Universidad Miguel Hernández
Escuela Politécnica Superior de Orihuela
Departamento de Tecnología Agroalimentaria

CARACTERIZACIÓN DE ACEITES ESENCIALES DE PLANTAS AROMÁTICAS MEDITERRÁNEAS Y SU APLICACIÓN A FILMS DE QUITOSANO PARA LA CONSERVACIÓN DE PRODUCTOS CÁRNICOS

Memoria realizada para optar al titulo de Doctor, presentada por

Yolanda Ruiz Navajas

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TESIS DOCTORAL

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CERTIFICA:

Que la Tesis Doctoral Titulada "**Caracterización de aceites esenciales de plantas aromáticas mediterráneas y su aplicación a films de quitosano para la conservación de productos cárnicos**" de la que es autora la Licenciada en Ciencia y Tecnología de los Alimentos **Yolanda Ruiz Navajas** ha sido realizada bajo la dirección de la Dra. Juana Fernández López, el Dr. Manuel Viuda Martos y la Dra. Esther Sendra Nadal la cual considero conforme en cuanto a forma y contenido para que sea presentada para su correspondiente exposición publica.

Y para que conste a los efectos oportunos firmo el presente certificado en Orihuela a veintinueve de Enero de dos mil catorce.

Fdo.: Dr. José Ramón Díaz Sánchez

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CERTIFICAN:

Que la Tesis Doctoral Titulada "**Caracterización de aceites esenciales de plantas aromáticas mediterráneas y su aplicación a films de quitosano para la conservación de productos cárnicos**" llevada a cabo por la Licenciada en Ciencia y Tecnología de los Alimentos **Yolanda Ruiz Navajas** ha sido realizada bajo nuestra dirección y autorizamos a que sea presentada para optar a la obtención del grado de Doctor por la Universidad Miguel Hernández.

Y para que conste a los efectos oportunos se firma el presente certificado en Orihuela a veintinueve de enero de dos mil catorce.

Fdo.: Dr. Juana Fernández López

Fdo.: Dr. Manuel Viuda Martos

Fdo.: Dr. Esther Sendra Nadal

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A mis Padres

Es absolutamente imposible
demostrarlo todo.

Aristóteles
(384 a. C.-322 a. C)

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UNIVERSITAS Miguel Hernández

ESTRUCTURA DE LA TESIS

1.- ESTRUCTURA DE LA TESIS

Para la realización de la presente Tesis Doctoral se ha seguido una metodología basada en la publicación de un compendio de artículos, tanto de investigación como bibliográficos.

La estructura de esta Tesis Doctoral consta de una breve introducción (capítulo segundo) en la que se incluye una revisión bibliográfica sobre la composición y propiedades, tanto tecnológicas como funcionales, de las especias y los aceites esenciales y su posible uso en la industria de alimentos. También incluye una revisión sobre el quitosano y su potencial uso en la elaboración de films para la aplicación en la conservación de alimentos.

En el capítulo tercero se describen los objetivos propuestos y en el capítulo cuarto, se presenta un resumen de los materiales y métodos utilizados para poder entender los distintos procesos de elaboración que se han llevado a cabo y las determinaciones analíticas practicadas.

En el capítulo quinto se recoge un resumen global de los resultados más relevantes obtenidos en los diferentes estudios realizados.

Seguidamente, en el capítulo sexto se recogen las conclusiones de todos los estudios que forman parte de la presente memoria de Tesis Doctoral, mientras que el capítulo séptimo corresponde a la bibliografía consultada.

En el último capítulo (octavo) de la presente Tesis Doctoral se incluyen las publicaciones que componen la base de la misma. El primer grupo de publicaciones se centra en la caracterización química y en la determinación de las propiedades antimicrobianas y antioxidantes de los aceites esenciales de hierbas aromáticas objeto de estudio. Forman parte de este primer grupo 3 publicaciones: la primera, en la revista *Food Control* donde se identifica la composición química, mediante cromatografía de gases y espectrometría de masas (GC/MS), y las propiedades antibacterianas de dos de los aceites esenciales, *Thymus moroderi* y *Thymus piperella*, objeto de estudio; la segunda, en la revista *Journal of Food Safety*, donde se identifica la composición química, mediante GC/MS, y las propiedades antibacterianas de los otros dos aceites esenciales, *Sideritis angustifolia* y *Santolina chamaecyparissus*, sometidos a estudio; la

tercera y última publicada en la revista *Journal of Food Protection* sobre la actividad antifúngica (mohos y levaduras) y antioxidante de los cuatro aceites esenciales sometidos a estudio.

El segundo grupo de publicaciones incluye 2 artículos donde se recogen los resultados de la aplicación de estos aceites esenciales a films de quitosano. La primera publicación, realizada en la revista *Food Control*, se centra en determinar las propiedades antioxidantes y antibacterianas de los films de quitosano adicionados con aceites esenciales de *T. moroderi* y *T. piperella*. La segunda publicación se refiere al efecto de dichos films de quitosano sobre la vida útil de un producto cárnico cocido. Esta última publicación está en proceso de revisión en la revista *Meat Science*.

De forma complementaria a estas publicaciones, se presentan una serie de comunicaciones sobre resultados de esta Tesis Doctoral, presentados a Congresos Internacionales donde se examinan las propiedades antioxidantes y antimicrobianas de los aceites esenciales analizados y de los films de quitosano adicionados con dichos aceites esenciales.

INTRODUCCION

2.- INTRODUCCION

2.1.- Hierbas aromáticas

Las hierbas aromáticas y las especias son una parte muy importante de la nutrición humana y su empleo se da en todas las culturas del mundo. La literatura describe cómo son utilizadas para impartir sabor y reducir la necesidad del empleo de sal y condimentos grasos, para mejorar la digestión, y proporcionar al organismo una carga de antioxidantes adicionales que pueden prevenir la aparición de alteraciones fisiológicas y metabólicas (Pérez-Alvarez et al., 2002).

Gran parte de las especias y, en menor medida, las hierbas aromáticas tienen su origen en países orientales, mientras otras han sido introducidas en Europa tras el descubrimiento del Nuevo Mundo. La Cuenca Mediterránea también ha aportado un buen número de hierbas aromáticas y especias, como el cilantro, romero, tomillo o el orégano (Díaz-Maroto et al., 2002) los cuales han sido utilizados desde la antigüedad por civilizaciones como la Egipcia o la Romana. Las principales hierbas aromáticas y especias procedentes de la Cuenca Mediterránea pertenecen, fundamentalmente, a dos familias, la familia *Lamiaceae* y la familia *Apiaceae* (Tabla 1).

Uno de los géneros más importantes de hierbas aromáticas presentes en la Cuenca Mediterránea es el género *Thymus*, perteneciente a la familia *Laminaceae*, del que se conocen más de 215 especies distintas. Son plantas que se adaptan muy bien a climas calurosos y secos, propios de la Cuenca Mediterránea, habiéndose extendido por la zonas áridas de la Península Ibérica (Horwath et al., 2008). Esta hierba aromática se emplea como condimento culinario, además de ser ampliamente utilizada en la medicina popular por su acción estimulante sobre todas las funciones del organismo (Viuda-Martos et al., 2011a). Como se ha mencionado, es una planta de crecimiento silvestre en la Cuenca Mediterránea, aunque también puede ser cultivada, existiendo distintos ecotipos los cuales difieren en sus características morfológicas y en la composición de los aceites esenciales que producen (Corticchiato et al., 1998; Tedone et al., 2001), aunque todos están caracterizados por un fuerte y penetrante olor y un sabor balsámico y especiado muy pronunciado.

Tabla 1. Principales hierbas aromáticas y especias procedentes de la Cuenca Mediterránea

Familia	Nombre común	Nombre Científico	Parte de la planta usada
Laminaceae	Tomillo	*Thymus vulgaris*	Brote terminal, hojas
	Romero	*Rosmarinus officinalis*	Brote terminal, hojas
	Orégano	*Origanum vulgare*	Hojas, flores
	Albahaca	*Ocimum basilicum*	Brote terminal, hojas
	Hierbabuena	*Mentha spicata*	Brote terminal, hojas
	Salvia	*Salvia officinalis*	Brote terminal, hojas
	Menta	*Mentha piperita*	Brote terminal, hojas
	Mejorana	*Origanum majorana*	Hojas, botones florales
	Melisa	*Melissa officinalis*	Brote terminal, hojas
	Ajedrea	*Satureja hortensis*	Brote terminal, hojas
	Hisopo	*Hyssopus officinalis*	Hojas, flores
	Lavanda	*Lavandula angustifolia*	Hojas, flores
Apiaceae	Hinojo	*Foeniculum vulgare*	Hojas, tallos, flores
	Perejil	*Petroselinum crispum*	Hojas, raíz
	Eneldo	*Anethum graveolens*	Fruto, hojas
	Perifollo	*Anthriscus cereifolium*	Hojas
	Cilantro	*Coriandrum sativum*	Hojas, frutos
	Comino	*Cuminum cyminum*	Semillas
	Alcaravea	*Carum carvi*	Frutos
	Anís	*Pimpinella anisum*	Semillas

Dos especies pertenecientes al género *Thymus* son el *Thymus piperella* y el *Thymus moroderi*. El primero de ellos, *T. piperella*, en un ecotipo endémico de la Península Ibérica. Se extiende aproximadamente sobre 800 km^2 en las provincias de Alicante, Murcia, y sur de Valencia. Esta especie de tomillo se emplea

fundamentalmente como especia para condimentar diversos platos tradicionales. El segundo, *T. moroderi*, está estrechamente relacionado con el tomillo común (*Thymus vulgaris* L.), también se trata de un ecotipo endémico del sureste de la Península Ibérica, encontrándose únicamente en las provincias de Alicante y Murcia. Esta planta se utiliza sobre todo para la elaboración de bebidas espirituosas además de como especia en la condimentación de algunos platos tradicionales.

