee *et al.,* 2018). The tested rosemary EO main component was 1,8 – cineole (or eucalyptol), which according to Kumar Sahoo *et al.* (2011) is an effective antimicrobial against *M. luteus, S. epidermis, B. subtilis, E. coli, S. aureus and P. aeruginosa.* The antimicrobial effect of eucalyptol has been attributed to its lipophilic character enabling it to enter the membrane structures, resulting in membrane expansion, enhanced permeability and fluidity; furthermore, it makes iron transport processes difficult and inhibits respiration (Zengin and Baysal, 2014). The antibacterial activity of EOs can be associated to the interactions among all their components and not only due to their major ones (Lobritz *et al.,* 2014). Beside their main components, the tested allspice, thyme, or rosemary EOs have other components with antimicrobial properties, such as α-terpineol, α-cadinol, caryophyllene, camphor, and borneol (Table 1). Finally, according to the obtained results of the vapor phase antibacterial activity in vivo and on alfalfa seeds, it could be said that allspice, thyme and rosemary EOs antibacterial activity are more effective against Gram-positive bacteria, making their performances as antimicrobial agents, dependent on bacteria type. Despite this, we determined that the studied EOs are effective antimicrobial agents for alfalfa seeds disinfection.

3.3. Seed germination

Table 2 presents the germination percentage of alfalfa seeds treated with tested EOs; the results show that none of the treatments applied to the seeds affected their germination, despite the different concentrations of EOs used. In all cases, 100% of seeds germinated. Evidently, no significant ($p > 0.05$) difference was found between the applied treatments.

Table 2. Percentage of germinated alfalfa seeds treated with the vapor phase of an essential oil (EO).

Bacteria inoculated in seeds	EO utilized for the antibacterial treatment	Concentration of the EO (mL/L_{air})	Germinated seeds (%)
L. monocytogenes	Allspice	6.0	100[a]
	Thyme	0.8	100 [a]
	Rosemary	4.0	100 [a]
S. Typhimurium	Allspice	6.7	100 [a]
	Thyme	3.6	100 [a]
	Rosemary	11.7	100 [a]

[a] Indicates that there were not significant differences ($p > 0.05$) between samples

3.4. Sensory analysis

The triangle test was performed with the objective of determining whether sprouted seeds treated with EOs were different from the nontreated ones. Results are shown in Table 3; treatment of alfalfa seeds with vapor phase EOs did not significantly ($p \geq 0.05$) affect the sensory acceptability of the sprouts (Stone *et al*., 2012).

Table 3. Number of panelist responses that identify the odd sample during the triangle test among samples of sprouted seeds treated or not with tested essential oils (EOs)

Comparison	Number of correct answers/Total tests
Sprouts from seeds treated with allspice EO *vs* non-treated seeds sprouts	15/51[a]
Sprouts from seeds treated with thyme EO *vs* non-treated seeds sprouts	17/51[a]
Sprouts from seeds treated with rosemary EO *vs* non-treated seeds sprouts	20/51[a]

[a] Indicates that there were not significant differences ($p > 0.05$) between samples

For most judges who identified the different sample; the degree of difference among the samples was moderate for those samples treated with allspice or thyme EO, and slightly for those sprouted seeds treated with rosemary EO. Furthermore; most of the panelists preferred sprouts from seeds treated with allspice or rosemary EOs over untreated samples. In the case of the sprouts treated with thyme EO, panelists preferred untreated samples. Finally, additional comments from a few panelists suggested that sprouts treated with rosemary EO had acid and bitter flavors, while sprouts treated with thyme EO had bitter flavors. Gabrovská *et al*. (2005) mentions acid flavor as a descriptor of alfalfa sprouts, while Troszyńska *et al.* (2007) describe bitter as a basic taste in germinated seeds.

Thus, our findings indicate that tested EOs exhibited vapor phase antibacterial activity against *L. monocytogenes* or *S.* Typhimurium in culture media and in alfalfa seeds without significantly ($p > 0.05$) impacting the sensory acceptability of the sprouts; this is useful for further investigation regarding other sprouts, it is necessary to evaluate these EOS at semi-commercial level, as well as for studying inactivation of different foodborne pathogens with EOs in vapor phase in diverse food products.

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Conflict of interest

No conflict of interest declared.

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RESEARCH PAPER II

"Antimicrobial, cytotoxic, and anti-inflammatory activities of *Pimenta dioica* and *Rosmarinus officinalis* essential oils"

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Antimicrobial, cytotoxic, and anti-inflammatory activities of *Pimenta dioica* and *Rosmarinus officinalis* essential oils

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Abstract

Essential oils (EOs) are natural products composed of a mixture of volatile and aromatic compounds extracted from different parts of plants that have shown antimicrobial activities against pathogens. In this study, EOs extracted from *Pimenta dioica* (Myrtaceae) and *Rosmarinus officinalis* (Lamiaceae) were assessed for their antimicrobial activities using a panel of pathogenic Gram-positive, Gram-negative, and fungal strains. The antimicrobial activity was measured by the minimal inhibitory concentration required for the growth inhibition of the microorganisms. The cytotoxicity of the EOs was tested *ex vivo* using the model of human-derived macrophage THP-1 cells. In addition, an inflammatory response was evaluated using the anti-inflammatory cytokine IL-10, and the pro-inflammatory cytokines IL-6 and TNF-α. Results showed that both EOs had antimicrobial activity different pathogens were exposed to concentrations ranging between 600-2000 μg/mL. In addition, the EOs showed no inflammatory activity when exposed to human macrophages, but a potent anti-inflammatory activity was measured when the oil from Rosmarinus officinalis was exposed to macrophages. This study demonstrates that the use of EOs is an effective alternative for pathogenic bacterial and fungal control, alone or in combination with antibiotic therapy. Moreover, the oil extracted from *Rosmarinus officinalis* could be used as potent anti-inflammatory agent.

Keywords: essential oils; antimicrobial activity; anti-inflammatory activity; cytotoxic activity; Myrtaceae; Lamiaceae; *Pimenta; Rosmarinus*;

1. Introduction

Antibiotics are molecules used to treat infectious diseases. The appearance of multidrug-resistant strains of pathogens has alerted the scientific community and health care systems worldwide because of the lack of treatment for microbial-related illnesses [1, 2]. This threat has also been increased because of the misuse of antibiotics [3].

Natural products have been used in traditional medicine to treat infectious diseases since ancient times. Over the last few decades, the antimicrobial activity of these products has been scientifically validated [4].

Essential oils (EOs) are a mixture of volatile and aromatic compounds extracted from different parts of plants. EOs extracted from plants such as basil, cilantro, eucalyptus, and oregano have shown antimicrobial activities [5–7], including their potential to protect foods against pathogenic microorganisms [4, 8, 9].

Leaves of the tree *Pimenta dioica* (PD) (Myrtaceae) are used as ingredients in many cuisines worldwide. In addition, it has been used in traditional medicine to treat different illnesses [10–12]. *Rosmarinus officinalis* (RO) (Lamiaceae) is an herb used worldwide in cuisine, and it can also be used in traditional medicine for its antimicrobial, antiparasitic and anti-nociceptive activities, as well as, it is a strong candidate as an anti-inflammatory and a wound-healing agent [13–18].

Several compounds extracted from EOs have been reported to have antimicrobial activity. For example, citronellol, estragole, eudesmol, eugenol, geraniol, linoleic acid, and phytol have all shown significant antimicrobial activities against human and plant pathogens [19–24].

Following our program of investigation with the purpose of exploring new alternatives for antimicrobial activities based on EOs, we evaluated the antimicrobial activities of the EOs extracted from allspice (PD), and rosemary (RO) against a panel of pathogenic bacteria and fungi. The bacterial strains included Gram-positive and Gram-negative species, and the fungal strains included filamentous and yeast species. In addition, the cytotoxic and inflammatory activities of the EOs were assessed with a human macrophage cell line.

2. Experimental section

2.1. EOs and plant material.

RO EO was obtained from Hersol® laboratories (San Mateo Atenco, Estado de México, Mexico). Dried berries of PD were purchased from Condimentos Naturales Tres Villas S.A. de C.V. Puebla, Mexico.

2.2. PD EO extraction and sample preparation.

The dried berries of PD were first ground (NutriBullet, Magic Bullet, USA) and sieved (number 20 mesh, 850 µm). The EO was extracted using a microwave-assisted extraction (MAE) method after mixing the ground material with water at a ratio of 1:20 (w/v). The microwave (NEOS System equipment, Milestone, Shelton CT, USA) was operated at 800 W and 600 W for 30 min each. The extracted oil was placed in hermetically sealed amber vials to avoid any volatilization of the component. Stock solution of the two EOs at concentrations of 20 mg/mL DMSO were prepared and stored at 4°C until needed.

The chemical analysis of both EOs were analyzed by gas chromatography equipped with a mass spectrometer, as published [25]. The main components of the PD EO were eugenol (~90%) and α-terpinol (2%), and the main components of RO EO were α-pinene (27%), camphor (21%) and 1,8 cineole (~21%) [25].

2.3. Strains and culture media.

The pathogenic bacterial strains assayed in this study were *Acinetobacter baumannii* (ATCC BAA-747), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 14210), methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 700698), and *Staphylococcus aureus* (ATCC 25923). The pathogenic fungal strains included the yeast *Candida albicans* (ATCC 10231), and *Cryptococcus neoformans* var. *grubii* (kindly provided by Dr. Karen Bartlett, University of British Columbia, BC, Canada). The filamentous fungi *Aspergillus fumigatus* (ATCC 1022) and *Trichophyton rubrum* (ATCC 18758) were also tested in this study. Bacterial stocks were maintained in Mueller-Hinton broth (Becton & Dickinson) supplemented with 1.5% agar (Becton & Dickinson) at 4°C. Bacterial strains were cultured in a shaker at 37°C with the same broth. Fungal strains were maintained in Sabouraud broth (Becton & Dickinson) supplemented with 1.5% agar and incubated at 28°C. In the case of the filamentous fungi, spores were harvested in 1 mL of Sabouraud broth containing 10% glycerol, aliquoted, and maintained at -20°C until further use [26].

2.4. Minimal inhibitory concentration determination.

The minimum inhibitory concentration (MIC) was defined as the minimum concentration at which no growth was observed (no turbidity observed in the well). MICs were determined by a microdilution assay using a 96-well plate, according to previous published protocols [27]. The EO concentrations of 20, 30, 40, 50, 100 and 200 µg/mL) were assayed in a final volume of 100 µL/well. Bacterial strains were grown at 37°C overnight and their densities were

adjusted to an optical density of 0.05 at 600 nm, while 5 µL of a spore suspension ($1x10^6$ spores/mL) was used as inoculum for fungal strains, which were incubated at 28°C for 48 h. Untreated cells and DMSO were used as negative controls, whereas amikacin and gentamicin (for bacteria), and amphotericin and terbinafine (for fungi) were used as positive controls. Experiments were performed in triplicate.

2.5. Cytotoxic assay.

The cytotoxicity of the EOs was performed using human-derived THP-1 monocytic cells (ATCC 202), following published protocols [9]. Briefly, $5x10^4$ cell were dispensed per well in a 96-well plate with a final volume of 100 µL. EOs were tested at final concentrations of 2000, 1000, 600, 200, 100, 50, and 10 µg/mL. The detergent Tween-20 (10 µL of a 10% solution) was used as a positive control, whereas untreated cells and DMSO were used as negative controls. The analysis of the EO toxicity was performed with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) following published protocols [9]. The half-maximal lethal concentration (LC50) was calculated by plotting the EO concentrations against the damaged cells. Experiments were performed in triplicate. Final concentrations of DMSO per well were always ≤ 1%.

2.6. Anti-inflammatory assay.

The anti-inflammatory assay was performed as previously published using activated THP-1 cells at a final concentration of $7.5x10^4$ cells/well [9]. Cells treated with 1% DMSO served as negative control, whereas 100 ng/mL of lipopolysaccharide (LPS) from E. coli (Sigma-Aldrich) was used as a positive control. Experiments were carried out in triplicate and the final concentrations of DMSO per well were always ≤ 1%. EOs were tested at a final concentration of 7.5 µg/mL, which was selected based on the survival of the cell in the cytotoxic experiments.

2.7. Statistical analysis.

A t-test was used for statistical analysis. The statistical analysis was performed with Prism 4 (GraphPad Software, Inc.). A p-value <0.05 was considered statistically significant.

3. Results and discussion

3.1 Antimicrobial activities

The EOs were tested against two panels of pathogenic bacteria and fungi. Results showed that the EO extracted from PD was the most effective to kill five strains, including *A. baumannii*, MRSA, *P. aeruginosa*, *S. aureus*, and the yeast *C. albicans*, with MICs ranging between 500-2000 μg/mL (Table 1). The bacterial strain *E. coli* was resistant to the concentrations tested in this study.

Table 1. Antimicrobial activity of PD, and RO EOs expressed as MIC (µg/mL).

EO	Bacteria					Fungi			
	AB	EC	MRSA	PA	SA	AF	CA	CN	TR
PD	500	R	500	500	2000	R	600	R	R
RO	500	R	R	R	R	R	600	R	R
Control	0.1^{ak}	10^{g}	60^{g}	10^{ak}	1^{g}	2^{am}	2^{am}	2^{am}	1^{tb}

AB, *Acinetobacter baumannii*; EC, *Escherichia coli*; MRSA, methicillin-resistant *Staphylococcus aureus*; PA, *Pseudomonas aeruginosa*; SA, *Staphylococcus aureus*; AF, *Aspergillus fumigatus*; CA, *Candida albicans*; CN, *Cryptococcus neoformans* var. *grubii*; TR, *Trichophyton rubrum*. R, resistant strain. PD, *Pimenta dioica* EO; RO, *Rosmarinus officinalis* EO. Ak, amikacin; Am, amphotericin; G, gentamicin; Tb, terbinafine.