Otros géneros, también muy comunes en la Cuenca Mediterránea son, el género *Santolina* y el género *Sideritis.* El género *Santolina*, perteneciente a la familia Asteraceae, está representado por más de 10 especies, las cuales están ampliamente distribuidas por todo el Mediterráneo (Derbesy et al., 1989), siendo las especies más representativas: *Santolina viridis* W. (presente en el sur de Francia y norte de España), *Santolina pectinata* Lag. (Presente en la Península Ibérica) y *Santolina chamaecyparissus* L. (crece en forma silvestre en toda la Cuenca Mediterránea). Esta última es un arbusto perenne, nativo de la zona oeste y central del Mediterráneo, que crece de forma silvestre en España, Italia, Túnez y Marruecos. A menudo se cultiva como planta ornamental debido a sus tallos lanudos y a sus flores amarillas, aunque se ha utilizado en la medicina popular debido a sus propiedades analgésicas, antiinflamatorias, antisépticas, antiespasmódicas, bactericidas, fungicidas y para el tratamiento de diferentes dermatitis (El-Sahhar et al., 2011).

El género *Sideritis*, que pertenecen a la familia Lamiaceae, subfamilia *Lamioideae*, comprende al menos 150 especies. Las plantas de este género, principalmente *Sideritis angustifolia*, han sido ampliamente utilizadas en la medicina popular, a modo de infusión, como antiinflamatorios, antiulcerosos, antimicrobianos, astringentes, para el tratamiento contra la gripe y como agentes estimulantes circulatorios (Senatore, 2000).

Todas las plantas anteriormente mencionadas tienen en común, sobre todo las dos especies de tomillos, la producción de altas concentraciones de aceite esencial.

2.2.- Aceites esenciales de hierbas aromáticas

En general, los aceites esenciales (AEs) son productos formados por la mezcla de numerosas sustancias, con una composición química bastante compleja, que se

obtienen a partir del metabolismo secundario de las plantas. Son lípidos simples, sin ácidos grasos, y están compuestos por sustancias volátiles a diferencia de los aceites fijos que contienen ácidos grasos como componentes estructurales fundamentales y que no son volátiles (Burt, 2004). En términos generales los AEs están compuestos por más de setenta componentes, fundamentalmente: terpenos, monoterpenos y sesquiterpenos, hidrocarburos, alcoholes, cetonas, etc. Éstos pueden ser acíclicos, monocíclicos, bicíclicos o tricíclicos (Russo et al., 1998).

Los componentes mayoritarios pueden constituir por encima del 85% de la composición del AE, mientras que otros componentes se encuentran en forma de trazas (Bauer et al., 2001). Es muy importante el papel que juegan estos compuestos minoritarios, ya que existen evidencias de que estos componentes contribuyen, de manera significativa, a las propiedades funcionales que el AE pueda presentar, debido al posible sinergismo entre distintos componentes (Burt, 2004). Las hierbas aromáticas y especias sintetizan y acumulan estos AEs en estructuras glandulares, las cuales pueden estar distribuidas por toda la parte aérea de la planta, aunque esta distribución no es uniforme, ya que generalmente se suelen encontrar en las hojas y en la flores (Faleiro et al., 2002).

Los AEs presentan múltiples propiedades, entre las que destacan sus propiedades antioxidantes y sus propiedades antimicrobianas.

2.2.1.- Propiedades Antimicrobianas de los aceites esenciales

Están ampliamente documentadas en la literatura científica las propiedades antimicrobianas de los AEs de hierbas aromáticas y especias, tanto frente a bacterias como frente a mohos y/o levaduras. Sin embargo, no se han encontrado referencias sobre la actividad antimicrobiana de los AEs obtenidos de las dos especies de tomillo (*T. piperella* y *T. moroderi*) analizadas en este trabajo de investigación. Así como, tampoco se ha encontrado información de la actividad antimicrobiana de los AEs de las especies del género *Sideritis* y del género *Santolina* analizadas. No obstante, la actividad antimicrobiana de los AEs obtenidos de plantas del género *Thymus* está ampliamente establecida.

De Martino et al. (2009) analizaron la actividad antibacteriana de los AEs obtenidos de *Thymus longicaulis* y *Thymus pulegioides* recolectados en Italia, frente a una serie de bacterias, tanto Gram-negativas como Gram-positivas, como son: *Sthaphylococcus aureus, Streptococcus faecalis, Bacillus subtilis, Bacillus cereus, Proteus mirabilis, Escherichia coli, Salmonella typhi Ty2* and *Pseudomonas aeruginosa*. Se obtuvieron halos de inhibición comprendidos entre 9 y 18 mm para *T. longicaulis* y halos comprendidos entre 11 y 20 mm para *T. pulegioides*. En un estudio similar, Pinto et al. (2006), determinó la actividad antifúngica del AE obtenido a partir de *T. pulegioides* recolectado en Portugal, consiguiendo unos valores de concentración mínima inhibitoria (CMI) de entre 0,16 y 0,32 µL/mL frente a dermófitos y diversas cepas de *Aspergillus*. Gonçalves et al. (2010) evaluaron la actividad antifúngica de cuatro AEs de *Thymus zygis* subsp. *sylvestris* cultivados en Portugal, frente a dos hongos como son *Aspergillus niger* y *Aspergillus fumigatus*, obteniendo valores de CMI comprendidos entre 0,16 y 1,25 µL/mL. Zouari et al. (2011) analizaron la actividad antimicrobiana del AE de *Thymus algeriensis* Boiss cultivado en Túnez, frente a seis bacterias (*E. coli, P. aeruginosa, B. cereus, Klebsiella pneumoniae, Salmonella typhimurium* y *Enterococcus faecalis*) y dos hongos (*Fusarium solani* y *Aspergillus niger*). Los halos de inhibición obtenidos y los valores de CMI obtenidos estaban en un rango de 13,5-64 mm y 1-6 µL/mL, respectivamente. Viuda-Martos et al. (2011b) analizaron la actividad antibacteriana del AE de *Thymus vulgaris* cultivado en Egipto, frente a diversas bacterias relacionadas con el deterioro de alimentos como son *Listeria innocua, Serratia marcescens* y *Pseudomona fluorescens*, obteniendo halos de inhibición, con un volumen de 40 µL, de 41,00; 20,25 y 23,50 mm, respectivamente.

Como se ha descrito para el género *Thymus*, la actividad antibacteriana del género *Sideritis* ha sido, relativamente, analizada. Kiliç et al. (2003) describieron que los AEs de *Sideritis athoa, Sideritis trojana, Sideritis dichotoma, Sideritis spilyea* y *Sideritis argyrea*, eran activos frente a *E. coli, S. aureus, P. aeruginosa, K. pneumoniae* y *E. faecalis*. Basile et al. (2006) analizaron el AE de *Sideritis italic* (Miller) frente a 9 cepas bacterianas, tanto Gram-negativas como Gram-positivas, encontrando unos valores de CMI comprendidos entre 3,9 y 250 µg/mL. Köse et al. (2010) analizaron la actividad antibacteriana de los AEs de dos variedades de *Sideritis erythrantha*

endémicas de Turquía. Estos autores concluyeron que el AE de *S. erythrantha* var. *cedretorum* era efectivo como antibiótico frente a *Staphylococcus aureus* meticilina-resistente, *E. faecalis* vancomicin-resistente, *Haemophilus influenza* ampicilina-resistente *y E. faecalis* vancomicina-sensitivo. Por otro lado, el AE de *S. erythrantha* var. *erythrantha* fue también activo frente *E. faecalis* vancomicina-resistente y *H. influenza* ampicilina-resistente.

Diversos trabajos han tratado de describir los posibles modos de acción de los constituyentes de los AEs (Davidson y Naidu, 2000; Davidson, 2001), sin embargo, el mecanismo concreto todavía no ha sido completamente dilucidado (Lambert et al., 2001). Teniendo en cuenta el gran número de componentes químicos presentes en los AEs, es muy probable que su actividad antimicrobiana no se deba a un único mecanismo específico, sino a la acción conjunta de diferentes mecanismos que actúan sobre distintos "Targets" de la célula (Skandamis et al., 2001; Carson et al., 2002). La Figura 1 muestra los distintos mecanismos de acción propuestos a este efecto.

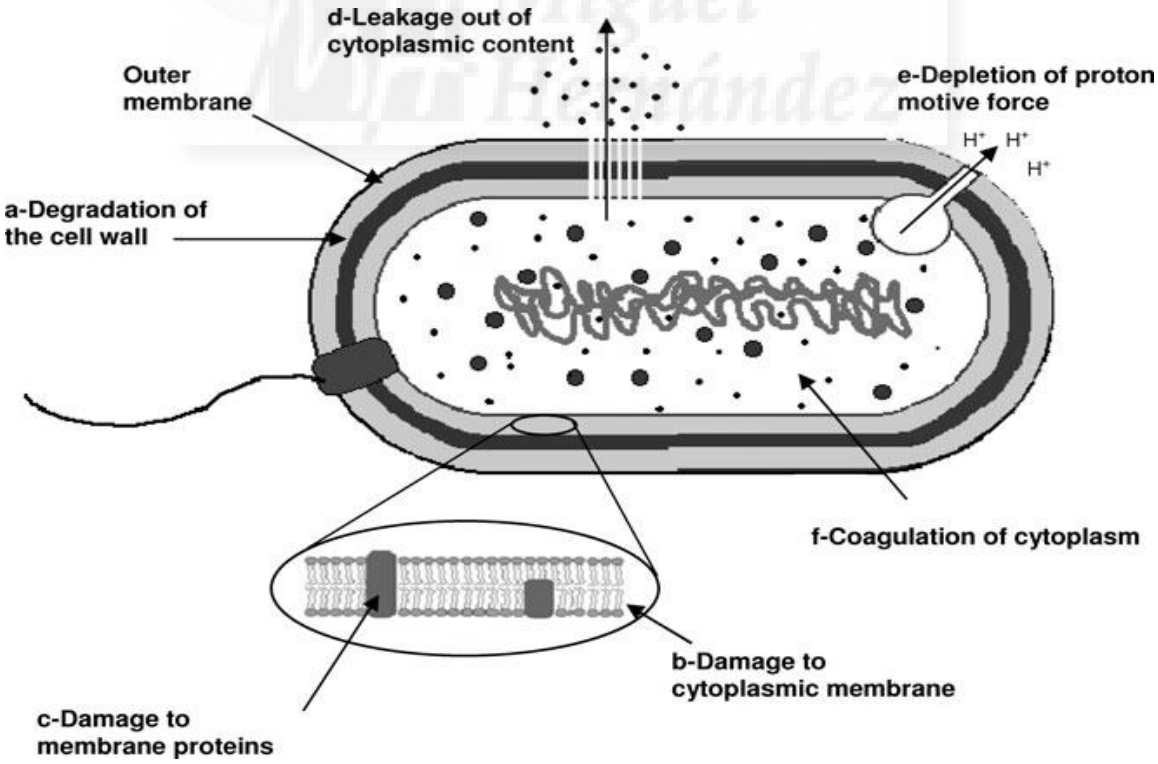

Figura 1. Mecanismos de acción propuestos para los aceites esenciales y sus componentes frente a las células microbianas.
Fuente: Raybaudi-Massilia et al. (2009).

Generalmente, la composición, la estructura así como los grupos funcionales de los componentes que integran el AE desempeñan un papel muy importante a la hora de determinar su actividad antimicrobiana.

Los compuestos con grupos fenólicos tipo timol o carvacrol son los principales responsables de las propiedades antimicrobianas, aunque también pueden existir otros componentes, presentes en los AEs que pueden presentan las mismas propiedades antimicrobianas y que son del tipo aldehídos, cetonas, alcoholes, etc. Tratar de identificar cada uno de los compuestos químicos del AE y determinar cuál es el posible responsable del efecto antimicrobiano es una tarea muy complicada y difícil de abordar. Por esta razón es muy importante conocer la composición química que presenta el AE objeto de estudio para así intentar predecir sus propiedades antimicrobianas.

2.2.2.- Propiedades Antioxidantes de los aceites esenciales

La actividad antioxidante de las hierbas aromáticas, de las especias, de los AEs y de sus componentes, ha sido objeto de múltiples estudios (Mata et al., 2007; Milan et al., 2008; Bozin et al., 2008; Alves-Silva et al., 2013), aunque gran parte de estos ensayos se hayan realizado *in vitro*. Como sucedía en el caso de las propiedades antimicrobianas, no existen estudios donde se determine la actividad antioxidante de los AEs obtenidos de las dos especies de tomillo (*T. piperella* y *T. moroderi*) analizadas en este trabajo de investigación. Así como, tampoco se han encontrado referencias sobre la actividad antioxidantes de AEs de las especies del género *Sideritis* y del género *Santolina* utilizadas en este trabajo. Sin embargo, la actividad antioxidante de los AEs obtenidos de plantas del género *Thymus* ha sido ampliamente referenciada. Tepe et al. (2005) compararon el potencial antioxidante, utilizando el ensayo del secuestro del radical 2,2'-difenil-1-picrilhidrazil (DPPH), de los AEs obtenidos de dos especies de tomillos, como son, *Thymus sipyleus* subsp. *sipyleus* var. *sipyleus* y *Thymus sipyleus* subsp. *sipyleus* var. *Rosulans*. Estos AEs presentaron un valor de IC_{50} (concentración de AE que inhibe el 50% del radical DPPH) de 0,22 y 2,67 mg/mL, respectivamente. Viuda-Martos et al. (2010) determinaron la actividad antioxidante del AE de *Thymus vulgaris* cultivado en Egipto, empleando distintos ensayos; con el ensayo del DPPH se obtuvo un valor de IC_{50} de 4,50 mg/mL; con el análisis de las especies reactivas del ácido tiobarbitúrico (TBARs) se obtuvo un valor de IC_{50} de 4,09 mg/mL; y con el

ensayo de la actividad quelante de metales se obtuvo un valor de IC_{50} de 0,27 mg/mL.

De igual modo, no existe ninguna referencia científica de la actividad antioxidante del AE de *S. angustifolia*. Sin embargo, la actividad antioxidante de los AEs obtenidos de otras especies del género *Sideritis* ha sido ampliamente estudiada. Köse et al. (2010) analizaron la actividad antioxidante de los AEs de *Sideritis erythrantha* var. *erythrantha* y *S. erythrantha* var. *cedretorum*. Estos autores concluyeron que dichos AEs presentaron una baja capacidad de secuestro de radicales libres (% de inhibición de 5,12 y 4,62, respectivamente, a concentraciones de 2,0 mg/mL). Basile et al. (2006) estudiaron la actividad antioxidante de los AEs de *Sideritis itálica*, obtenidos de las hojas o de las cabezas florales, mostrando una actividad antioxidante, expresada como equivalentes de ácido ascórbico, de 4,29 y 1,98 mg/mL, respectivamente.

No se han encontrado referencias bibliográficas sobre la actividad antioxidante de AEs obtenidos a partir de plantas del género *Santolina*.

El mecanismo de acción que provoca esta actividad antioxidante no está todavía esclarecido. Existen diversos mecanismos de acción para dicha capacidad antioxidante: el secuestro de radicales libres, la donación de hidrógenos, la quelación de iones metálicos o incluso pueden actuar como sustrato de radicales como el superóxido o el hidroxil (Al-Mamary et al., 2002). Como ocurría en el caso de la actividad antimicrobiana, la actividad antioxidante de los aceites esenciales está relacionada con la presencia y concentración de distintos componentes en dicho aceite esencial y fundamentalmente aquellos que presentan grupos fenólicos en su estructura.

La determinación de la capacidad antioxidante que las hierbas aromáticas y las especias, así como sus derivados (fundamentalmente los AEs), ejercen en los alimentos, es un tema que actualmente está teniendo un importante auge, tanto por parte de los investigadores como por parte de la industria agroalimentaria. No hay que olvidar que la oxidación lipídica constituye una de las principales causas de deterioro de los alimentos y se produce, tanto durante el almacenamiento de materias primas, como durante su procesado o incluso durante el almacenamiento de los productos finales (Tepe et al., 2005). Dicha oxidación conlleva una pérdida significativa del valor nutricional del alimento, ya que existe una pérdida de vitaminas y ácidos grasos esenciales, además de

una pérdida de calidad sensorial, ya que se producen cambios en el color, la textura y el sabor, lo que da como resultado una disminución de la vida útil del alimento y el consiguiente rechazo por parte del consumidor (Fernández-López et al., 2007).

Uno de los principales problemas que se plantean cuando se recurre a la aplicación directa de los AEs para que ejerzan, en los alimentos a los que son añadidos, efectos antioxidantes o antimicrobianos, reside en que para la obtención de dichos efectos inhibitorios se necesitan elevadas concentraciones, lo cual puede alterar las características sensoriales de los productos sobre los que son añadidos. Esta limitación puede superarse, potencialmente, a través del control de su liberación desde una matriz polimérica adecuada, diseñada como un film o recubrimiento comestible.

2.3.- Recubrimientos comestibles

Actualmente, hay un creciente interés por el desarrollo de materiales que puedan mejorar la vida útil de los alimentos y también, por la seguridad microbiológica que se les otorga. Los recubrimientos, películas o films comestibles formados por polímeros biodegradables, son uno de estos materiales que ha logrado mayor interés y por lo tanto se le atribuye un alto potencial comercial.

Una película o film comestible se puede definir como una capa fina y continua de material comestible, que se dispone sobre una superficie alimentaria para mejorar la calidad y aumentar la vida útil del alimento (Fernández-Pan y Maté-Caballero, 2011). En la formulación de films debe presentarse al menos un componente capaz de formar una matriz estructural estable, como los hidrocoloides clasificados en proteínas (colágeno, gelatina, zeina, gluten de trigo, proteína de soja, proteínas lácteas, etc.) (Morillon et al., 2002) o carbohidratos (derivados de celulosa, almidones, extractos de algas, pectinas, gomas o quitosano) (Ponce et al., 2008), los cuales se caracterizan por formar films con propiedades de barrera al oxígeno, aromas y lípidos, presentan biocompatibilidad, además de mejorar la apariencia estética del alimento (Han, 2000; Kalemba y Kunicka, 2003). Las características de este tipo de materiales están afectadas por diversos parámetros como son la formulación, la tecnología de formación de la película, las características del solvente y los aditivos (Gocho et al., 2000). Como se ha mencionado, son muchas las sustancias que son capaces de formar una matriz estable;

una de ellas, la cual presenta unas magníficas propiedades formadoras de dichas películas, además de mostrar ciertas propiedades antioxidantes y antimicrobianas por sí misma, es el quitosano (Sayas-Barberá et al., 2011).

2.3.1.- Quitosano

El quitosano, poli-β-(1,4)-D-glucosamina-N-acetil-D-glucosamina, (Figura 2A) se obtiene por desacetilación de la quitina, poli-β-(1,4)-N-acetil-D-glucosamina, (Figura 2B), la cual constituye el segundo polisacárido natural, más abundante, después de la celulosa. La quitina es uno de los componentes principales de las paredes celulares de los hongos, y del exoesqueleto de crustáceos e insectos. Es altamente insoluble en agua y solventes orgánicos, lo cual, restringe sus aplicaciones (Rinaudo, 2006).