Our study addresses the control of human pathogens that have developed antimicrobial resistance and have caused hospital outbreaks and healthcare-associated infections in recent years, such as *A. baumannii* [28]. In addition, the EO also showed antibacterial activity against MRSA and *P. aeruginosa*, which have been public health problems worldwide because of their resistance to commonly used antibiotics [29, 30].

A previous study from Oussalah *et al.* [31], related to the antibacterial activity of the EO of PD, reported that the EO extracted from leaves showed antibacterial activity against *E. coli, Listeria monocytogenes, S. aureus,* and *Salmonella* Typhimurium, with MICs ranging between 0.1%-0.2% [31]. Although these results indicate that a higher activity was shown in that study, the methodology was based on mixing the EO in molten agar, whereas our experiment was based on dissolving the EO in DMSO with direct supplementation to the bacterial broth. In addition, the PD EO used in Oussalah's study may have different percentages of the major components (data not shown in that study) of the EO, compared to our study (as described in Materials and Methods). This chemotypic difference depends on the geographic location of the plants, the methodology used for the EO extraction, season

of the year, and environmental conditions in the region, with profound effect on the bioactivity of the EOs [32].

Regarding the antifungal activity, the PD EO was able to inhibit the growth of *C. albicans*, a yeast resistant to antifungal drugs [33]. Another study reported that the antifungal activity of the PD EO tested against *Fusarium oxysporum, F. verticillioides, Penicillium expansum, P. brevicompactum, Aspergillus flavus,* and *A. fumigatus* at a mean value of 0.6 µL/mL [34]. These results cannot be compared to our results because of the different technique and fungal strains used in that study.

In the case of RO, the EO was able to inhibit the growth of *A. baumannii* at concentrations of 500 μg/mL but was unable to inhibit the growth of the rest of the bacterial strains tested (Table 1). Interestingly, other studies have reported antibacterial activities against *E. coli, P. aeruginosa*, and *S. aureus*, with variable MICs ranging between 0.3 mg/mL to 1.72 mg/mL [35–37], which include our MIC of 0.5 mg/mL for *A. baumannii*. The different chemotypes of the RO EOs used in the different studies may suggest the reason why no activities against *E. coli, P. aeruginosa,* and *S. aureus* were observed in our study with concentrations < 2 mg/mL.

In our study, the RO EO was able to inhibit the growth of *C. albicans* in a similar concentration as PD (Table 1). A few studies reported the activity of RO EO against this yeast with discrepancies. For example, although in our study a MIC of 0.6 mg/mL was measured, higher MICs ranging between 5 mg/mL and ~10 mg/mL (1%) were measured in other reports [38, 39]. Also, a very low MIC of 5.6 µL/mL was measured in a different study [40], but it is noteworthy that this low MIC was expressed as MIC80 and not MIC100 as in our study. Again, different chemotype oils may be the cause of the large difference in the MICs. Another study reported antifungal activity of RO EO against *F. verticillioides* with a calculated MIC of 150 µg/mL [41]. Again, our results are not comparable to this study because the strain used was not in our screening panel of fungi.

The composition of essential oils is correlated with their antimicrobial activity. Phenolic compounds are known to have a major antibacterial activity compared to other chemical groups. The chemical function of the component could also decrease the EO antimicrobial activity, since phenols are usually more effective than cinnamic aldehydes, followed by alcohols, aldehydes, ketones, ethers and hydrocarbons [42]. As mentioned in the Materials and Methods section, a previous study from our group reported that the

eugenol was the major compound (~90%) of the PD EO [25]. Eugenol is a phenolic compound with reported antimicrobial activities [4] and was likely responsible for the antimicrobial activity in our study. Previous studies in which phenolic groups were assessed against plaque formations in the oral cavity show that eugenol significantly reduces the number of the plaques, compared to the placebo group [43]. In addition, eugenol at concentrations of 1000 µg/mL inhibited the growth of *Streptococcus oralis*, a known oral pathogen responsible for cavities and periodontal disease development [44, 45]. Moreover, eugenol was able to inhibit the growth of *S. typhi* at a final concentration of 0.0125% after 60 min exposure [46]. In this report, the mechanism of eugenol was reported to increase the bacterial membrane permeability of the pathogen, [46] as reported in *E. coli* and *L. monocytogenes* [47]. Another study reported that the mechanisms of action were due to a leakage of K+ from the cytosol of *E. coli* and *S. aureus* [48]. Both mechanisms can be connected to a leakage of K+ from the cytoplasm, which produces a shrinking of the cell as a result of changes in the turgor tension.

Eugenol was also reported as an antifungal agent against different pathogenic fungi. For example, in an in vivo study, guinea pigs were infected with Microsporum gypseum and thereafter treated with 0.01-0.03% of eugenol mixed in petroleum jelly. This formulation was effective not only to control the infection with concentrations similar to the nystatin used as a positive control, but also to improve the skin lesions [49]. Other studies using C. albicans were also reported. For example, an *in vivo* study of candidiasis performed in immunosuppressed rats showed that a daily treatment of eugenol (24 mM) reduced ~96% the number of CFU after 4 days treatment [50]. Moreover, a broad study including the exposure of 31 clinical isolates of C. albicans strains to eugenol revealed that an averaged MIC of 625 µg/mL inhibited the growth of all the tested strains [51]. Interestingly, our study reported that the same pathogen was inhibited by similar concentrations of the PD EO, suggesting that eugenol (95%) is responsible for the antifungal activities.

In the case of RO, the major components of the EO were α-pinene, 1,8-cineole or eucalyptol, and camphor [25]. The antifungal activity of α-pinene has been reported against *E. coli* and *S. aureus*. Although no activity was found against *E. coli* as reported in our study as well, *S. aureus* was inhibited at concentrations of 13.6 µg/mL [52]. Moreover, the mechanism of toxicity of this compound against *C. albicans* is based on the rupture of the membranes and cell wall, and the impairment of the production of DNA, RNA, ergosterol, and polysaccharides involved in the construction of the cell wall [53].

The second most abundant compound in the EO is 1,8-cineole, or eucalyptol, which has been reported as an antimicrobial agent. For instance, antimicrobial activities against a panel of bacteria and fungi ranging between 8-64 mg/mL were reported [54]. Microorganisms in this panel included the microorganisms used in our study. It is noteworthy that these MICs are elevated compared to our study, but we used the EO that contains only a fraction of eucalyptol compared to the pure compound used in this study. Similarly, other studies reported MICs ranging between 2-23 mg/mL and 8-64 mg/mL using panels of microorganisms that also included the strains reported in our study [55, 56].

3.2 Cytotoxic and inflammatory activities

The cytotoxic and anti-inflammatory activities were assayed on the human macrophage cell line THP-1. When the cytotoxicity was assayed, the results showed that the EOs from PD and RO were toxic at concentration of 10 μg/mL and 5 μg/mL, respectively (Figure 1A and 1B). LD50 values of 29.63 µg/mL and 14.15 µg/mL were calculated for PD and RO EOs, respectively. A previous study performed in Egypt has reported lower IC50 than seen in our study. Although that study used the EO extracted from the same Mexican berries, the IC50s ranged between 3-12 µg/mL when a panel of colon, hepatic, pulmonary, and intestinal cancer cell lines were treated [57]. This difference may be due to the use of the human macrophage cell line in our study or due to the Mexican berries gathered from different regional sources under different environmental conditions.

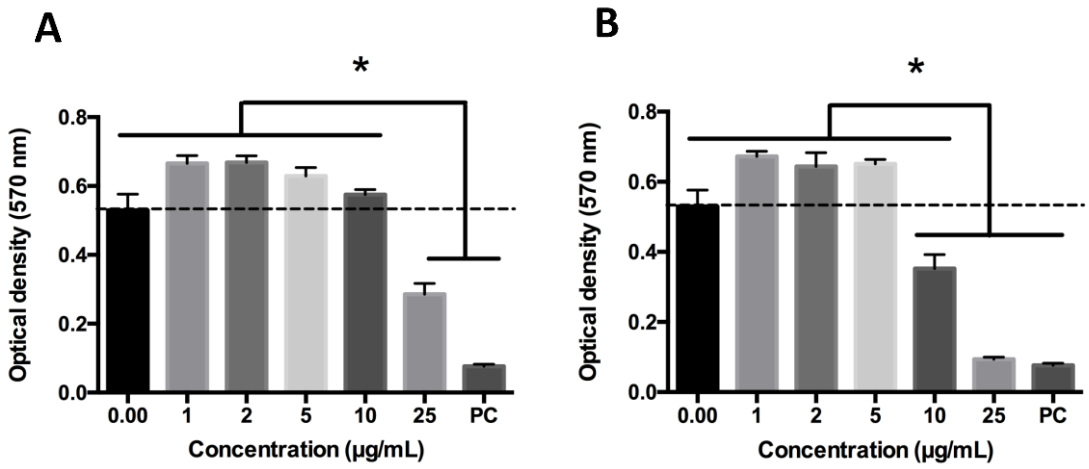

Fig. 1. Cytotoxicity of EO. The cytotoxicity of the (A) *Pimenta dioica* and (B) *Rosmarinus officinalis* EOs were assessed on human-derived macrophage THP-1 cell line using the

MTT assay. PC = positive control. Shown is the mean ± S.D. of three independent experiments. * = P-value <0.05.

The RO EO cytotoxicity has also been reported in the literature. Interestingly, high IC50 > 250 µg/mL was reported when the oil was exposed to a panel of ovarian and hepatic cancer cell lines [58], whereas a low IC50 of 8.5 µg/mL, similar to our 14.15 µg/mL, was calculated after exposure to pulmonary cancer cell line [36]. Again, all these discrepancies can be attributed to the composition of the EOs.

In the case of the anti-inflammatory activity, both EOs were not able to elicit a pro-inflammatory response because the levels of IL-6 and TNF-α were not significantly different from the untreated control (Figure 2A and 2C). Surprisingly, the levels of IL-10 (anti-inflammatory activity) of the RO EO showed a 4-fold increase compared to the untreated control (Figure 2B).

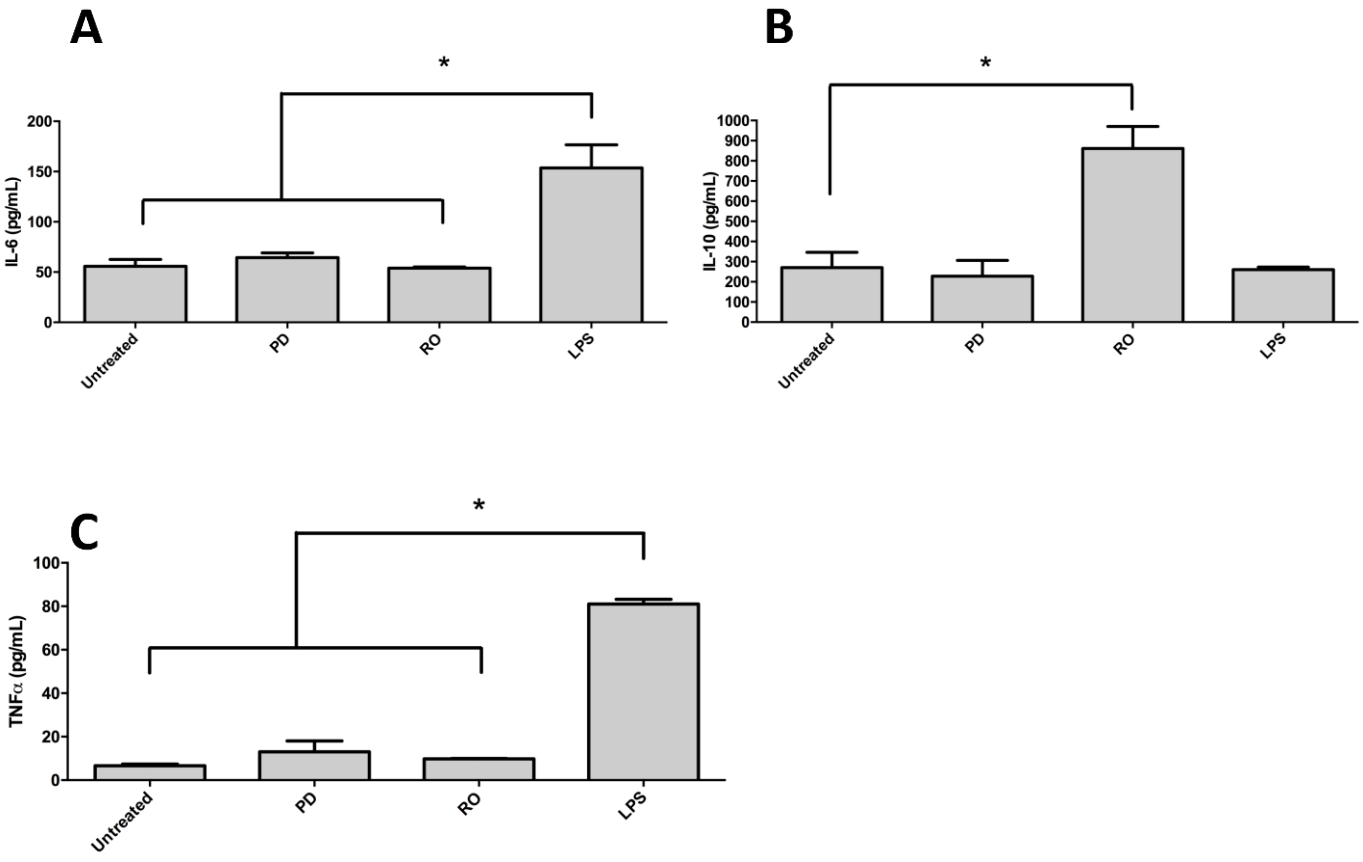

Fig. 2. Immunological response of EO. The immunological response of the *Pimenta dioica* and *Rosmarinus officinalis* EOs were assessed on human-derived macrophage THP-1 cell

line using ELISA for (A) IL-6, (B) IL-10, and (C) TNF-α. PD = *Pimenta dioica*. RO = *Rosmarinus officinalis*. LPS = lipopolysaccharide (positive control). Shown is the mean ± S.D. of three independent experiments. * = P-value <0.05.

A previous study using ground extracts of PD reported an increase of 150% and 166% of the pro-inflammatory cytokines IL-6 and TNF-α, respectively [59]. The discrepancies with our studies are based on (1) the different source of the material used (plant extract vs. EO in our study), and (2) the reported percentages of increase were normalized to the cytotoxicity values (using MTT), which cannot be compared to our results expressed in pg/mL. Eugenol, the major component of the PD EO, has been shown to modulate the inflammatory response when macrophages and lung tissues were challenged with LPS [60, 61]. The inhibition of the inflammatory response was based on the inhibition of the IL-6 and TNF-α as a result of its interference in the activation of the transcription factor nuclear factor-κB as measured in a murine model [61].

Regarding the anti-inflammatory activity of RO EO, a high concentration of the anti-inflammatory cytokine IL-10 was measured in our study, but an increase of the pro-inflammatory cytokines was not observed. Another study has reported a reduction of the IL-6 cytokine measured in the mice's colons [62], but no information was provided related to the amount of IL-10 secreted. Interestingly, another study reported a reduction of carrageenan-induced edema in a rat model, suggesting that the RO EO activates another anti-inflammatory pathway [63]. This fact is supported by other studies that showed that eucalyptol (one of the major compounds) in the RO EO reduced the inflammation in a carrageenan paw edema induced in mice and rats [18, 64]. Similarly, human gingival fibroblasts showed a decrease between 67-76% in the expression of IL-6 when exposed to eucalyptol and camphor, another compound identified in the EO [65]. Finally, a decrease in the level of TNF-α was measured when guinea pigs were challenged with ovalbumin and treated with eucalyptol [66]. These results are not surprising because eucalyptol and camphor are ingredients in over-the-counter medicines to treat coughs, such as VapoRub® and Buckley's®.

In our study, we found that the EOs show cytotoxicity when exposed to the cell line THP-1. It is clear that the addition of oils to the culture will have a direct contact with the cell membranes and always alter their composition, with detrimental effects to the viability of the cell. However, in vivo experiments showed different results. For example, a wound treatment of diabetic mice showed a better recovery when the animals were treated with the RO EO,

compared to the aqueous extraction [16]. Also, an anti-inflammatory effect was observed when eucalyptol alone was used to treat patients with severe asthma [67]. Lastly, human lymphocytes and macrophages treated with eucalyptol showed a significant decrease in the secretion of pro-inflammatory cytokines [68].

4. Conclusions

The bioactivities of the EOs extracted from PD and RO were assessed. Results of these experiments showed that both EOs have antimicrobial activity and the RO EA was able to significantly increase the level of the anti-inflammatory cytokine IL-10. In summary, the novelty of this study is the antifungal activity of the EOs against the fungal pathogen C. albicans together with the absence of an inflammatory activity when EOs were exposed to macrophages. In addition, the RO EO showed a potent IL-10-dependent anti-inflammatory activity. Taken together, both oils can be used not only for topical applications as antimicrobials but also as anti-inflammatory agents. In addition, both oils can be used as antiseptics, such as in mouthwashes, topical creams or gels, or disinfectants.

Conflict of interest

Authors declare no conflict of interest.

Data availability

Authors declare that the data used in this study is fully available.

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RESEARCH PAPER III

"Vapor phase antibacterial effect of thyme (*Thymus vulgaris*) and rosemary (*Rosmarinus officinalis*) essential oils and their major components at selected pH's and temperatures"

Submitted to Letters in Applied Microbiology and rejected. The information of this paper was used for the first and forth research paper presented in this thesis.

Vapor phase antibacterial effect of thyme (*Thymus vulgaris*) and rosemary (*Rosmarinus officinalis*) essential oils and their major components at selected pH's and temperatures

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Abstract

Chemically synthesized preservatives have been used as food antimicrobials for a long time. However, consumer demand for more natural products has increased over the years; for that reason, there is a need to find natural sources of food preservatives, such as essential oils. Therefore, the aim of this study is to evaluate the vapor phase antibacterial effect of thyme and rosemary essential oils (EOs) and their major components (thymol and 1,8-cineole, respectively) against three different bacteria (Salmonella enterica, Listeria monocytogenes and Pseudomonas fluorescens) at selected pH's and temperatures. The chemical composition was analyzed by gas chromatography-mass spectrometry, and the minimum inhibitory concentration (MIC) of the two EOs and their major components were determined at different pH's (6.0 or 6.5) and temperatures (10, 15, 25 or 35 °C) in culture mediums. The lowest MIC found was 0.13 mL Lair-1 for thyme EO or thymol against the three bacteria, and the highest was 25.30 mL Lair-1 for rosemary EO against P. fluorescens. In most of the cases, L. monocytogenes showed less resistance to EO's compared to S. enterica and P. fluorescens, which was expected, because Gram negative bacteria (Salmonella and P. fluorescens) are more resistant to EOs than Gram positive (L. monocytogenes).

Keywords

Antibacterial effect, vapor phase, thyme, rosemary, essential oils, pH and temperature.

1. Introduction

Bacteria are the main cause of foodborne illnesses (among chemicals, virus, bacteria and parasites) and the most difficult microorganisms to control in the food industry (USDA, 2012). Foodborne illness is a serious health problem that could be related to poor eating habits and low hygienic practices (Mekonnen and Sisay, 2015), but also in food products that received minimal processing and/or without antimicrobial agents or proper cold temperature storage.

On the other hand, chemically synthesized preservatives have been used as food antimicrobials for a long time. However, consumer demand for more natural products and clean labels has increased over the years; therefore, there is a need to find natural sources of food preservatives, such as herbs and spices, or their extracts and/or essential oils (Hyldgaard *et al*., 2012). Essential oils (EOs) are complex mixtures of volatile and aromatic compounds, extracted from different parts of plants: flowers, seeds, leaves, herbs, fruits, roots, rhizomes, among others. 85 – 95% of the main volume of EOs is constituted by their major components, with the remainder being minor components (Burt, 2004; Preedy, 2016). Alternative natural preservatives are EOs extracted form thyme (*Thymus vulgaris*) and rosemary (*Rosmarinus officinalis*). Both EOs have shown antimicrobial activity against different microbial strains, such as: Listeria monocytogenes, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus feacalis*, *Vibrio alginolyticus*, *Salmonella* Typhimurium, *Rhizoctonia solani*, *Macrophomia phaseolina*, *Salmonella* Senftenberg, and *Salmonella* Give (Mattos de Oliveira *et al*., 2013; Miladi *et al*., 2013; Han *et al*., 2014; Khaledi *et al*., 2015; Boskovic *et al*., 2016; Giarratana *et al*., 2016) in liquid and vapor phase.

However, when EOs are applied in liquid phase (directly), they confer a significant impact on food sensory attributes, because of their strong aroma and flavor (Preedy, 2016). In contrast, vapor phase application (indirectly), requires lower concentrations for their use as antimicrobials. Therefore, vapor phase application, could be a solution to the adverse effects of the intense aroma and flavor of EOs in food.

To avoid the mentioned effects, culture medium (*in-vitro*) studies have helped to simulate the antibacterial behavior of EOs in food systems (Hadjilouka *et al*., 2015; Mejía-Garibay *et al*., 2015). Culture medium and food system studies, and the microorganism's sensitivity could be influenced by intrinsic (protein and water content, aw, pH, salt concentration, among others) and/or extrinsic (atmosphere composition, temperature) factors. These factors can be controlled during *in vitro* studies to simulate different storage conditions and intrinsic properties, such as pH, of food products (Burt, 2004).

There are few studies that mention the use of thyme and rosemary EOs and its major components as antimicrobials against Salmonella enterica, Listeria monocytogenes and Pseudomonas fluorescens in vapor phase, and even fewer evaluate their antibacterial activity at different pH's and temperatures. Thus, the aim of this study is to evaluate the vapor phase antibacterial effect of thyme (*Thymus vulgaris*) and rosemary (*Rosmarinus*

officinalis) essential oils and their major components against three different bacteria (*Salmonella* enterica, *Listeria monocytogenes* and *Pseudomonas fluorescens*) at selected pH's and temperatures.

2. Results and discussion

2.1 Chemical composition of the essential oils

The main components identified by GC-MS in thyme and rosemary EOs, and their calculated retention index, are reported in Table 1. Burt (2004) mentions that thyme EO, may contain 10–64% of thymol, 2–10% of carvacrol, and 2–31% γ–terpinene; and rosemary EO may contain 6–14% of 1,8–cineole, and 2–10% of β–pinene, which agrees within the results obtained in this study. In the other hand, Miladi *et al*. (2013), found in thyme EO that thymol, p-cymene and γ-terpinene were the main components; and in rosemary EO these were α–pinene, 1,8–cineole, β–pinene, and camphene. The main components of thyme EO where similar to the ones reported in this study (except for p-cymene), and in the case of rosemary EO, the exception was α–pinene.

Table 1. Main components of thyme and rosemary essential oils (EOs) determined by gas chromatography-mass spectrometry.

Compound	Retention Index	Percentage in total Thyme oil	Percentage in total Rosemary
α-Pinene	930	5.38	-
γ-Terpinene	1055	7.83	-
Thymol	1288	16.98	-
Carvacrol	1299	6.11	-
Camphene	946	-	7.16
β-pinene	973	-	6.41
1,8-cineole	1025	-	19.86
α-Terpineol	1187	-	4.25

2.2 Antibacterial activity of essential oils

The antibacterial activity of both EOs and their major components (thymol and 1,8-cineole) against *L. monocytogenes*, *S*. enterica and *P. fluorescens*, were tested by the inverted Petri dish technique, and the obtained results are presented in Tables 2, 3 and 4. It can be observed that the two EOs and their major components had antibacterial effects (at different concentrations) against the three studied bacteria. Among the EOs, thyme EO showed the strongest antibacterial activity, at the different levels of pH (6.0 or 6.5) and temperature (10, 15, 25 or 35°C) studied, and followed by its major component thymol. A lower antibacterial activity was exhibited by rosemary EO and its major component (1,8–cineole). These results agree with those obtained by Mattos de Oliveira *et al*. (2013), who tested the vapor phase antimicrobial activity of thyme and rosemary EOs against *L. monocytogenes*, with thyme EO being more effective than rosemary EO.

Table 2. Minimum inhibitory concentrations (MICs[1]) of the thyme and rosemary essential oils and their major components against *Listeria monocytogenes*, at different pHs and temperatures.

pH	Temperature (°C)	Thyme EO (mL L_{air}^{-1})	Thymol (mL L_{air}^{-1})	Rosemary EO (mL L_{air}^{-1})	1,8–cineole (mL L_{air}^{-1})
6.0	10	0.13	0.13	0.40	0.40
	15	0.13	0.13	0.40	0.40
	25	0.13	0.40	2.40	2.00
	35	0.13	0.40	3.99	2.66
6.5	10	0.13	0.13	0.40	0.40
	15	0.13	0.13	0.40	0.40
	25	0.13	0.53	2.66	2.66
	35	0.13	0.53	4.66	3.33

[1] average of three replicates.

Table 3. Minimum inhibitory concentrations (MICs[1]) of the thyme and rosemary essential oils and their major components against *Salmonella enterica*, at different pHs and temperatures.

pH	Temperature (°C)	Thyme EO (mL L_{air}^{-1})	Thymol (mL L_{air}^{-1})	Rosemary EO (mL L_{air}^{-1})	1,8–cineole (mL L_{air}^{-1})
6.0	10	0.13	0.13	1.33	0.67
	15	0.13	0.13	1.33	1.33
	25	0.13	1.80	12.68	9.99
	35	0.20	1.86	13.32	10.65
6.5	10	0.13	0.13	1.33	1.33
	15	0.13	0.13	1.33	1.33
	25	0.33	1.80	13.33	9.99
	35	0.33	1.86	13.33	10.65

[1] average of three replicates.

Table 4. Minimum inhibitory concentrations (MICs[1]) of the thyme and rosemary essential oils and their major components against *Pseudomonas flourescens*, at different pHs and temperatures.

pH	Temperature (°C)	Thyme EO (mL L_{air}^{-1})	Thymol (mL L_{air}^{-1})	Rosemary EO (mL L_{air}^{-1})	1,8–cineole (mL L_{air}^{-1})
6.0	10	0.13	0.13	2.00	1.33
	15	0.13	0.13	2.00	1.33
	25	1.07	1.33	23.97	8.66
	35	1.20	1.79	23.97	9.32
6.5	10	0.13	0.13	2.66	2.00
	15	0.13	0.13	2.66	2.00
	25	1.13	2.00	25.30	14.65
	35	1.20	2.66	25.30	15.98

[1] average of three replicates.