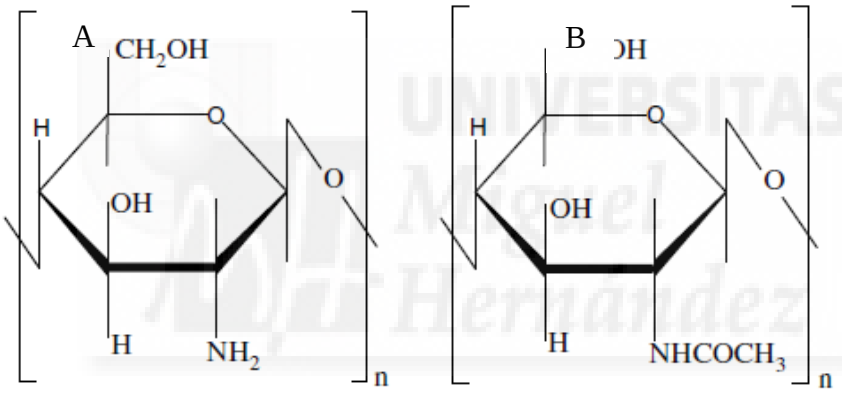

Figura 2. Estructura química del quitosano (A) y de la quitina (B).

Sin embargo, el quitosano es un compuesto que exhibe unas características fisicoquímicas de notable interés, como son, una elevada proporción de grupos amino libres, mayor solubilidad comparada con la quitina, biocompatibilidad y biodegradabilidad, lo cual hace que presente múltiples aplicaciones en medicina, industria cosmética, agricultura, biotecnología, industria alimentaria, industria papelera y en el tratamiento de aguas (Tabla 2).

El quitosano se define en términos del grado de acetilación, peso molecular, viscosidad y solubilidad. La importancia de este biopolímero, para la industria de alimentos, está fundamentalmente, en su propiedad para formar films (Fernández-Saiz et al., 2009).

Tabla 2. Principales aplicaciones del quitosano

Industria	Propiedad	Referencia
Agricultura	Estimulación del crecimiento de las plantas	Rahman et al., 2013
	Recubrimiento de semillas	Ziani et al., 2010
	Aumenta el tiempo de liberación de los fertilizantes y nutrientes en el suelo	Wu y Liu, 2008
Tratamiento de aguas	Eliminación de iones metálicos	Laus et al. 2007
	Floculante	Zeng et al. 2008
Cosmética	Tratamiento frente al acné	Ridolfi et al. 2012
	Cuidado bucal	São Pedro et al. 2009
Biomedicina	Sistemas liberadores de fármacos	Giri et al. 2012
	Suturas quirúrgicas	Ono et al. 2001
	Piel Artificial	Kim et al. 2008
	Reconstrucción ósea	Muzzarelli et al. 2009
	Implantes dentales	Arnaud et al. 2010
Alimentos	Reducción del colesterol	Liu et al. 2008
	Estabilizante de salsas	Laplante et al. 2005
	Conservante	Li et al. 2011

2.3.2.- Films de Quitosano

Como ya se ha comentado, el quitosano tiene una excelente capacidad formadora de films. Generalmente para la formación de films y coberturas de quitosano se utilizan concentraciones de 1%-3% de quitosano en soluciones acuosas acidificadas (ácido acético o láctico entre un 1-3%). A estos ingredientes básicos, generalmente se les añade un plastificante (habitualmente glicerol 0,2-0,5g/g quitosano) y/o un surfactante (Tween 80 al 0,1-0,5%) (Leceta, 2011).

En el mercado se puede encontrar quitosano con distinto grado de deacetilación y peso molecular, lo que dará lugar a la formación de films con distintas propiedades. En el estudio realizado por Leceta et al. (2011) se ponen de manifiesto las diferencias existentes entre el quitosano de alto peso molecular y el de bajo peso molecular, a los que se les adiciona glicerol como plastificante. Las propiedades de barrera frente a la humedad, oxígeno y la migración de solutos, del quitosano de alto peso molecular con glicerol como plastificante, muestra mejores valores de permeabilidad que el quitosano de bajo peso molecular. Esto puede deberse a que, el quitosano de bajo peso molecular

interacciona en menor medida con el glicerol empleando como plastificante, permitiendo que las moléculas de agua puedan escapar más fácilmente fuera del film.

Respecto a las propiedades mecánicas, también se ha observado que la resistencia a la tracción se ve aumentada y la rotura por alargamiento disminuida, cuando el quitosano tiene un peso molecular mayor, lo que puede atribuirse a la formación de una red más compacta en el quitosano; sin embargo, para la deformación por punción no se observaronn diferencias significativas en cuanto al peso molecular del quitosano, aunque sí se muestran con el contenido en glicerol de las muestras (Vargas et al., 2009; Sánchez-González et al., 2010; Leceta et al., 2011).

Aparte de actuar como barreras selectivas frente a la humedad, gases y la migración de solutos, estos films podrían actuar como vehiculizantes de una gran cantidad de componentes funcionales (Ojagh et al., 2010). Estos componentes funcionales incluyen agentes antioxidantes, agentes antimicrobianos, saborizantes, colorantes, etc., los cuales mejorarían la funcionalidad de los materiales de envasado debido a la "aparición" de nuevas funciones (Salmieri y Lacroix, 2006).

Uno de estos componentes que pueden ser adicionados a los films de quitosano para mejorar sus propiedades funcionales, son los aceites esenciales obtenidos de hierbas aromáticas y/o especias.

2.3.3.- Films de quitosano adicionados con aceites esenciales de hierbas aromáticas

Los films de quitosano adicionados con aceites esenciales de hierbas aromáticas o especias, además de exhibir determinadas propiedades de barrera (oxigeno, lípidos, aromas, humedad) presentan unas excelentes propiedades antioxidantes y/o antimicrobianas provocadas por la presencia de los AEs. Debido a todas estas propiedades, este tipo de films se convierten en una seria alternativa para lograr el objetivo de aumentar la vida útil y mantener la calidad del producto en el cual son utilizados.

Los films que incorporan AEs, los cuales presentan propiedades antioxidantes y/o antimicrobianas, permiten que la migración de sus componentes desde el recubrimiento a la superficie del alimento sea realice lentamente, permitiendo controlar esta migración (Figura 3) tal y como describe Fernández-Pan et al. (2010).

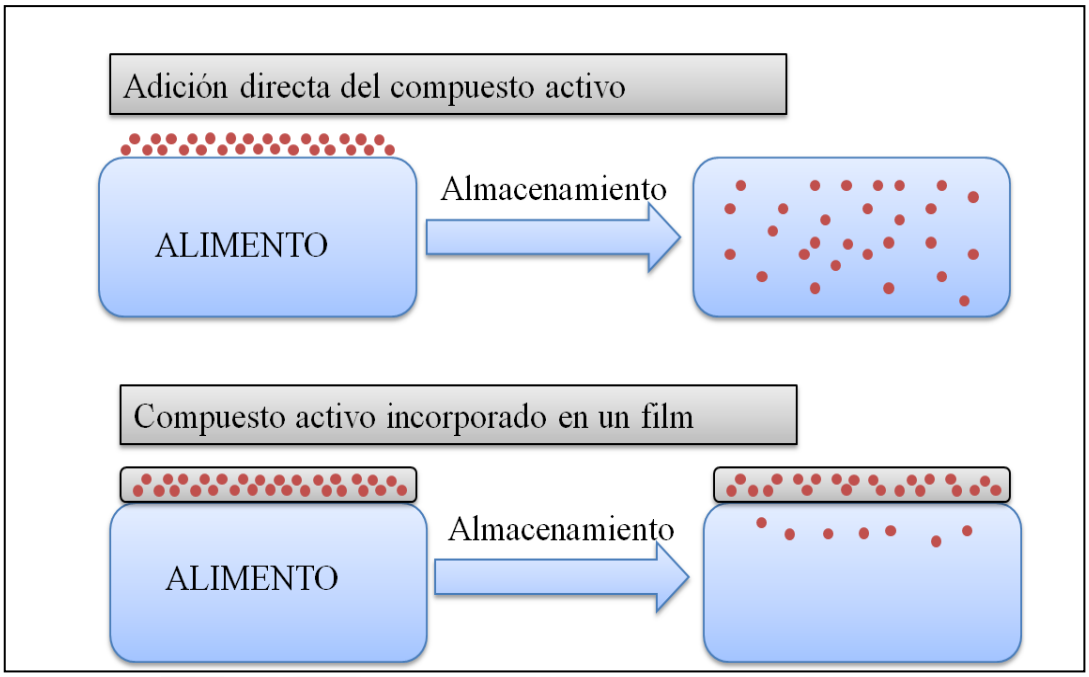

Figura 3. Evolución de la concentración de compuestos activos añadidos directamente sobre la superficie del sistema alimentario o a través de una película comestible.

El único inconveniente de la aplicación de este tipo de films adicionados con AEs de hierbas aromáticas sobre el alimento es que, cualquier pequeña modificación en los atributos organolépticos sea percibida o detectada por parte de los consumidores. Es por ello que se debe tener una gran certeza de que las características finales del producto varían lo menos posible y de que no son percibidas por el consumidor.

2.3.3.1.- Propiedades antimicrobianas de los films de quitosano adicionados con aceites esenciales de hierbas aromáticas

Una de las tecnologías emergentes más novedosas, que pueden aplicarse en la conservación de los alimentos, es el empleo de films o recubrimientos comestibles activos que pueden funcionar como matrices portadoras de agentes antimicrobianos (Martin-Belloso et al., 2009). Durante la última década, la investigación sobre los films de quitosano como portadores de AEs con propiedades antimicrobianas se ha incrementado notablemente (Altiok et al., 2010; Avila-Sosa et al., 2012; Kurek et al., 2013; Leceta et al., 2013). Moradi et al. (2011) analizó la actividad antibacteriana frente a *Listeria monocytogenes* de films de quitosano adicionados con AE de *Zataria multiflora* Boiss, obteniendo unos halos de inhibición de 92 y 162 mm^2 para

concentraciones de 5 y 10 mg/g de film. Zivanovic et al. (2005) desarrollaron films de quitosano enriquecidos con AEs de anís, albahaca, cilantro y orégano, y los evaluaron *in vitro* frente a los patógenos *L. monocytogenes* y *E. coli* O157:H7. Los films más activos resultaron ser los que incorporaban AE de orégano, seguidos por los de AE de cilantro, albahaca y finalmente anís. Altiok et al. (2010) desarrollaron films de quitosano adicionados con distintas concentraciones de AE de *T. vulgaris*, analizando posteriormente su actividad antibacteriana frente a distintas bacterias como *E. coli*, *K. pneumoniae*, *P. aeruginosa* y *S. aureus*. Estos autores encontraron que todas las bacterias fueron sensibles a los films desarrollados pero solo a la máxima concentración (1,2% (v/v)) obteniendo halos de inhibición comprendidos entre 16 y 19 mm. En otro estudio, Sánchez-González et al. (2011) analizaron la actividad antimicrobiana de films de quitosano preparados con diferentes concentraciones de AE de bergamota, AE de limón o AE de árbol del té. Estos autores concluyeron que los films analizados mostraron un efecto bacteriostático frente a diversas bacterias como *E. coli*, *L. monocytogenes* y *S. aureus*.

La efectividad antimicrobiana de los films de quitosano adicionados con AEs reside en la lenta migración de sus agentes activos hacia la superficie del producto que recubren, ayudando al mantenimiento de altas concentraciones de ingrediente activo donde son necesarias (Kristo et al., 2008).

2.3.3.2.- Propiedades antioxidantes de los films de quitosano adicionados con aceites esenciales de hierbas aromáticas

Otra de las propiedades que presentan los films de quitosano adicionados con AEs de hierbas aromáticas o especias y que puede ser muy importante en la industria agroalimentaria es su actividad antioxidante. Sin embargo, existen pocos estudios donde se determine la actividad antioxidante de los films de quitosano adicionados con AEs (Altiok et al., 2010; Moradi et al., 2012).

Esta actividad antioxidante podría estar relacionada con la presencia, en los films de quitosano, de compuestos bioactivos tales como compuestos fenólicos o ácidos terpénicos procedentes de los AEs. Así, los compuestos fenólicos y terpenoides presente en la composición química de los AEs están estrechamente relacionados

con las propiedades antioxidantes, fundamentalmente debido a sus propiedades redox ejercidas siguiendo distintos mecanismos, como el secuestro de radicales libres, la donación de átomos de hidrogeno y/o quelación de metales de transición (Liyana-Pathirana y Shahidi, 2006).

2.4.- Aplicación de los films de quitosano adicionados con aceites esenciales de hierbas aromáticas, en alimentos.

Tras la comprobación de la actividad antimicrobiana y antioxidante de los films de quitosano adicionados con AEs en ensayos de laboratorio, una variedad de ellos se han aplicado en sistemas alimentarios, fundamentalmente de origen vegetal. En el desarrollo de películas y recubrimientos comestibles antimicrobianos dirigidos al mantenimiento de la calidad y aumento de la vida útil de los productos cárnicos, las principales dianas microbianas empleadas, por su poder alterante, pertenecen a *Pseudomonas* spp. y *Listeria* spp. ya que son los responsables directos más comunes del daño producido en productos cárnicos frescos y cocidos, respectivamente, almacenados a bajas temperaturas (Fernández-Pan y Maté-Caballero, 2011). El grupo de bacterias ácido-lácticas también son una diana fundamental puesto que se reconocen como la microflora resistente y predominante en los productos envasados a vacío en condiciones de refrigeración (Emiroğlu et al., 2010).

Así, recientemente, Beverlya et al. (2008) analizaron la actividad antibacteriana frente a *L. monocytogenes,* de películas comestibles de quitosano en carne asada "*Ready-to-eat*". Estos autores informaron que tras catorce días de almacenamiento a 4 ºC las muestras recubiertas con los films de quitosano presentaban un reducción en los recuentos de *L. monocytogenes* de 2-3 UFC/g. Zivanovic et al. (2005) estudiaron el efecto antibacteriano frente a *L. monocytogenes* de films de quitosano y films de quitosano adicionado con distintos aceites esenciales (anís, albahaca, cilantro y orégano) empleados como separadores entre lonchas de mortadela. Las películas de quitosano adicionadas con AEs resultaron más eficaces que las películas formadas solo por el quitosano, ya que las primeras provocaron una reducción en los recuentos de *L. monocytogenes* de 3,6-4 ciclos logarítmicos mientras que las de segundas provocaron una reducción en los recuentos de 2 ciclos logarítmicos. Khanjari et al. (2013) llevaron a cabo un estudio donde se analizó el efecto combinado de film de quitosano con aceite

esencial de orégano, aplicado sobre filetes de pechuga de pollo, inoculados con *L. monocytogenes* mediante inmersión. Estos autores indican que tras catorce días de almacenamiento a 4 ºC las muestras no tratadas alcanzaron unos recuentos de aerobios totales de 7 log UFC/g (recuentos en los que se detecta alteración) en los primeros 6 días, mientras que en las muestras tratadas con quitosano y aceite esencial de orégano nunca alcanzaron los recuentes de 7 log UFC/g. Del mismo modo, en las muestras inoculadas con *L. monocytogenes* y tratadas con los films de quitosano más AE de orégano se produjo la inhibición completa de esta bacteria tras dos días de almacenamiento. Siripatrawan y Noipha (2012) analizaron el efecto, sobre la extensión de la vida útil de salchichas elaboradas con carne de cerdo, de films de quitosano a los que se incorporó extractos de té verde que eran empleados como envoltorios de dichas salchichas. Estos autores informaron que la incorporación del film de quitosano con el té verde mejoró las propiedades antimicrobianas de la película, obteniendo recuentos para aerobios totales, levaduras y mohos, inferiores en las salchichas tratadas que en las muestras control. Moradi et al. (2012) estudiaron la eficacia, frente a bacterias ácido-lácticas, aerobios totales y *L. monocytogenes*, de los films de quitosano que contenían AE de *Zataria multiflora Boiss* (ZEO) y extracto de semilla de uva (GSE) empleados como recubrimientos en mortadela. En este caso, el crecimiento de *L. monocytogenes* fue inhibido significativamente por los films que contenían ZEO-GSE. Así mismo, las bacterias aerobias mesófilas y las bacterias lácticas fueron los grupos más sensibles y resistentes a la acción de los films, con reducciones en los recuentos de 0,1-1,1 y 0,1-0,7 ciclos logarítmicos, respectivamente.

OBJETIVOS

3.- OBJETIVOS

3.1- Objetivos generales

El objetivo general del estudio es evaluar el potencial tecnológico de los aceites esenciales obtenidos de diferentes hierbas aromáticas endémicas del sureste de la península Ibérica, y su aplicación en films de quitosano para aumentar la vida útil de un sistema modelo cárnico cocido.

Para alcanzar este objetivo general, se plantearon los siguientes objetivos particulares.

3.2.- Objetivos particulares

- Caracterizar químicamente los aceites esenciales obtenidos de diferentes hierbas aromáticas endémicas del sureste de la península Ibérica.
- Determinar la actividad antibacteriana de estos aceites esenciales frente a diversas cepas bacterianas relacionadas con la degradación de alimentos o indicadoras de la presencia de patógenos.
- Determinar la capacidad de inhibición del crecimiento fúngico, tanto frente a mohos como a levaduras.
- Conocer la capacidad antioxidante de los aceites esenciales mediante la utilización de diferentes técnicas analíticas.
- Aplicar los aceites esenciales obtenidos de las hierbas aromáticas sometidas a estudio en films de quitosano.
- Determinar la capacidad antioxidante de los films de quitosano adicionados con los aceites esenciales sometidos a estudio.
- Determinar la actividad antibacteriana de los films de quitosano adicionados con los aceites esenciales sometidos a estudio.
- Evaluar la vida útil de un producto cárnico cocido loncheado en el que se han utilizado, como separadores de lonchas, films de quitosano adicionados con los aceites esenciales de estudio.

MATERIALES Y METODOS

4.- MATERIALES Y METODOS

En este apartado se recoge un resumen de los materiales utilizados, de los procesos de elaboración aplicados y de los análisis efectuados. La información completa de toda esta metodología se ha desarrollado en los respectivos artículos publicados en revistas internacionales y que se adjuntan a esta memoria.

4.1.- Material vegetal.

El material vegetal empleado para la extracción de los aceites esenciales utilizados en el presente estudio, ha sido: cantueso (*Thymus moroderi*), pebrella (*Thymus piperella*), abrotano (*Santolina chamaecyparissus*) y rabo de gato (*Sideritis angustifolia*). Todas estas plantas eran de crecimiento silvestre, fueron recolectadas en la provincia de Alicante durante su periodo de floración y fueron identificadas por la Dra. Concepción Obón del Área de Botánica del Departamento de Biología Aplicada. En la figura 4 se muestran las plantas utilizadas para la obtención de los aceites esenciales.