Thyme EO antibacterial activity was not significantly affected by the different pH and temperature conditions; the MIC was the same (against the three bacteria). In the specific case of *L. monocytogenes*, the MIC of thyme EO never changed despite the increase in temperature. Although when it was applied against to *S*. enterica and *P. flourescens*, the concentration did increase as both temperature and pH increased. The effect of thymol was very similar to thyme EO, needing the same concentrations as the EO (pH's 6.0 or 6.5 at 10 or 15 °C) against the three microorganisms, but the MIC increased when the temperature rose up to 25 or 35 °C. Also, the concentration of thyme EO needed to inhibit *S*. enterica and *P. fluorescens* was higher when the pH increased (Tables 2, 3 and 4).

Regarding rosemary EO, the MICs against *L. monocytogenes* were the lowest, followed by the ones against *S*. enterica and then by the ones against *P. fluorescens*. As with thyme EO, the lowest concentrations needed to inhibit the three studied bacteria, were at 10 and 15 °C in both pH's (in almost every condition studied). However, with this EO, when the temperature rose up to 25 or 35 °C (at both pH's) the MIC for the three bacteria,

increased considerably. The same effect was observed for 1,8–cineole, but in lower concentrations.

It is well known, that microorganisms have an optimum pH and temperature for growth, and when this level is moved in either direction (lower or higher), microbial growth could be delayed. Also, when a microorganism is exposed to different factors, such as temperature, pH and/or preservatives, such as EOs or its major components, there is an interaction of factors that affect microbial growth. The results of these interactions could give us an idea of how bacterial growth would behave when exposed to these factors in food systems. However, further research in food systems would be needed to ensure the observed behavior. Based on the different conditions EOs and its components where exposed to in this study, temperature only showed a lower inhibition effect when it increased to 25 and 35 °C. This could be related to the fact that the optimum growth temperature, for the three bacteria, is between 25 – 37 °C. The pH had a lower impact, compared to the temperature; but, in some cases as it increased, the MIC also increased. To summarize, when the temperature and the pH increased, in most of the cases the MIC was higher.

Observing the differences obtained between the MICs of the EOs and the MICs of the major components in this study, it can be deduced that the antibacterial activity of essential oils is related to the interaction between all of their components, and not only due to their major ones. Also, phenolic compounds are known to be the majorly responsible for the antibacterial activity of EOs. In thyme oil, for example, the antimicrobial effect is mostly mediated thymol and carvacrol, both phenolic compounds seem to cause the bacterial cell membrane to become permeable, because they are capable of disintegrating the outer membrane of Gram negative bacteria, making the cytoplasmic membrane more permeable to adenosine triphosphate (ATP) which depolarizes the cytoplasmic membrane (Brenes and Roura, 2010; Lobritz *et al*., 2014). For thyme EO, the antibacterial effect can be attributed mainly to its major component, thymol. But in the case of rosemary EO, the observed antibacterial effects cannot be correlated solely to the presence of 1,8 cineol, since in many of the evaluated conditions the concentration needed to inhibit the evaluated bacteria was higher than those observed for the EO.

Furthermore, it has been reported that Gram negative bacteria are more resistant to EOs than Gram positive bacteria (Soković *et al*., 2010; Han *et al*., 2014; Techathuvanana *et al*., 2014). This was also observed in this study, since L. monocytogenes (Gram positive) was more susceptible to EOs than S. enterica and P. fluorescens (Gram negative). This

effect could be due to the interaction between the cellular membrane of Gram-positive bacteria and the hydrophobic components of the EOs. Whereas Gram negative bacteria show more resistance because of their hydrophilic cell wall, which helps them against penetration of hydrophobic compounds (Rivera Calo *et al*., 2015).

Finally, the bacteriostatic effect refers to the inhibition of cell growth, and the bactericidal effect to cell death (Lobritz *et al*., 2014). In the results obtained, there were only six cases of bacteriostatic effect, most of which were against *L. monocytogenes*: 1) 1,8–cineole MIC in pH 6 at 25 °C, 2) rosemary EO MIC in pH 6.5 at 25 °C, 3) thymol MIC in pH 6.5 at 35 °C and 4) rosemary EO MIC in pH 6.5 at 35 °C. The only bacteriostatic effect against *S*. enterica was for rosemary EO in pH 6.5 at 25 °C, and against *P. fluorescens* was for 1,8 – cineole in pH 6.5 at 35 °C.

3. Materials and methods

3.1 Essential oil and its major components

Essential oils of thyme and rosemary were purchased from Hersol® laboratories (Hersol, Mexico City, Mexico) and the compounds 1,8–cineole and thymol were obtained from Sigma-Aldrich® (Sigma, St. Louis, MO).

3.2 Gas chromatograph/mass spectrometry (GC/MS) analysis

The EOs were analyzed by a gas chromatographer with a 6850 Series Network (Agilent Technologies, Santa Clara CA), equipped with a mass selective detector (5975C VL) and with triple-axis detector (Agilent Technologies). The column, used for the separation of the components, was an HP-5MS (5% phenyl – 95% polydimethylsiloxane) capillary column (30 m by 0.35 mm, 0.25 µm film thickness). Helium was the carrier gas at a constant flow mode of 1.5 mL min-1. The temperature of the column was initially maintained at 60 °C for 10 minutes, then increased every 5 minutes until reaching 240 °C and maintained at 240 °C for 50 minutes. The injector temperature was 240 °C. Retention indices were calculated by a homologous series of n-alkanes C8 to C18 (Sigma, St. Louis, MO) and compounds were found by comparing their retention indices from the US NIST library (National Institute of Standard Technology Library) and with Shimadzu retention index (RI) isothermal equation (Reyes-Jurado *et al*., 2016; Shimadzu, 2017).

$$RI = 100\left(\frac{\log(t_{rs}) - log(t_{rn})}{\log(t_{rn+1}) - log(t_{rn})} + n\right) \qquad \text{(Shimadzu, 2017)}$$

Where t_{rs} is retention time of the target component, t_{rn} is the previous alkane to the target component, t_{rn+1} is the alkane after the target component and n is the carbons number of the alkane t_{rn+1}.

3.3 Microorganisms, culture maintenance and incubation

Bacterial strains (*Salmonella enterica* serovar Typhimurium ATCC 14028, *Listeria monocytogenes* Scott A and *Pseudomonas fluorescens*) were obtained from the Food Microbiology Laboratory strain collection of Universidad de las Americas Puebla (Mexico, Puebla), and were maintained on Trypticasein Soy Agar (TSA; Difco, BD, Sparks, MD) slants at 5 °C.

Cultures were prepared by inoculating the bacteria (*L. monocytogenes*, *S. enterica* or *P. flourescens*) strains into 10 mL of Trypticasein Soy Broth (TSB; Difco, BD, Sparks, MD), and incubated at 35 °C for 24 h. Inoculum cell concentration was adjusted to 10^7 CFU mL^{-1} (Catherine *et al*., 2012; Reyes-Jurado *et al*., 2016).

3.4 Culture medium preparation and inoculation

Culture mediums were prepared with TSA. The pH of the culture medium, was adjusted to the established values (6.0 or 6.5) with hydrochloric acid (Meyer S.A. de C.V., Mexico City, Mexico), using a previously calibrated potentiometer pH10 (Conductronic S.A. de C.V., Mexico City, Mexico). The pH-adjusted culture media were sterilized (15 min at 121 °C), and allowed to solidify in sterile Petri dishes. Afterwards, culture media were inoculated with 50 µL of inoculum of each strain, using a spiral plater Autoplate 4000 (Spiral Biotech, Norwood, MA) (Claxton *et al*., 2001).

3.5 Inverted Petri dish method and incubation

The antibacterial activity was evaluated determining the minimum inhibitory concentration (MIC) using the inverted Petri dish technique. A sterile paper disc (Whatman No. 1, diameter 55 mm) was placed on the lid of the dish and it was impregnated with a known volume of EO or major component. The volumes tested varied from 5 to 1200 µL, depending on the tested bacteria, pH and temperature. Then the culture medium with the paper disc was immediately inverted on top of the lid, sealed with Parafilm® and incubated (Krisch *et al*., 2013; Miladi *et al*., 2013; Kim *et al*., 2016). There were four different combinations of temperature and time of incubation: 1) 35 °C for 24 hours, 2) 25 °C for 48 hours, 3) 15 °C

for 8 days and 4) 10 °C for 9 days. These conditions were selected from previous experiments that corroborate that tested bacteria can grow at the studied temperatures after those incubation times.

MIC refers to the minimum concentration necessary to inhibit the visible growth of the studied strain (López-Malo *et al*., 2005.) and was expressed as mL of EO or compound per L of air. When growth was detected, colony forming units (CFU mL-1) were quantified using the Q-Count counter and software (Spiral Biotech, Norwood, MA). If no growth was observed, the systems were incubated again for the same corresponding period and temperature (35 °C/24 h, 25 °C/48 h, 15 °C/8 days or 10 °C/9 days), removing them from the generated antibacterial atmosphere, through the change of lid with a new sterile one (with non-impregnated paper disc). This was made with the aim of proving a bacteriostatic or a bactericidal effect. If the bacteria grew after the removal, it was reported as bacteriostatic effect, whereas if no growth was found, it was reported as a bactericidal effect (Mejía-Garibay *et al*., 2015). Tests were performed in triplicate.

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Conflict of Interest

No conflict of interest declared.

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RESEARCH PAPER IV

“Vapor phase antibacterial activity, composition and diffusion of allspice, thyme and rosemary essential oils and their major components”

In Progress

Vapor phase antibacterial activity, composition and diffusion of allspice, thyme and rosemary essential oils and their major components

Ana Cecilia Lorenzo-Leal, Enrique Palou, and Aurelio López-Malo

Abstract

Foodborne pathogens are a global concern that could cause outbreaks associated with a great variety of food, and their control is an important issue for regulatory agencies and the food industry. The control of these microorganisms is mainly done through preservatives, which could be classified as chemical or natural antimicrobial agents. Therefore, the aim of this work was to evaluate the vapor phase antibacterial activity, composition and diffusion of allspice, thyme and rosemary essential oils and their major components. Generally lower concentrations of eugenol or eucalyptol were needed to inhibit the growth of the three bacteria, compared to their corresponding EO, while higher concentrations of thymol were needed to achieve the antibacterial effect against the bacteria compared to thyme EO. At higher temperatures, the adsorption percentages of eugenol in allspice EO, thyme in thymol EO and thymol in thymol, were higher, while the adsorption percentage of eugenol in eugenol was lower. The adsorption percentage of eucalyptol in eucalyptol and in rosemary EO did not have a lot of changes.

1.Introduction

Foodborne pathogens are a global concern that could cause outbreaks associated with a great variety of food, and their control is an important issue for regulatory agencies and the food industry. The control of these microorganisms is mainly done through preservatives, which could be classified as chemical or natural antimicrobial agents (Liu *et al*., 2017). Recently, there has been an increase in consumers and in the food industry demand for more natural products as a trend for “clean labels”, substituting chemical preservatives for natural antimicrobials (Anžlovar *et al*., 2014; Davidson *et al*., 2015).

Natural preservatives could be obtained from herbs and species or their extracts and/or essential oils. Essential oils (EOs) are complex mixtures of volatile compounds extracted from different parts of the plant (flowers, roots, bark, leaves, seeds, fruits, wood, among others) and they could have antiseptic, antimicrobial, antiviral, antioxidant, anti-parasitic, antifungal or insecticidal effects (Anžlovar *et al*., 2014; Chouhan *et al*., 2017; Davidson *et*

al., 2015; Giarratana *et al*., 2016). EOs contain different components, of which the main ones represent 85-95% of the total volume, while the other components are known as minority components. (Adelakun, Oyelade and Olanipekun, 2016). Some of the main components in different spices and their corresponding EOS are: eugenol in allspice EO, thymol in thyme EO and eucalyptol in rosemary EO (López-Malo, Barreto-Valdivieso, Palou and San Martin 2006; Jiang *et al*., 2015).

Essential oils extracted from (*Pimenta dioica*), thyme (*Thymus vulgaris*), and rosemary (*Rosmarinus officinalis*) are recognized to have antibacterial effects, in liquid and vapor phase against different microbial strains (Kim *et al*., 2016; Mattos de Oliveira *et al*., 2013). However, when EOs are applied in liquid phase, the organoleptic properties and the antimicrobial activity could be affected due to their strong odor and flavor and their high volatility and hydrophobicity. Compared to liquid phase, vapor phase application could not affect sensorial properties because they are not added directly into food and offers more reliable results when the activity of the volatiles compounds present in EOs are evaluated (Lee *et al*., 2018; Mejía-Garibay *et al*., 2015).

There are some studies that mention the antimicrobial efficacy of different EOs, but only a few mentions the antimicrobial activity EOs and their major components in vapor phase and their diffusion. Therefore, the aim of this work was to evaluate the vapor phase antibacterial activity, composition and diffusion of allspice, thyme and rosemary essential oils and their major components.

2.Material and methods

2.1 Microorganisms

Listeria monocytogenes Scott A, *Salmonella enterica* serovar Typhimurium ATCC 14028 and *Pseudomonas fluorescens* were obtained from the Food Microbiology Laboratory of Universidad de las Americas Puebla (UDLAP, Mexico, Puebla). and were maintained on slants of Trypticasein Soy Agar (TSA; Difco, BD, Sparks, MD) at 4 °C.

Inoculum were prepared by inoculating each microorganism (*L. monocytogenes, S. Thyphimurium* or *Pseudomonas fluorescens*) into 10 mL of Trypticasein Soy Broth (TSB; Difco, BD, Sparks, MD) and incubated at 35 °C for 24 h. Inoculum cell concentration was adjusted to 10^7 CFU/mL (Catherine *et al*., 2012; Reyes-Jurado *et al*., 2016).

2.2 Essential oils and major components

Thyme and rosemary essential oils were purchased from Hersol® laboratories (San Mateo Atenco, Estado de México, Mexico), and allspice (*Pimenta dioica*) essential oil was obtained from Liquid Gold® (Evansville, IN). Eugenol, thymol and eucalyptol were obtained from Sigma-Aldrich® (Sigma, St. Louis, MO).

The chemical analysis of the EOs were analyzed by gas chromatography equipped with a mass spectrometer as published (Lorenzo-Leal *et al.,* 2019).

2.3 Vapor phase antibacterial activity *in vitro*

2.3.1 Culture medium

TSA was prepared adjusting its pH values (6.0 or 6.5) using hydrochloric acid (Meyer S.A. de C.V., Mexico City, Mexico), with a previously calibrated potentiometer pH10 (Conductronic S.A. de C.V., Mexico City, Mexico). Then, sterilized TSA was poured in sterile Petri dishes and allowed to solidify overnight. Subsequently, a spiral plater Autoplate 4000 (Spiral Biotech, Norwood, MA) was used to inoculate culture mediums, applying 50 µL of inoculum of each bacteria (Claxton *et al*., 2001).

2.3.2 Inverted Petri dish method

The antibacterial activity was evaluated through the inverted Petri dish technique, which consists in placing a sterile paper disc (Whatman No. 1, diameter 55 mm) impregnated with a known volume of each EO or major component (that varied from 5 to 2000 µL), on the Petri dish lid. Culture mediums were then immediately inverted on top of the lid, sealed with Parafilm® and incubated at different temperatures and time periods: 1) 35 °C for 24 hours, 2) 25 °C for 48 hours, 3) 15 °C for 8 days and 4) 10 °C for 9 days (Krisch *et al*., 2013; Miladi *et al*., 2013; Kim *et al*., 2016). The incubation conditions mentioned, were selected from experiments made to corroborate that the microorganisms of study could grow.

The results obtained from each EO or major component were expressed as the minimum inhibitory concentration (MIC), which means minimum concentration necessary to inhibit the visible growth of the studied microorganisms (López-Malo *et al*., 2005). MICs were expressed as mL of EO per L of air. The quantification of colony forming units (CFU mL^{-1}) were made by a Q-Count counter and software (Spiral Biotech, Norwood, MA), when growth was observed. Tests were performed in triplicate.

2.3.3 Determination and quantification of essential oil major components

The chemical analysis of the EOs was performed using gas chromatographer 6850N (Agilent Technologies, Santa Clara, CA), equipped with a 5975C mass spectrometry detector, and with triple-axis detector (Agilent Technologies) following published protocols (Lorenzo-Leal *et al.,* 2019).

Eugenol (purity 98%), thymol (purity 99.5%) and eucalyptol (purity 99%), were studied as standards to quantify its concentrations in the studied EO's, allspice, thyme and rosemary (respectably), and in eugenol, thymol and eucalyptol when they were used to determine their antimicrobial activity. A calibration curve was made by plotting the peak areas determined for different eugenol (1.05, 0.52, 0.27, 0.13 and 0.06 mg/mL), thymol (0.91, 0.45, 0.23, 0.11 and 0.06 mg/mL) and eucalyptol (0.95, 0.47, 0.24, 0.12 and 0.06 mg/mL) concentrations. The solvent used to prepare the solutions from the standard was ethanol (Mejía-Garibay *et al*.,2015).

2.3.4 Quantification of components in culture mediums

Major components (eugenol, thymol or eucalyptol) quantifications were made through the absorption of each EOs (allspice, thyme or rosemary) or component (eugenol, thymol or eucalyptol) in the TSA contained in the culture medium (after their exposure to EOs or components vapors). The exposed agar, was removed from the Petri dish and dissolved in 10 mL of ethyl acetate (Baker SOLUSORB, Avantor Performance Materials, Mexico). The mixture of agar and ethyl acetate was stirred (120 rpm) for 1 h, and filtrated with a 0.45 µm membrane filter, to inject 1 mL of the solution into the gas chromatographer. The quantification was performed with eugenol, thymol or eucalyptol as major components present in the culture mediums by using the previously obtained standard curve, to determine the diffusion of the allspice, thyme or rosemary EOs and eugenol, thymol and eucalyptol into the TSA (Mejía-Garibay *et al*.,2015).

2.3.5 Statistical analysis

Balanced ANOVA comparison tests ($p \leq 0.05$), was used for the statistical analysis of the vapor phase antimicrobial effect obtained data. Statistical analysis of the data obtained from the Quantification of components in culture mediums was performed by ANOVA and Tukey's mean comparison tests. For both analysis Minitab statistical package (Minitab 17, Minitab Inc., College, PA) was used.

3. Results and discussion

3.1 Chemical composition of essential oils

According to the chemical analysis of the EOs previously published (Lorenzo-Leal *et al.,* 2019). Eugenol represented the major component in allspice EO, while m-Cymene and thymol were the major ones in thyme EO and in the rosemary EO, the main components were α-Pinene, eucalyptol and camphor. Stewart *et al*. (2016), report that allspice EO contains 61.2% of eugenol, 4.6% β-caryophyllene, and 1.9 % of α-humulene. According to Giarratana *et al* (2016), rosemary EO main components were 23.98 % of α-pinene, 22.62 % of camphor and 18.76 % of eucalyptol, and regarded to thyme EO, Gavaric *et al*. (2015), found that thymol was the major component (41.6%) followed by carvacrol (12.0%) and borneol (8.9 %), all of which coincide with the results obtained in the present work. Also, the antimicrobial activity of terpenes, such as p-Cymene and α-Pinene, is not as effective as the one showed by terpenoids (Hyldgaard *et al*., 2012). Therefore, in this study, we decided to use as major component in thyme EO, thymol and in rosemary EO eucalyptol.

The quantitative analysis confirmed the concentration of eugenol in allspice EO was 823.51 mg/mL, 368.46 mg/mL of thymol in thyme EO, 479.59 mg/mL of eucalyptol in rosemary EO, 927.35 mg/mL of eugenol in eugenol, 953.28 mg/mL of thymol in thymol and 916.76 mg/mL.

3.2 Vapor phase antibacterial activity *in vitro*

Table 1 shows the essential oils and major components different concentrations that probed their antibacterial effect against *L. monocytogenes, S.* Typhimurium or *P. fluorescens*. Thyme EO and its major component thymol, presented the strongest antibacterial activities against the three studied microorganisms, compared to allspice or rosemary EOs and their major components eugenol and eucalyptol.

Table 1. Minimum inhibitory concentrations (MICs) of allspice, thyme or rosemary essential oils and their major components eugenol, thymol and eucalyptol to inhibit the growth of *L. monocytogenes, S.* Typhimurium or *P. fluorescens*.

Microorganisms	pH	T (°C)	MIC (mL L of air^{-1})					
			Allspice EO	Eugenol	Thyme EO	Thymol	Rosemary EO	Eucalyptol
L. monocytogenes	6	10	0.93	0.13	0.13	0.13	0.40	0.40
		15	0.93	0.40	0.13	0.13	0.40	0.40
		25	3.06	2.40	0.13	0.40	2.40	2.00
		35	3.33	3.99	0.13	0.40	3.99	2.66
	6.5	10	1.33	0.40	0.13	0.13	0.40	0.40
		15	1.60	0.40	0.13	0.13	0.40	0.40
		25	11.98	7.99	0.13	0.53	2.66	2.66
		35	13.32	7.99	0.13	0.53	4.66	3.33
Salmonella	6	10	4.66	1.33	0.13	0.13	1.33	0.67
		15	4.66	1.33	0.13	0.13	1.33	1.33
		25	11.32	5.99	0.13	1.80	12.68	9.99
		35	9.32	6.66	0.2	1.86	13.32	10.65
	6.5	10	5.33	2.00	0.13	0.13	1.33	1.33
		15	6.66	2.00	0.13	0.13	1.33	1.33
		25	13.32	7.99	0.33	1.80	13.33	9.99
		35	17.31	7.99	0.33	1.86	13.33	10.65
P. fluoresces	6	10	4.66	1.33	0.13	0.13	2.00	1.33
		15	4.66	1.33	0.13	0.13	2.00	1.33
		25	15.98	11.32	1.07	1.33	23.97	8.66
		35	14.65	11.98	1.20	1.79	23.97	9.32
	6.5	10	5.33	2.00	0.13	0.13	2.66	2.00
		15	6.66	2.00	0.13	0.13	2.66	2.00
		25	17.31	13.32	1.13	2.00	25.30	14.65
		35	17.98	13.32	1.20	2.66	25.30	15.98

Generally lower concentrations of eugenol or eucalyptol were needed to inhibit the growth of the three bacteria, compared to their corresponding EO, while higher concentrations of thymol were needed to achieve the antibacterial effect against the bacteria compared to thyme EO. Related to the obtained results, it can be deduced that the antibacterial effects cannot be correlated solely to the presence of major components, since

in most of the evaluated conditions, the concentration of the major components needed to inhibit the bacterial growth were different than those observed for the EOs. According to Chouhan *et al.* (2017), the presence of major components may not be the only responsible factor for the antimicrobial activity of EOs, but it is also important the interaction between these and the minor components

The main component of tested allspice EO was eugenol (representing 89.55% of the total EO), which has been used to protect foods from different microorganisms during storage, and it has been reported as an effective antibacterial against *Bacillus cereus, B. subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhi* trough the inhibition of amino acid decarboxylase, the interruption of amylases and proteases production, and by the deterioration of the cell wall (Lee *et al*., 2018). The tested thyme EO major component was thymol, which also has demonstrated antibacterial effects against *B. subtilis, E. coli, Klebsiella pneumonia* and *S. aureus*; this compound causes lipid perturbation in the microbial plasma membrane and penetrates the microorganism's cell to exert antimicrobial effects (Lee *et al*., 2018). The tested rosemary EO main component was 1,8 – cineole (or eucalyptol). Eucalyptol's antimicrobial effect has been attributed to its lipophilic character; hence: it will enter the membrane structures, resulting in membrane expansion, enhanced permeability and fluidity; furthermore, it makes difficult iron transport processes and inhibits respiration (Zengin and Baysal, 2014).

The three EOs and their major components, were more effective against *L. monocytogenes*, despite the pH (6.0 or 6.5) or the evaluated temperatures (10, 15, 25 or 35 ° C), compared to *S.* Typhimurium or *P. fluorescens*. Which could be related with the fact that Gram-positive (*L. monocytogenes*) bacteria response to plant extracts, such as EOs, is greater than the one of Gram-negative bacteria (*S.* Typhimurium *and P. fluorescens*). Gram-negative bacteria resistance to EOs has been related to the presence of lipopolysaccharide in their cell wall which prevents hydrophobic compounds diffusion, while Gram-positive bacteria, have lipophilic ends of lipoteichoic acid in their cell wall which could ease the hydrophobic compounds infiltration (Chouhan *et al*., 2017; Gayawali *et al*., 2015; Han *et al*., 2014; Soković *et al*., 2010; Techathuvanana *et al*., 2014; Zengin and Baysal, 2014).

Antimicrobial activity of allspice EO in vapor phase against *L. monocytogenes, Escherichia coli O157:H7* and *Salmonella enterica* was tested by Du *et al*. (2009), where *L. monocytogenes* showed less resistant to the vapor of the EO than the other studied microorganisms, which also probes the effect of EOs on Gram-negative and Gram-positive

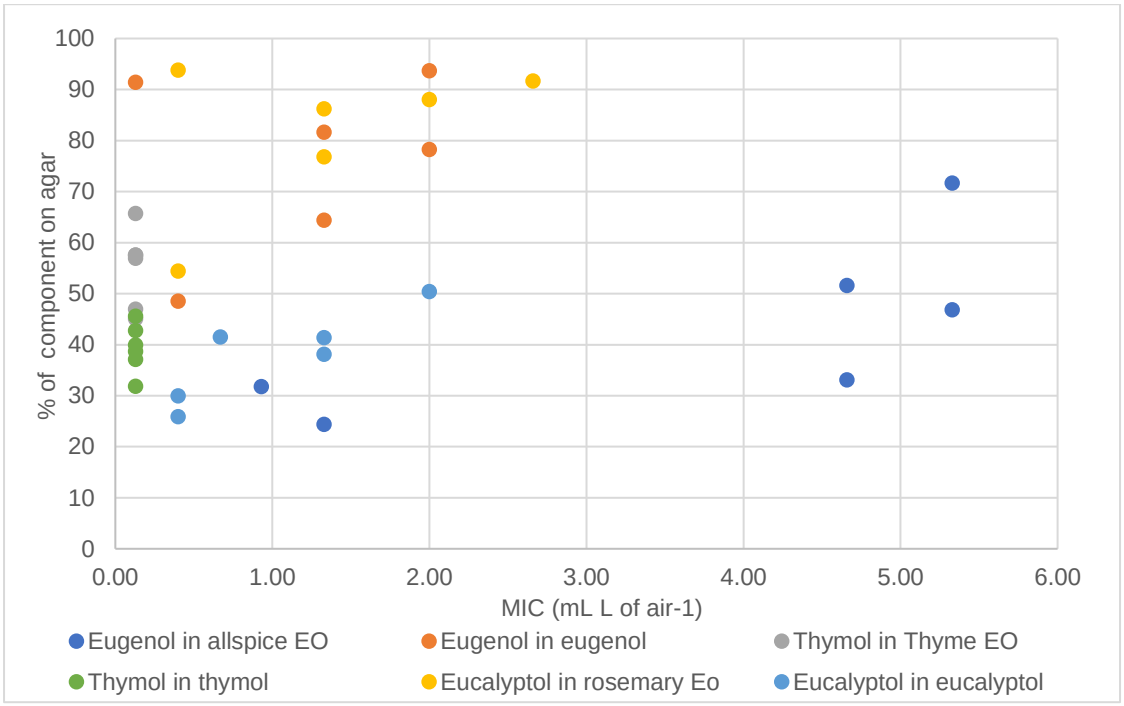
bacteria. Related to thyme and rosemary EOs, Han *et al*. (2014), probed the inhibitory effect of the released volatiles (of the mentioned EOs) against *L. monocytogenes*, where thyme EO was more effective as antibacterial than rosemary EO, coinciding with our findings

On the other hand, pH levels did not have an important impact on the antimicrobial effect of the essential oils or major components, but different temperature conditions did have an impact on the antimicrobial effect; specially when the temperature increase up to 25 and 35 °C, as shown in Table 2. Microorganisms are known to have an optimum pH and temperature for their growth, and when these conditions move in any direction (lower or higher), microbial growth could be stop or delayed. Furthermore, the interaction between different factors such as: temperature, pH, antimicrobials (essential oils), between others, could affect microbial growth. In summary, when the temperature and pH increased, in most cases the MICs were higher. When allspice EO was tested to inhibit the growth of the three studied bacteria, the pH and the temperature had a significant ($p \leq 0.05$) impact in the resulted MICs. In the case of major components eugenol, thymol and eucalyptol and the rosemary EO only the temperature had a significant ($p \leq 0.05$) impact, while thyme EO did not have a significative ($p \geq 0.05$) impact of the factors (temperature or pH) over its resulted MICs.