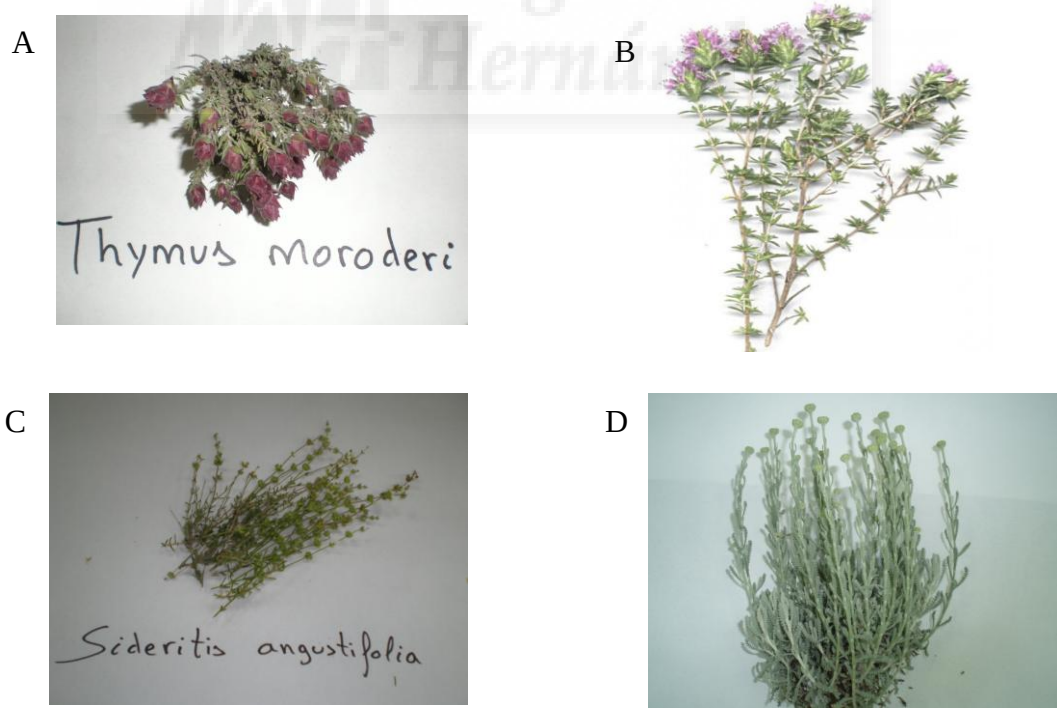

Figura 4. Plantas utilizadas para la obtención de los aceites esenciales analizados. A: *Thymus moroderi*; B: *Thymus piperella*; C: *Sideritis angustifolia*; D: *Santolina chamaecyparissus*

4.2.- Extracción de los aceites esenciales

Los aceites esenciales (AE) de *T. moroderi, T. piperella, S. chamaecyparissus y S. angustifolia* se extrajeron de la planta completa (tallos, hojas y flores) mediante hidrodestilación, utilizando un equipo tipo Clevenger, durante 3 horas. La capa oleosa obtenida en la parte superior del destilado acuoso se separó y se secó con sulfato de sodio anhidro (0,5 g). Los AE así extraídos se guardaron a 4 ºC en viales de vidrio opacos y sellados herméticamente hasta su posterior análisis. Los rendimientos obtenidos para los distintos AEs fueron *T. moroderi* 1,90%, *T. piperella* 1,66%, *S. chamaecyparissus* 1% y *S. angustifolia* 0,90%.

4.3.- Composición química de los aceites esenciales de especias

La determinación de la composición de química de los distintos aceites esenciales estudiados se realizó mediante cromatografía de gases/espectrometría de masas (CG/MS) en un cromatógrafo de gases Shimadzu GC-17A (Shimadzu Corporation, Tokio, Japón), acoplado a un detector selectivo de masas Shimadzu GCMS-QP5050A (Shimadzu Corporation) equipado con una columna TRACSIL Meta X5 (Teknokroma S. Coop. C. Ltd, Barcelona, España), 30 m x 0.25 mm i.d., y un espesor de relleno de 0.25 µm. Las Tª del inyector y del detector fueron de 250 y 300 ºC, respectivamente. La Tª del horno se mantuvo a 40 ºC durante 5 min incrementándose gradualmente hasta los 200 ºC en un ratio de 3 ºC/min manteniéndose a esta Tª durante 1 min. Transcurrido este tiempo se volvió a aumentar la Tª hasta los 280 ºC en un ratio de 15 ºC/min manteniéndose a 280 ºC durante 10 min. Como gas portador se utilizó Helio con un flujo de 1 mL/min. La identificación de los compuestos se realizó mediante comparación de los tiempos de retención y el espectro de masas con los de los estándares previamente inyectados, así como, con la biblioteca Wiley 229 del sistema, los valores del índice de Kovats y valores presentes en la literatura científica.

4.4.- Actividad antibacteriana de los aceites esenciales

Para la determinación de la actividad antibacteriana se seleccionaron cepas bacterianas relacionadas con el deterioro de alimentos en refrigeración y cepas indicadoras de cepas patógenas: *Listeria innocua* CECT 910, *Serratia marcenscens* CECT 854, *Pseudomonas fragi* CECT 446, *Pseudomonas fluorescens* CECT 844,

Aeromonas hydrophila CECT 5734, *Shewanella putrefaciens* CECT 5346, *Achromobacter denitrificans* CECT 449, *Enterobacter amnigenus* CECT 4078, *Enterobacter gergoviae* CECT 587, *Alcaligenes faecalis* CECT 145 *y Leuconostoc carnosum* CECT 4024. Todas ellas fueron adquiridas en la Colección Española de Cultivos Tipo de la Universidad de Valencia (España). Para la determinación de la actividad antibacteriana se empleó el método de difusión de disco en agar, siguiendo las recomendaciones de Tepe et al. (2005). También se determinó el efecto de la concentración siguiendo el método descrito por Viuda-Martos et al. (2005).

4.5.- Actividad antifúngica de los aceites esenciales

La actividad antifúngica de los aceites esenciales se ensayó frente a seis cepas de hongos, seleccionadas por su relación con el deterioro de alimentos, como son: *Aspergillus niger* CECT 2091, *Aspergillus flavus* CECT 2685, *Mucor racemosus* CECT 2670, *Mucor circinelloides* CECT 20765, *Penicillium chrysogenum* CECT 2784 y *Alternaria alternata* CECT 20560. Esta actividad antifúngica también se ensayó frente a seis levaduras: *Yarrowia lipolytica* CECT 1240, *Saccharomyces cerevisiae* CECT 1383, *Candida zeylanoides* CECT 10048, *Debaryomyces hansenii* CEPT 11369, *Rhodotorula mucilaginosa* CECT 10011, *Pichia carsonii* CECT 10227. Todas las cepas se adquirieron en la Colección Española de Cultivos Tipo de la Universidad de Valencia (España). Para analizar el efecto inhibidor de los AE frente a los hongos se empleó el método de dilución en agar, siguiendo las recomendaciones de Fraternale et al. (2003). Las concentraciones analizadas fueron 0,2; 0,1; 0,05 y 0,025 mL/100 mL. Para la determinación de la actividad antifúngica frente a las levaduras se empleó el método colorimétrico de microdilución propuesto por Abate et al. (1998). Las concentraciones analizadas estaban comprendidas entre 0,04 y 40 µL/mL.

4.6.- Actividad antioxidante de los aceites esenciales

La determinación de la actividad antioxidante *in vitro* se realizó siguiendo cuatro métodos analíticos diferentes: (i) secuestro del radical 2,2'-difenil-1-picrilhidrazil (DPPH), siguiendo las recomendaciones de Brand-Williams et al. (1995); (ii) poder antioxidante de la reducción de hierro (FRAP), según el método descrito por Oyaizu (1986); (iii) actividad antioxidante de las especies reactivas del ácido tiobarbitúrico

(TBARS), siguiendo las indicaciones de Daker et al. (2008); y (iv) capacidad quelante del ion ferroso (FIC), según el método utilizado por Carter (1971). También se determinó el contenido en fenoles totales utilizando el reactivo Folin-Ciocalteu's, siguiendo las recomendaciones de Singleton y Rossi (1965).

4.7.- Elaboración de films de quitosano con aceites esenciales

Para la elaboración de los films de quitosano adicionados con los aceites esenciales se siguió la metodología descrita por Ojagh et al. (2010). Se utilizó quitosano de alto peso molecular con un grado de deacetilacion de 75-85% (Sigma-Aldrich Chemical Co., Steinheim, Alemania) al 2% en una disolución de ácido láctico al 1%, utilizando Tween 80 como emulsionante al 0,2% y glicerol (0,75 mL/g quitosano) como plastificante. Se utilizaron, como moldes, placas de Petri estériles de 6 cm de diámetro. Se dosificaron 7 g de dilución a cada placa y se llevaron a deshidratar a 37ºC durante 48 h en estufa de convección. Transcurrido ese tiempo se almacenaron a 25 ºC y una humedad relativa de 51% hasta su uso. Los diferentes AEs se adicionaron a los films de quitosano a diferentes concentraciones (Tabla 3).

Tabla 3. Concentraciones y aceites esenciales empleados en la elaboración de films de quitosano.

Formulación	*T. piperella* (%)	*T. moroderi* (%)	*S. chamaecyparissus* (%)	*S. angustifolia* (%)
CH	0	0	0	0
CH+0,5TP	0,5	0	0	0
CH+1TP	1	0	0	0
CH+2TP	2	0	0	0
CH+0,5TM	0	0,5	0	0
CH+1TM	0	1	0	0
CH+2TM	0	2	0	0
CH+0,5SC	0	0	0,5	0
CH+1SC	0	0	1	0
CH+2SC	0	0	2	0
CH+0,5SA	0	0	0	0,5
CH+1SA	0	0	0	1
CH+2SA	0	0	0	2

CH: quitosano; CH+0,5TP: quitosano más 0,5% de AE de *T. piperella*; CH+1TP: quitosano más 1% de AE de *T. piperella*; CH+2TP: quitosano más 2% de AE de *T. piperella*; CH+0,5TM: quitosano más 0,5% de AE de *T. moroderi*; CH+1TM: quitosano más 1% de AE de *T. moroderi*; CH+2TM: quitosano más 2% de AE de *T. moroderi*; CH+0,5SC: quitosano más 0,5% de AE de *S. chamaecyparissus*; CH+1SC: quitosano más 1% de AE de *S. chamaecyparissus*; CH+2SC: quitosano más 2% de AE de *S. chamaecyparissus*; CH+0,5SA: quitosano más 0,5% de AE de *S. angustifolia*; CH+1SA: quitosano más 1% de AE de *S. angustifolia*; CH+2SA: quitosano más 2% de AE de *S. angustifolia*.