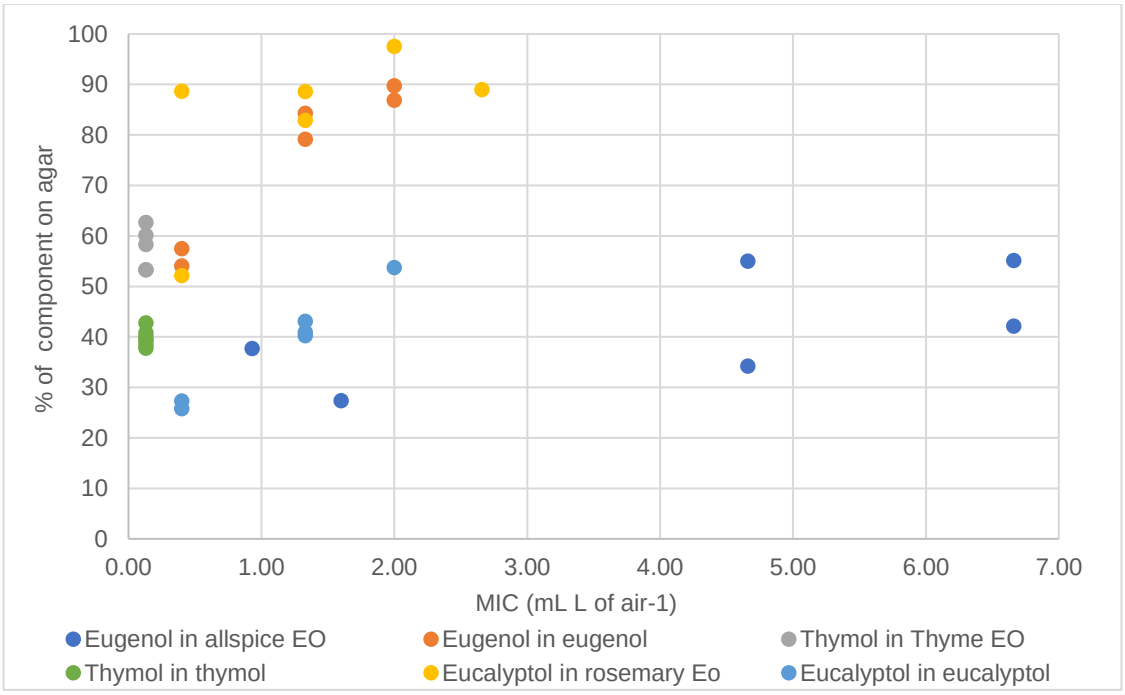
3.3 Quantification of major components in culture mediums

The quantity of each component (eugenol, thymol or eucalyptol) absorbed into TSA, was made after the exposure to the vapors of allspice, thyme and rosemary EOs and eugenol, thymol and eucalyptol. Therefore, the MICs of each EO or component were the studied concentrations where the measurement of the diffusion of the components was made, and the results are presented in Figure 1.

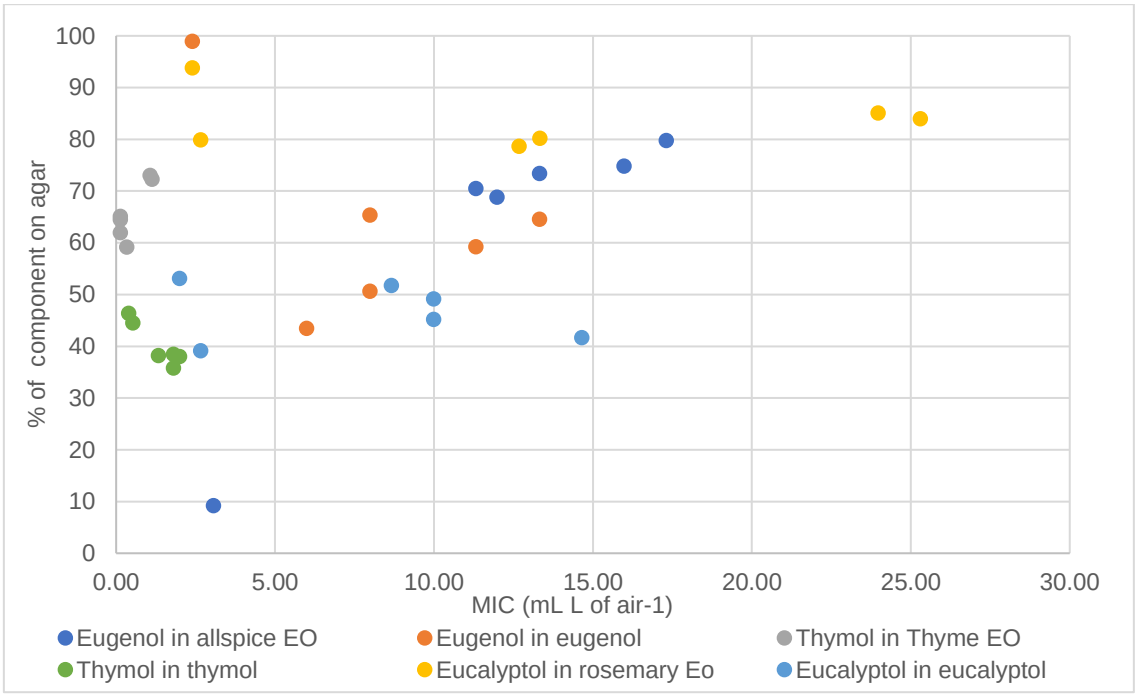
a)


100
90
80
70
60
50
40
30
20
10
0
% of component on agar
0.00
1.00
2.00
3.00
4.00
5.00
6.00
MIC (mL L of air-1)
Eugenol in allspice EO
Eugenol in eugenol
Thymol in Thyme EO
Thymol in thymol
Eucalyptol in rosemary Eo
Eucalyptol in eucalyptol

b)


100
90
80
70
60
50
40
30
20
10
0
% of component on agar
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
MIC (mL L of air-1)
Eugenol in allspice EO
Eugenol in eugenol
Thymol in Thyme EO
Thymol in thymol
Eucalyptol in rosemary Eo
Eucalyptol in eucalyptol

c)



d)

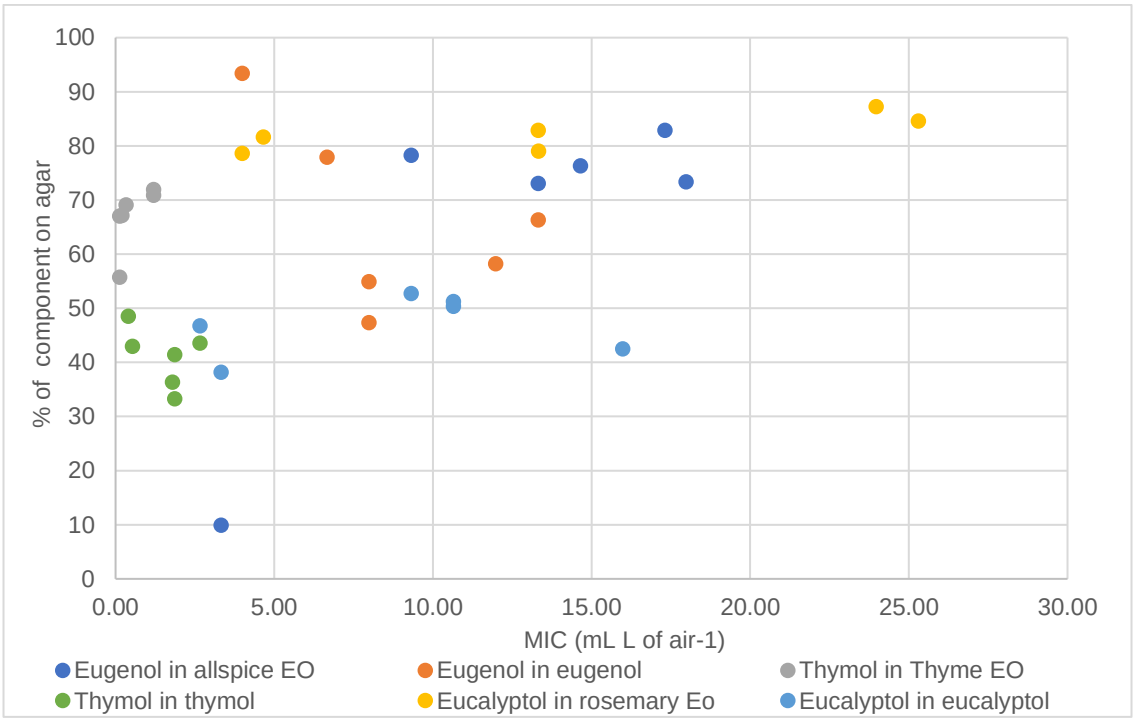


Figure 1. Percentage of eugenol, thymol and eucalyptol on agar of allspice, thyme or rosemary EOs and their major components eugenol, thymol or eucalyptol in their respective concentrations (mL L of air^{-1}) after the vapor phase exposure at different temperatures, a) at 10°C, b) at 15°C, c) at 25 °C and d) at 35 °C.

As explained before, temperature had an important impact in the MICs required to inhibit bacterial growth, and as it can be seen in Figure 1, temperature also generate differences on the diffusion of the components. At higher temperatures, the adsorption percentages of eugenol in allspice EO, thyme in thymol EO and thymol in thymol, were higher, while the adsorption percentage of eugenol in eugenol was lower. The adsorption percentage of eucalyptol in eucalyptol and in rosemary EO did not have a lot of changes. However, no significant ($p \geq 0.05$) impact of the temperature was found in the adsorption percentage of the different components. Also, the different pH in the culture media did not significantly ($p \geq 0.05$) affected the adsorption percentage of the components but the different EOs and components studied, did have a significative ($p \leq 0.05$) impact in their respective adsorption percentage as expected.

In the results obtained in this work, generally, the percentage of eugenol absorbed into the agar was higher than thymol. When thymol was studied alone, the percentage of the component on agar was lower than when it was measured in thymol EO, opposite to the results of eugenol. The adsorption of eucalyptol, was higher than the other two components in most of the cases and as thymol, the percentage of component on agar was lower than when it was measured in thymol EO (Figure 1). Related to this, Suhr and Nielsen (2003) mention that thymol is a smaller, more volatile and is more effective as antimicrobial compared with eugenol, which coincides with our findings in this study.

Differences in the diffusion of the components could be due to factors like volatility, molecular weight, temperature, among other. Also, volatile compounds could have been lost during the incubation time (despite the systems were sealed with parafilm).

Preliminary conclusion

It was observed that temperature and pH do not have a significant effect on the diffusion of the major components. However, the diffusion of the components, in most cases, was higher when the diffusion of essential oils was tested compared to the diffusion of the major components when they were used individually.

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GENERAL CONCLUSIONS

- The vapors of allspice, thyme, and rosemary essential oils probed to be effective for their use as natural preservatives, inhibiting the growth of different microbial strains. When the essential oils were tested against bacteria, they were more effective inhibiting the growth of Gram-positive bacteria compared to Gram-negative bacteria.
- The most effective essential oil to inhibit the growth of the pathogenic bacteria *L. monocytogenes* and *S.* Typhimurium were thyme, followed by rosemary and allspice essential oils. In addition, when the essential oils were tested against molds and yeasts, allspice essential oil was more effective than rosemary essential oil.
- This study demonstrates that the use of essential oils is an effective alternative for pathogenic bacterial and fungal control
- Moreover, the oil extracted from Rosmarinus officinalis could be used as potent anti-inflammatory agent.
- Vapors of allspice, thyme and rosemary essential oils, could be used to disinfect alfalfa seeds, without having any problems in the process of seed germination to obtain alfalfa sprouts.
- Regarding the general acceptance of 51 untrained panelists of the alfalfa sprouts, there was no significant difference between the sprouts obtained from untreated seeds and the sprouts obtained from seeds treated with essential oils.