4.8.- Determinación de la actividad antibacteriana de los films de quitosano

Para la determinación de la actividad antibacteriana de los films de quitosano adicionados con los AE de *T. moroderi, T. piperella, S. chamaecyparissus y S. angustifolia* se siguió la metodología descrita en el apartado 4.4

4.9.- Determinación de la actividad antioxidante de los films de quitosano

Para la determinación de la actividad antioxidante de los films de quitosano adicionados con los AE de *T. moroderi, T. piperella, S. chamaecyparissus y S. angustifolia* se siguió la metodología descrita en el apartado 4.6

4.10.- Aplicación de los films en un producto cárnico tipo Jamón cocido

El Jamón Cocido (JC) fue adquirido directamente de un supermercado y transportado inmediatamente, en condiciones de refrigeración, al laboratorio del grupo de Industrialización de Productos de Origen Animal (IPOA) localizado en el Departamento de Tecnología Agroalimentaria de la Escuela Politécnica Superior de Orihuela. Posteriormente se procedió a lonchear el producto, obteniendo lonchas con un espesor de 3 mm y un peso aproximado de 12,5 g. Los films elaborados con las distintas formulaciones se colocaron como separadores entre dos lonchas de JC y fueron introducidas en bolsas estériles de polietileno y poliamida laminada con las siguientes características: permeabilidad al vapor de agua a 23 ºC 1,1 g/m^2/24 h; permeabilidad al nitrógeno a 23 ºC 10 cm^3/m^2/24 h; permeabilidad al dióxido de carbono a 23º C 140 cm^3/m^2/24 h y permeabilidad al oxígeno a 23 ºC 30 cm^3/m^2/24 h (Fibran, Girona, España). De igual modo se envasaron dos lonchas sin ningún tipo de película, que actuarían como muestras control.

Las bolsas se sellaron y fueron almacenadas a temperatura de refrigeración 4±1 ºC durante 21 días. El análisis de las muestras para cada tratamiento se realizó a los 0, 7, 14, y 21 días (tiempo de almacenamiento) y dichos análisis se llevaron a cabo el mismo día del muestreo.

4.11.- Efecto de los films de quitosano con aceites esenciales sobre las características y vida útil de un producto cárnico tipo Jamón cocido.

4.11.1.- Determinaciones Físico-Químicas.

4.11.1.1.- Color.

Las determinaciones de color se efectuaron de acuerdo a las normas de la Asociación Americana de la Carne (Hunt et al., 1991). Dichas determinaciones se realizaron mediante un colorímetro Minolta CM-2600 (Minolta Camera Co., Osaka, Japón) con iluminante D_{65}, y el observador 10º. En todas las determinaciones de color se interpusieron cristales de baja reflectancia Minolta CR-A51/1829-752 (Minolta Co., Osaka, Japón) entre las muestras y el equipo (Hunt et al., 1991).

4.11.1.2.- pH.

El pH de cada una de las unidades de muestras se determinó bajo las directrices del Ministerio de Agricultura Pesca y Alimentación (1994), mediante disolución acuosa en una proporción 1:10 de producto y agua destilada. Las lecturas de pH se efectuaron con un equipo Crison modelo 507 (Crison, Barcelona, España).

4.11.2.- Oxidación lipídica

La determinación de la oxidación lipídica se realizó mediante el test del ácido 2-tiobarbiturico siguiendo las directrices descritas por Buege y Aust (1978). Los resultados se expresaron como mg malonaldehido/kg de muestra.

4.11.3.- Análisis microbiológico.

Para el recuento microbiológico, se tomaron 25 g de muestra que se homogeneizaron con 225 mL de agua de peptona al 1,5% en un Stomacher 400 (Colworth, Londres, Reino Unido) durante 2 minutos. Los medios de cultivo utilizados y las condiciones de incubación se detallan a continuación:

- Recuento total de aerobios mesófilos en agar de recuento en placa (PCA) a 35 ºC durante 48 horas.
- Recuento total de bacterias ácido lácticas en una doble capa de MRS (Man, Rogosa and Sharpe Agar) a 35 ºC durante 48 horas.
- Recuento total de enterobacterias en Agar Glucosa Bilis Rojo Violeta (VRBG) a 35 ºC durante 48 horas.
- Recuento total de mohos y levaduras en Agar Rosa de Bengala con cloramfenicol a 26 ºC durante 5 días.

4.11.4.- Determinaciones del contenido en fenoles totales de los films

Para la evaluación de la liberación de compuestos bioactivos de los films durante el almacenamiento refrigerado de las muestras se procedió de la siguiente manera: en el momento del muestreo se recuperó el film, se pesó y se realizó una extracción con 5 mL de metanol en un baño de ultrasonidos durante 1 hora. Posteriormente se centrifugaron las muestras y con el sobrenadante obtenido se procedió a la determinación del contenido en fenoles totales utilizando el reactivo Folin-Ciocalteu's siguiendo las recomendaciones de Singleton y Rossi (1965).

4.12. Metodología estadística

La metodología estadística se diseñó para cada uno de los estudios a analizar. La totalidad de los análisis se realizaron mediante el paquete estadístico Statgraphics Plus para Windows versión 5.1 (Statical Graphics Corp., Rockville, USA) utilizando el programa Analisis of Variance.

Para la determinación de las media y la desviación estándar se siguieron métodos estadísticos convencionales. El análisis estadístico empleado en cada ensayo fue la aplicación de un análisis de la varianza (ANOVA) de uno, dos o tres factores, dependiendo del ensayo realizado.

Para estudiar entre qué variables de los factores principales las diferencias fueron estadísticamente significativas se realizaron contrastes entre las medias, aplicando el test de Tukey siguiendo las recomendaciones de Afifi y Azen (1979).

RESULTADOS

5.- RESULTADOS Y DISCUSION

Este capítulo recoge los principales resultados y una breve discusión de los diferentes trabajos realizados. Las versiones completas de los mismos se encuentran en los correspondientes artículos publicados o en proceso de revisión en revistas internacionales incluidas en el *Journal Citations Reports* y se adjuntan al final de esta memoria (capítulo octavo).

5.1.- Composición química de los aceites esenciales sometidos a estudio

El objetivo de estos trabajos, publicados en las revistas "*Food Control*" (2012, 27:294-299) y "*Food Safety*" (2012, 32:426-434), fue la determinación de la composición química de los aceites esenciales (AE) objeto de estudio *Thymus moroderi*, *Thymus piperella*, *Santolina angustifolia* y *Sideritis chamaecyparissus* mediante el empleo de cromatografía de gases acoplado a espectrometría de masas.

En la Tabla 4 se muestran los principales componentes químicos, así como su porcentaje relativo con respecto al total y el índice de Kovats, de los distintos aceites esenciales objeto de estudio.

En el caso del AE obtenido de *T. moroderi* se identificaron 51 compuestos distintos, representando el 92% de los componentes del aceite. Los compuestos principales fueron canfor (26,74%), 1,8-cineol (24,99%) y mirceno (5,63%). Para el AE de *T. piperella* se identificaron 48 compuestos, representando el 90,5% de los componentes del aceite. Los componentes mayoritarios identificados en este AE fueron el carvacrol (31,92%) el *para*-cimeno (16,18%) y el γ-terpineno (10,11%). En el análisis del AE de *S. chamaecyparissus*, se identificaron 58 componentes representando el 90,1% del total de componentes, siendo el componente principal la artemisa-ketona (27,19%) seguido por dihidro-aromadendreno (18,21%) y β-felandreno (7,49%). Para el AE de *S. angustifolia* se identificaron 77 compuestos, los cuales representaban el 94,6% del total de componentes del aceite. El componente mayoritario fue el α-pineno (12,71%). Otros componentes importantes fueron el β-felandreno (11,97%) y el 1,8-cineol (7,41%).

Existe una gran variabilidad de composición entre los AE pertenecientes al mismo género y especie, tal y como se muestra en la literatura científica. Esta

variabilidad puede atribuirse a diversos factores como las condiciones climáticas y medioambientales del lugar de recolección, la temporada del año en la que se realizó dicha recolección, la situación geográfica, la disponibilidad de agua, la altura sobre el nivel del mar, la presencia de enfermedades causadas por hongos e insectos, la parte de la planta empleada, el secado y almacenamiento post-cosecha o el método utilizado para obtener el AE (Viuda-Martos et al., 2008).

Tabla 4. Principales componentes químicos, así como, su porcentaje relativo con respecto al total e índice de Kovats de los distintos aceites esenciales objeto de estudio.