GENERAL RECOMENDATIONS

- Design a new method or apparatus to perform treatments with vapors of essential oils in food products.
- Evaluate the quality of the seed use to generate the sprouts, before and after the treatments with essential oils vapors.
- Evaluate the effectiveness of the essential oils vapors as natural preservatives in alfalfa seeds against other microbial strains.
- Research on the residual effect of essential oils on alfalfa sprouts.
- Evaluate the diffusion of the major components of essential oils, when they are applied in vapor phase to inhibit microbial growth in alfalfa seeds.

ANNEXES

ANNEXE I. Evidence of other publications

Vapor phase antibacterial effect of ROSEMARY (*ROSMARINUS OFFICINALIS*) ESSENTIAL OIL and its major component at selected pH's and temperatures

Por: Ana Cecilia Lorenzo-Leal · Enrique Palou · Aurelio López-Malo

ABSTRACT

Consumer demand for natural preservatives, such as essential oils (EOS) from plants has increased over the years. Therefore, the aim of this study was to evaluate the vapor phase antibacterial effect of rosemary essential oil (EO) and its major component (1,8-cineole), against *Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes*, or *Pseudomonas fluorescens* at selected pHs and temperatures. The minimum inhibitory concentrations (MICS) of the EO and 1,8-cineole were determined at different pH's and temperatures in culture mediums; resulting that in most of the cases *L. monocytogenes* exhibited less resistance to tested natural antimicrobials when compared to *S. enterica* and *P. fluorescens*.

KEY WORDS:

Antibacterial effect · Vapor phase · Rosemary · 1,8-cineole · Essential oils.

INTRODUCTION

Bacteria are the main cause of foodborne illnesses and difficult to detect and control by the food industry (USDA, 2012). Foodborne illnesses are important health concerns for governments, nowadays commonly attributed to minimally (or not appropriately) processed (or stored) food products.

Chemical preservatives have been used as food antimicrobials for a long time; however, consumer search for food products with «clean» labels has increased over recent years. Therefore, there is an increasing need to find natural preservatives such as herbs and spices, and/or their extracts or essential oils (Hyldgaard *et al.*, 2012). Essential oils (EOS) are defined as mixtures of volatile and aromatic compounds. EOS could be extracted from different parts of plants, where 85-95% of the main volume of EOS is constituted by their major components (Burt, 2004). Among several alternative natural preservatives are EOS extracted from rosemary (Rosmarinus officinalis), which have shown antimicrobial activity against different microbial strains, when applied either in liquid or vapor phase.

Though, when EOS are applied in the liquid phase (directly to the food) they usually have a significant impact on food sensory attributes, because of their strong aromas and flavors. In contrast, vapor phase application (indirectly) of EOS typically requires lower concentrations for their use as antimicrobials; thus, vapor phase application could be a solution to the adverse effects of the intense aroma and flavor of applying EOS directly to foods. Several culture medium (in vitro) studies have helped to simulate the antibacterial behavior of selected EOs in food systems (Mejía-Garibay *et al.*, 2015). Thus, the aim of this study was to evaluate the vapor phase antibacterial effect of rosemary essential oil and its major component (1,8-cineole) against three different bacteria at selected pHs and temperatures.

Lorenzo-Leal A. C., Palou E. y López-Malo A. (2018). Vapor phase antibacterial effect of rosemary (Rosmarinus officinalis) essential oil and its major component at selected pH's and temperatures. Entorno UDLAP, 1, 28-40. **Recibido:** 5 de febrero de 2018. **Aceptado:** 20 de febrero de 2018.

METHODOLOGY

Rosemary essential oil was obtained from Hersol® laboratories (Hersol, Mexico City, Mexico) while its major component (1,8-cineole also known as eucalyptol) was obtained from Sigma-Aldrich® (Sigma, St. Louis, MO, USA). Tested essential oil was analyzed by a gas chromatographer coupled to a mass selective detector (GC/MSD, Thermo Fisher Scientific Inc., Waltham, MA, USA); compounds were identified by comparing their retention indices with the US National Institute of Standard Technology Library and with Shimadzu retention index (RI) isothermal equation.

WHEN EOS ARE APPLIED IN THE LIQUID PHASE THEY USUALLY HAVE A SIGNIFICANT IMPACT ON FOOD SENSORY ATTRIBUTES, BECAUSE OF THEIR STRONG AROMAS AND FLAVORS.

Salmonella enterica serovar Typhimurium ATCC 14028, Listeria monocytogenes Scott A, and Pseudomonas fluorescens were obtained from the Universidad de las Américas Puebla Food Microbiology Laboratory strain collection. They were maintained on Trypticase soy agar (TSA, Merck, Germany) slants at 5 °C. Cultures were prepared by inoculating the bacteria strains into 10 mL of Trypticase soy broth (TSB, Merck, Germany), incubated at 35 °C for 24 h, and adjusted to a cell concentration to 10⁷ CFU mL-1.

Culture mediums were prepared with tsa adjusting its pH (6.0 or 6.5) with hydrochloric acid, then sterilized (15 min at 121 °C), and allowed to solidify in sterile Petri dishes. Subsequently, sterilized culture media were inoculated with 50 µL of inoculum of each bacteria strain, using a spiral plater (Spiral Biotech, Inc., Norwood, MA, USA).

Minimum inhibitory concentration (MIC) refers to the minimum concentration necessary to inhibit the visible growth of the studied strain (López-Malo *et al.*, 2005), and it was utilized to evaluate the antibacterial activity of tested EO by means of the inverted Petri dish technique. This method consists in placing a sterile paper disc (Whatman No. 1), on the lid of the Petri dish, impregnated with a known volume of the tested EO or its major component. The volumes tested varied from 5 to 1900 µL, depending on the studied combinations of bacteria, pHs, and temperatures. The culture medium with the paper disc was immediately inverted on top of the lid, sealed with Parafilm® and incubated as follows: 1) at 35 °C for

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14

Turpentine or Pine Oil

Emma Mani-López, Ana C. Lorenzo-Leal, Enrique Palou, and Aurelio López-Malo

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10

Principles of Sensory Evaluation in Foods Containing Essential Oil

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10.1 Introduction

One of the most important goals of the food industry is to understand how food affects consumer's senses and acceptance. That is why consumer reaction, perceived by the five senses, is considered a vital measure in food development. Since senses can't be substituted with any apparatus for food analysis, humans are utilised as test subjects during several product development steps, especially for evaluating product quality and acceptability (Edelstein, 2014).

The term 'organoleptic testing' was first used to detonate an objective measurement of sensory attributes in the late 1990s, but in reality those tests were subjective, had few judges (tasters) and several interpretations were open to bias. In the early 1900s, ritualistic schemes for grading tea, wine, coffee, fish, butter and meat gave rise to professional tasters and consultants to beverage, food and cosmetic industries. Through the years, scientists have developed, formalised, structured and codified methodology for sensory analysis, continuing the development of new methods and refining existing ones (Meilgaard *et al.*, 2016).

Sensory analysis is known to apply the scientific method to identify, measure, evoke, analyse and interpret perceived attributes of a product. Like other assessable methods, sensory analysis is permanently implicated with precision, accuracy and sensitivity, as well as preventing false-positive results (Lawless & Heymann, 2010; Stone *et al.*, 2012; Edelstein, 2014). For a reliable sensory test, the sensory analyst has to fine-tune the following four factors: define the problem or item to be measured, design the appropriate test by minimising the number of tests required to produce the expected result, train the panelists (judges) and interpret the results by applying appropriate statistics (Edelstein, 2014).

Generally, panelists in sensory evaluations are usually placed in individual booths, tables or cubicles, so that the judgements given are theirs and do not imitate the choices of others. Samples are identified with random numbers and in a uniform fashion, with

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EVALUACIÓN DE LAS PROPIEDADES FÍSICAS, QUÍMICAS Y FUNCIONALES DE CLARA DE HUEVO FRESCA Y SECADAS POR ASPERSIÓN

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Resumen
La clara de huevo es un alimento alto en proteínas, dichas que le confieren propiedades que son importantes en la industria alimentaria. Este producto al ser sometido a tratamientos como el secado por aspersión, puede mejorar su conservación y algunas propiedades. Es por esto que el objetivo de este trabajo fue evaluar las propiedades físicas, químicas y funcionales de clara de huevo, obtenida de huevo comercial o de granja, frescas y secadas por aspersión. Se compararon cuatro muestras de clara: frescas, de granja (CG), comercial (CC), y las secadas por aspersión, de granja (PG) y comercial (PC). A éstas se les midieron los siguientes parámetros: color, densidad, contenido de humedad, proteínas, a_w, estabilidad y formación de espuma. Las muestras de polvo sólo presentaron cambios ($p < 0.05$) en el parámetro a* (rojo) de color. El pH, la a_w y la humedad se encontraron dentro de los valores establecidos por la NMX-F-330-S-1979. El tamaño de partícula, la densidad compactada y la densidad de bulto no mostraron diferencias significativas ($p > 0.05$) entre PG y PC. El contenido de proteína si tuvo una reducción significativa después del proceso de secado en la CG y CC, sin embargo, las propiedades funcionales de espumado no fueron diferentes entre PG y PC. Los resultados en las propiedades físicas y funcionales de los dos productos secados por aspersión fueron similares, por lo que las condiciones del secado por aspersión demuestra ser una alternativa para mejorar la conservación del producto.

Introducción
La clara de huevo está constituida, en su mayoría, por una solución de proteínas globulares con fibras, ricas en aminoácidos esenciales; es empleada como ingrediente en la industria de alimentos debido a las diferentes propiedades funcionales que confiere como el espumado y la gelificación. La clara de huevo en polvo, obtenido por secado por aspersión, podría ser una alternativa en la industria de alimentos, ya que permite una mejor conservación y manejo a diferencia del producto fresco. Sin embargo, es importante conocer el comportamiento del producto al ser sometido a un proceso de secado, ya que éste podría tener cambios en las propiedades físicas, químicas, funcionales o sensoriales del producto [1, 2].
El objetivo de este trabajo es evaluar las propiedades físicas (color, dimensiones, densidad), químicas (contenido de humedad y proteínas, a_w) y funcionales (estabilidad y formación de espuma) de la clara de huevo, obtenida de huevo comercial y de granja, fresca y por secado por aspersión.

Metodología
Materiales
Se emplearon huevo comercial (San Juan®) y de granja (Puebla, Pue.). Dichas muestras de huevo, fueron utilizadas sin exceder una semana de almacenamiento, a 5±2 °C. La clara de granja (CG) y clara comercial (CC) se separaron manualmente de la yema de huevo de origen.

Secado por aspersión
La CG y CC se añadieron (individualmente) en una solución al 20% (p/p) de maltodextrina 10ED (CP Ingredientes, México), en una proporción 1:2. El secado de CG y CC se llevó a cabo en un secador por aspersión (Büchi B-290, Suiza) utilizando una temperatura del aire de entrada 135 °C, temperatura del aire de salida 95.91±4.63 °C, velocidad de flujo de la solución de 0.38 L/h.

Seguridad Alimentaria: Aprovechamiento integral y calidad microbiológica de alimentos.

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Capítulo 4 Inhibición de bacterias patógenas y causantes de deterioro mediante aceites esenciales de tomillo (*Thymus vulgaris*), romero (*Rosmarinus officinalis*), o sus componentes mayoritarios en fase de vapor

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Resumen

Los conservantes sintetizados químicamente se han empleado como antimicrobianos alimenticios durante mucho tiempo. Sin embargo, la demanda por parte del consumidor de productos más naturales ha aumentado a lo largo de los años; por lo que surge la necesidad de encontrar fuentes naturales de antimicrobianos, como aceites esenciales (AEs). Por lo tanto, el objetivo de este estudio es evaluar la actividad antibacteriana en fase vapor del aceite esencial de tomillo, romero, o sus componentes mayoritarios timol o eucaliptol contra *Salmonella* Typhimurium, *Listeria monocytogenes* o *Pseudomonas fluorescens* en diferentes condiciones de pH y temperatura. Se determinó la concentración mínima inhibitoria (CMI) de los dos AEs y sus componentes mayoritarios a diferentes niveles de pH (6.0 o 6.5) y temperatura (25 o 35 ° C) en sistemas modelo. La CMI más baja encontrada fue de 0.13 mL L_{aire}^{-1} para el AE de tomillo contra las tres bacterias, y el más alto fue 25.30 mL L_{aire}^{-1} para el AE de romero contra *P. fluorescens*. En la mayoría de los casos, *L. monocytogenes* mostró menos resistencia a los AEs en comparación con *S.* Typhimurium y *P. fluorescens*, lo cual era de esperarse, ya que las bacterias Gram negativas (*Salmonella* y *P. fluorescens*) son más resistentes a los AEs que Gram positivas (*L. monocytogenes*).

Palabras clave:. Aceites esenciales, timol, eucaliptol, fase vapor, pH, temperatura.

Introducción

Los conservadores sintetizados químicamente, se han empleado desde hace mucho tiempo como antimicrobianos en la industria de alimentos, con la finalidad de alargar la vida útil de una gran diversidad de productos alimenticios. Sin embargo, la demanda de productos más naturales por parte de los consumidores ha aumentado, surgiendo la necesidad de encontrar fuentes de conservadores naturales, como es el caso de hierbas y especias, o sus extractos y aceites esenciales (Škrinjar y Nemet, 2009; Rodríguez Sauceda, 2011).

Una fuente alternativa de conservadores naturales son los aceites esenciales de tomillo (*Thymus vulgaris*) y romero (*Rosmarinus officinalis*). Dichos aceites esenciales han probado tener efectos antimicrobianos contra diferentes cepas, tales como: *Listeria monocytogenes, Staphylococcus aureus, Staphylococcus epidermidis, Micrococcus luteus, Bacillus cereus,*

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The aim of the Workshop: Technology, Science, and Culture: A Global Vision is to create a discussion forum on research related to the fields of Water Science, Food Science, Intelligent Systems, Molecular Biomedicine, and Creation and Theories of Culture. The workshop is intended to discuss research on current problems, relevant methodologies, and future research streams and to create an environment for the exchange of ideas and collaboration among participants.

This first edition of the workshop was held on November 6, 2018, at Universidad de las Americas Puebla. In this edition, we had four keynotes and nine posters presented in the poster session, which aimed to show selected research from doctoral students. At the end of the workshop, the best poster was awarded.

Keynote speakers are researchers with recognized trajectories, who have published in leading academic and scientific journals. In this edition, the invited speakers were: Dr. Horacio Bach, Dr. Andreas Linninger, Dr. Miguel Ángel Rico-Ramírez, and Dr. Theodore Gerard Lynn.

During keynotes, Dr. Horacio Bach discussed the problem of multidrug resistant bacteria and the lack of R&D in the development of new antibiotics in pharmaceutical companies. In his talk, Dr. Andreas Linninger focused on mathematical modeling, and he proposed a definition, explained applications on chemistry and biochemistry, and emphasized the benefits of viewing math as a language for scientific inquiry and math education. In his turn, Dr. Rico-Ramírez explained the importance of measuring and forecasting precipitations; he also discussed latest advances of the measurement and forecasting of precipitations with weather radars. Finally, Dr. Theodore Lynn explained the importance of Intelligent Systems in the Internet of Everything; he explained the building blocks of Intelligent Systems and research opportunities.

In the first edition of this workshop, the best poster was awarded to Omar López Rincón, a student of Intelligent System Doctorate, who presented his work "A 3D Spatial Visualization of Measures in Music Compositions."

The number and impact of water-related natural disasters have increased since the middle of last century. As a result of increased climate variability and the effects of global warming, the hydrometeorological risk has increased and spread, while the resilience of societies, in many cases, is not adequate. Consequently, the risk has increased. Floods and droughts, particularly in a changing climate, require greater understanding to generate better forecasts and proper management of these phenomena. Mexico, like other countries in the world, and of course in Latin America

Chapter

Extraction, Composition, and Antibacterial Effect of Allspice (*Pimenta dioica*) Essential Oil Applied in Vapor Phase

Ana Cecilia Lorenzo-Leal, Enrique Palou and Aurelio López-Malo

Abstract

The aim of this study was to extract and evaluate the composition and antibacterial effect of allspice (*Pimenta dioica*) essential oil applied in vapor phase against *Salmonella* Typhimurium, *Listeria monocytogenes*, and *Pseudomonas fluorescens* at selected levels of pH and temperature. Microwave assisted extraction (MAE) was tested at different conditions, and it was found that the best extraction conditions were ground allspice, allspice:water relation 1:20, 90 min of soaking before extraction, 800 W for 30 min, and 600 W for 20 min. Antibacterial activity was determined through the minimal inhibitory concentration (MIC) of EO in vapor phase in culture media. Allspice essential oil (AEO) was more effective against *L. monocytogenes* despite the pH or temperature level, compared with *S.* Typhimurium and *P. fluorescens*. Allspice essential oil was able to inhibit the growth of the three bacteria tested, and it was found that both the incubation temperature and pH are the factors that could influence the inhibitory effect of the EO tested in this study.

Keywords: microwave extraction, antibacterial effect, allspice essential oil, vapor phase

1. Introduction

The use of microwaves is an alternative to extract essential oils (EOs), and it can be used to assist a common method known as hydrodistillation. This method is achieved by adapting a distillation apparatus to a microwave oven, or with specialized equipment such as the NEOS System equipment (Milestone, Shelton CT, USA) [1]. The MAE is a method that uses microwave radiation as a heating source, for a mixture made with solvent and sample. This type of heating is instantaneous and occurs inside the sample, so the extraction is usually a very fast process [2].

Essential oils are substances extracted from different parts of aromatic plants (flowers, seeds, leaves, herbs, fruits, roots, and rhizomes, among others) and have antiviral, antibacterial, antifungal, and insecticidal properties [3]. EOs contain

ANNEXE II. Evidence of participation in congresses

AMEPAL A.C.

Latin Food 2016

IAFP´s 5th Latin American Symposium in Food Safety
7th Food Science, Biotechnology and Safety Meeting

The organizing committee certifies that :

AMECA

Ana Lorenzo Leal

Attended the Latin Food 2016
Meeting held in Cancún, Q. Roo. México,

November 9 - 11, 2016.

The duration of the meeting program was 30 hours.
On belhaf of AMECA and AMEPAL, we thank you for your participation.

Dr. Santos García.
President AMEPAL AC

Dr. Hugo Sergio García
President AMECA

UANL


AMEPAL A.C.
Latin Food 2016
IAFP´s 5th Latin American Symposium in Food Safety
7th Food Science, Biotechnology and Safety Meeting
AMECA
CERTIFICATE OF PARTICIPATION
This is to certify that the presentation entitled:
Vapor Phase Antibacterial Activity Evaluation of Allspice (Pimenta dioica), Thyme (Thymus vulgaris), and Rosemary (Rosmarinus officinalis) Essential Oils Against Pathogenic Bacteria at Selected pHs.
Authored by:
Lorenzo-Leal A.C., Palou E., López-Malo, A.
Was presented in Latin Food 2016, held in Cancun Q. Roo, México. November 9 - 11, 2016.
Dr. José Santos García Alvarado
President of AMEPAL
Dr. Hugo Sergio García Galindo
President of AMECA
UANL
BENEMERITA

AMIDIQ

La Academia Mexicana de Investigación y Docencia en Ingeniería Química A.C.

La Ingeniería Química en el Desarrollo Sostenible de Nuevos Procesos y Productos

Otorga el presente

RECONOCIMIENTO

A:

Mónica Dávila Rodríguez, Ana Cecilia Lorenzo Leal, Gabriela Ríos Corripio, María Teresa Jiménez Munguía

Por la presentación del trabajo:

EVALUACIÓN DE LAS PROPIEDADES FÍSICAS, QUÍMICAS Y FUNCIONALES DE CLARA DE HUEVO FRESCA Y SECADAS POR ASPERSIÓN

ID: 401

XXXVIII Encuentro Nacional de la AMIDIQ

Ixtapa Zihuatanejo, Gro., México, del 9 al 12 de mayo de 2017

Dr. Mauricio Sales Cruz
PRESIDENTE DE AMIDIQ

Dr. Jesús Alberto Ochoa Tapia
PRESIDENTE DEL COMITÉ TÉCNICO

IFT17

Certificate of Participation

This certifies that

Ana Cecilia Lorenzo Leal

Attended IFT17: Where Science Feeds Innovation® June 25-28, 2017 held in Chicago, Illinois, USA

The IFT17 scientific and applied sessions qualify for Certified Food Scientist (CFS) recertification contact hours (CH). CFS Certificants may claim a maximum of 22 CH for their participation in scientific and technical symposia and poster sessions related to the CFS Content Domains.

IFT is a Continuing Professional Education (CPE) accredited provider (IN141) with the Academy of Nutrition and Dietetics' Commission on Dietetic Registration (CDR).

Clare Keessey

Clare Keessey

IFT Meeting Planner

SEGURIDAD ALIMENTARIA 2017

CONGRESO INTERNACIONAL DE INOCUIDAD ALIMENTARIA
CONGRESO NACIONAL SOBRE SOSTENIBILIDAD ANTE EL DESPERDICIO DE ALIMENTOS

Otorgan la presente

CONSTANCIA

a: Ana Cecilia Lorenzo Leal

Por su participación como **ASISTENTE** en el
Congreso de Seguridad Alimentaria 2017, llevado a cabo
del 15 al 17 de Noviembre del 2017, Chihuahua, Chih.

Valor curricular 25 horas

Dr. Pedro Javier Martínez Ramos
Director de la Facultad de Ciencias Químicas

Dra. Guadalupe Virginia Nevárez Moorillón
Presidente AMEPAL, A.C

Dra. Juliana Morales Castro.
Coordinadora de la Red REDSA PDA

AMEPAL A.C.

SEGURIDAD ALIMENTARIA 2017

CONGRESO INTERNACIONAL DE INOCUIDAD ALIMENTARIA
CONGRESO NACIONAL SOBRE SOSTENIBILIDAD ANTE EL DESPERDICIO DE ALIMENTOS

Otorgan la presente

CONSTANCIA

a: *Lorenzo-Leal, A.C., López-Malo, A.*

Por su PARTICIPACIÓN con el trabajo titulado "**Inhibición de bacterias patógenas y causantes de deterioro mediante aceites esenciales de tomillo (*Thymus vulgaris*), romero (*Rosmarinus officinalis*), o sus componentes mayoritarios en fase de vapor**" en el Congreso de Seguridad Alimentaria 2017, llevado a cabo del 15 al 17 de Noviembre del 2017, Chihuahua, Chih.

Dr. Pedro Javier Martínez Ramos
Director de la Facultad de Ciencias Químicas

Dra. Guadalupe Virginia Nevárez Moorillón
Presidente AMEPAL, A.C

Dra. Juliana Morales Castro
Coordinadora de la Red REDSA PDA

AMEPAL A.C.

Certificate of Participation

This certifies that

Ana Lorenzo

Poster Attended

Individual and Combined Antibacterial Activity of Essential Oils in Vapor Phase Against Pathogenic and Spoilage Bacteria

Attended IFT18: Where Science Feeds Innovation® July 15-18, 2018 held in Chicago, IL USA

The IFT18 scientific and applied sessions qualify for Certified Food Scientist (CFS) recertification contact hours (CH). CFS Certificants may claim a maximum of 22 CH for their participation in scientific and technical symposia and poster sessions related to the CFS Content Domains.

IFT is a Continuing Professional Education (CPE) accredited provider (IN141) with the Academy of Nutrition and Dietetics' Commission on Dietetic Registration (CDR).

Daniel Gullicksen

IFT Meeting Planner

Certificate of Participation

This certifies that

Ana Lorenzo

Poster Attended

Vapor Phase Antibacterial Activity In Vitro and In Vivo of Thyme (Thymus Vulgaris) Essential Oil Against Pathogenic Bacteria

Attended IFT18: Where Science Feeds Innovation® July 15-18, 2018 held in Chicago, IL USA

The IFT18 scientific and applied sessions qualify for Certified Food Scientist (CFS) recertification contact hours (CH). CFS Certificants may claim a maximum of 22 CH for their participation in scientific and technical symposia and poster sessions related to the CFS Content Domains.

IFT is a Continuing Professional Education (CPE) accredited provider (IN141) with the Academy of Nutrition and Dietetics' Commission on Dietetic Registration (CDR).

Daniel Gullicksen

IFT Meeting Planner

CAIA
Comisión Argentina de Inocuidad Alimentaria
Filial IAFP / DAMyC - AAM

VI Simposio Latinoamericano de Inocuidad Alimentaria
III Simposio Argentino de Inocuidad Alimentaria

25 al 27 de setiembre de 2018
Buenos Aires, Argentina

asociación argentina de microbiología

International Association for Food Protection

Se certifica que

LORENZO-LEAL, ANA CECILIA; PALOU, ENRIQUE; LOPEZ-MALO, AURELIO

han presentado el Trabajo

EVALUACIÓN DE LA ACTIVIDAD ANTIMICROBIANA IN VIVO E IN VITRO DEL ACEITE ESENCIAL DE ROMERO ROSMARINUS OFFICINALIS EN FASE VAPOR CONTRA BACTERIAS PATÓGENAS

en el Simposio **IAFP Latino 2018** organizado por la Comisión Argentina de Inocuidad Alimentaria (CAIA), Filial argentina de la IAFP y Subcomisión de la División Alimentos, Medicamentos y Cosméticos de la Asociación Argentina de Microbiología.

Graciela Vaamonde — David Tharp — Fabiana Guglielmone

CAIA
Comisión Argentina de
Inocuidad Alimentaria
Filial IAFP / DAMyC - AAM

IAFP LATINO 2018

VI Simposio Latinoamericano de Inocuidad Alimentaria IAFP
III Simposio Argentino de Inocuidad Alimentaria
6th IAFP's Latin American Symposium on Food Safety

LIBRO DE RESÚMENES

25 al 27 de septiembre de 2018
Ciudad Autónoma de Buenos Aires, Argentina

asociación argentina de microbiología

International Association for Food Protection

Latin Food 2018

8th Food Science, Biotechnology & Safety Congress

MEXICAN ASSOCIATION OF FOOD SCIENCE

Food for Health 2018

AWARDS THE PRESENT CERTIFICATE TO:

Lorenzo-Leal, A. C., Palou, E., López-Malo, A.

IN RECOGNITION FOR THEIR PARTICIPATION AS

AUTHORS OF THE WORK:

Vapor phase antibacterial activity in vitro and in alfalfa seeds of rosemary (Rosmarinus officinalis) essential oil against pathogenic bacteria (ORAL)

DR. GUSTAVO HIDEL GUTIÉRREZ LÓPEZ
PRESIDENT OF THE AMECA DIRECTING COUNCIL 2017-2019

DR. NICOLÁS OSCAR SOTO CRUZ
VICE PRESIDENT OF THE AMECA DIRECTING COUNCIL PRESIDENT OF THE ORGANIZING DIRECTING COMMITTEE

DRA. LILIA ARELY PRADO BARRAGÁN
SECRETARY OF THE DIRECTING COUNCIL PRESIDENT OF THE SCIENTIFIC COMMITTEE

DR. SANTOS GARCÍA
PRESIDENT OF FOOD FOR HEALTH

Puerto Vallarta, Jalisco, México, 14-16 November 2018