	Indice de Kovats			Aceite esencial			
Compuesto	**Id.[1]**	**KI**	**Lit[2]**	***T. moroderi* (% área)**	***T. piperella* (% área)**	***S. angustifolia* (% área)**	***S. chamaecyparisus* (% área)**
α-pineno	KI,W	940	939	4,35	---	12,71	---
Canfeno	KI,W	960	953	8,8	---	---	---
Mirceno	KI,W	989	991	5,63	---	---	6,94
***Para*-cimeno**	KI,W,St	1032	1027	---	16,18	---	---
β-feladreno	KI,W	1033	1031	---	---	11,97	7,49
1,8-cineol	KI,W,St	1042	1039	24,99	---	7,41	---
Artemisia ketona	KI,W	1061	1062	---	---	---	27,19
γ-terpineno	KI,W	1064	1062	---	10,11	---	---
Ho-trienol	KI,W	1101	1101	---	---	5,33	---
Canfor	KI,W	1163	1143	26,74	---	---	3,88
Terpinen-4-ol	KI,W	1191	1189	2,36	7,29	2,46	---
Timol	KI,W,St	1294	1290	---	5,19	---	---
Carvacrol	KI,W,St	1307	1299	---	31,92	---	---
Trans-cariofileno	KI,W	1436	1430	---	6,09	6,33	---
Biciclogermacreno	KI,W	1511	1494	1,86	---	5,11	---
Isocitronellon	KI,W	1565	1563	---	---	5,83	---
Dihidroaromadendreno	KI,W	1669	1665	---	---	---	18,21

[1]"KI,W" significa que la identificación se basó en el índice de Kovats y la comparación con la biblioteca Wiley. "KI,W,St" significa que la identificación se basó en el índice de Kovats, la comparación con la biblioteca Wiley y la comparación con el compuesto estándar. [2]Base de Datos NIST.

5.2.- Actividad antibacteriana de los aceites esenciales sometidos a estudio

El objetivo de estos trabajos, publicados en las revistas "*Food Control*" (2012, 27:294-299) y "*Food Safety*" (2012, 32:426-434), fue la determinación de la actividad antibacteriana (mediante el empleo de la técnica de difusión en disco) de los AE obtenidos de *T. moroderi*, *T. piperella*, *S. angustifolia* y *S. chamaecyparissus*, frente a diversas bacterias relacionadas con el deterioro de alimentos o indicadoras del mismo.

La Tabla 5 muestra la inhibición del crecimiento bacteriano obtenido para cada uno de los AEs analizados. Como puede observarse, el AE de *T. piperella* mostró actividad inhibidora del crecimiento sobre 5 de las 11 bacterias analizadas, con halos de

inhibición comprendidos entre 16,00 mm para *A. denitrificans* y 45,00 mm para *A. hydrophila*.

Tabla 5. Valores de inhibición del crecimiento bacteriano obtenidos para los distintos aceites esenciales (40µL) impregnados en un disco de 9 mm de diámetro

Bacteria	Aceite esencial			
	T. moroderi	*T. piperella*	*S. chamaecyparissus*	*S. angustifolia*
S. marcescens	23,90±0,10[a¥Ж]	24,50±0,70[a]	13,50±0,07[b]	14,80±0,04[c]
P. fragi	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]
P. fluorescens	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]
A. hydrophila	20,50±0,70[a]	45,50±3,50[b]	16,50±0,07[c]	11,00±0,00[d]
S. putrefaciens	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]
E. gergoviae	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]
E. amnigenus	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]
A. faecalis	24,00±1,40[a]	45,50±0,70[b]	16,50±0,07[c]	18,00±0,70[d]
A. denitrificans	9,00±0,00[a]	16,00±0,00[b]	11,50±0,07[c]	12,50±0,07[d]
L. carnosum	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]
L. innocua	15,50±0,70[a]	27,30±1,20[b]	12,70±0,02[c]	12,10±0,01[d]

¥Para una misma cepa. Valores seguidos de la misma letra dentro de la misma fila no presentan diferencias estadísticamente significativas (p>0,05) de acuerdo con el Test de Rangos Múltiples de Tukey.
ЖDiámetro (media y desviación) del halo de inhibición (mm) incluyendo el diámetro del disco de 9 mm

Hay que destacar que el AE de *T. piperella* fue el que presentó un mayor halo de inhibición bacteriana para todas aquellas cepas bacterianas que resultaron sensibles a dichos AEs. En el caso del AE de *T. moroderi*, presentó actividad antibacteriana sobre 4 de las 11 cepas analizadas, con halos de inhibición que variaban entre los 15 mm para *L. innocua* y 24 mm para *A. faecalis*.

Cuando se analizó la actividad antibacteriana del AE de *S. chamaecyparissus*, este mostró, al igual que ocurría con el AE de *T. piperella*, acción inhibitoria del desarrollo sobre 5 de las 11 bacterias analizadas, con halos de inhibición comprendidos entre los 11,50 mm para *A. denitrificans* y 16,50 mm para *A. hydrophila* y *A. faecalis*. El AE de *S. angustifolia*, también presentó actividad inhibitoria del desarrollo bacteriano sobre 5 de las 11 bacterias analizadas, con halos de inhibición que oscilaban entre los 11 mm para *A. hydrophila* y los 18 mm para *A. faecalis*.

Es importante resaltar que ninguno de los aceites esenciales analizados presentó actividad antibacteriana frente a las siguientes cepas: *P. fragi*, *P. fluorescens*, *S. putrefaciens*, *E.* gergoviae, *E. amnigenus* y *L. carnosum*.

El efecto de la aplicación de diferentes volúmenes de AE (40, 20, 10, 4 y 2 µL), sobre su actividad antibacteriana, se puede observar en la Tabla 6. Dicha determinación solo se realizó sobre aquellas cepas bacterianas que presentaron inhibición a la máxima concentración ensayada, en este caso 40 µL (ver Tabla 5).

Tabla 6. Efecto del volumen adicionado de los distintos aceites esenciales objeto de estudio sobre la inhibición del crecimiento bacteriano.

Aceite esencial	Volumen (µL)	Bacteria				
		S. marcescens	*A. hydrophila*	*A. faecalis*	*A. denitrificans*	*L. innocua*
T. moroderi	2	9,00±0,00[a]Ж	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]
	4	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]
	10	11,30± 0,10[a]	12,50±0,70[a]	11,50±0,70[a]	9,00±0,00[a]	10,90±0,10[a]
	20	14,90± 0,10[b]	16,00±1,40[b]	13,50±0,00[b]	9,00±0,00[a]	12,20±0,10[b]
	40	23,90±0,10[c]	20,50±0,70[c]	24,00±1,40[c]	9,00±0,00[a]	15,50±0,70[c]
T. piperella	2	13,80±0,40[a]	32,00±0,00[a]	21,50±0,70[a]	11,00±0,00[a]	15,80±0,30[a]
	4	15,70±0,60[b]	35,50±2,10[b]	27,50±2,10[b]	12,00±0,00[b]	17,80±0,40[b]
	10	19,60±0,60[c]	38,50±2,10[b]	30,05±2,10[c]	13,00±0,00[c]	21,50±0,70[c]
	20	22,80±0,70[d]	43,00±2,80[c]	35,00±0,70[d]	14,50±0,70[d]	23,30±1,10[d]
	40	24,50±0,70[e]	45,50±3,50[c]	45,50±0,70[e]	16,00±0,00[e]	27,30±1,20[e]
S. chamaecyparissus	2	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]
	4	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]
	10	9,00±0,00[a]	9,00±0,00[a]	11,50±0,70[b]	9,00±0,00[a]	9,00±0,00[a]
	20	9,00±0,00[a]	10,00±0,00[b]	14,00±0,00[b]	9,00±0,00[a]	9,00±0,00[a]
	40	13,50±0,07[b]	16,50±0,07[c]	16,50±0,07[d]	11,50±0,07[b]	12,70±0,02[b]
S. angustifolia	2	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]
	4	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]
	10	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]
	20	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]	9,00±0,00[a]
	40	14,80±0,04[b]	11,00±0,00[b]	18,00±0,00[b]	12,50±0,07[b]	12,10±0,01[b]

¥Para un mismo AE. Valores seguidos de la misma letra dentro de la misma columna no presentan diferencias estadísticamente significativas (p>0,05) de acuerdo con el Test de Rangos Múltiples de Tukey.
ЖDiámetro (media y desviación estándar) del halo de inhibición (mm) incluyendo el diámetro del disco de 9 mm.

En el caso del AE de *T. moroderi*, no se observó ningún efecto antibacteriano de los discos impregnados con 4 y 2 µL, sobre ninguna de las cinco bacterias analizadas. Sin embargo, para los discos impregnados con 40, 20 y 10 µL si se apreció un efecto inhibidor del desarrollo de dichas bacterias, produciéndose dicho efecto inhibidor de forma concentración dependiente ($p<0,05$). Por lo que respecta al AE de *T. piperella*, a todas las concentraciones ensayadas, se obtuvieron halos de inhibición en las cepas analizadas (*S. marcescens*, *A. denitrificans*, *A. faecalis*, *A. hydrophila* y *L. innocua*). Apreciándose, además, diferencias estadísticamente significativas ($p<0,05$) para todos los volúmenes empleados, así como para las cepas. Para el AE de *S. chamaecyparissus* no hubo efecto inhibitorio del desarrollo de las bacterias seleccionadas con los discos impregnados con 10, 4 y 2 µL, excepto en el caso de *A. faecalis* que si se observó un ligero halo cuando se empleó un volumen de 10 µL. Para *S. marcescens*, *A. denitrificans* y *L. innocua*, no se apreció ninguna actividad antibacteriana en ninguno de los discos impregnados con 20, 10, 4 y 2 µL. *A. hydrophila* mostró halos de inhibición en los discos impregnados con 40 y 20 µL apreciándose diferencias significativas entre ellos ($p<0,05$). En el caso del AE de *S. angustifolia* solo se obtuvieron halos de inhibición cuando se ensayó el volumen máximo (40 µL). Los volúmenes de 20, 10, 5 y 2 µL no tuvieron ningún efecto sobre las bacterias seleccionadas.

Aunque está ampliamente demostrado que los AEs presentan una amplia actividad antibacteriana, la razón por la que ésta se produce aún no está bien establecida. Dicha actividad podría estar provocada por los componentes principales de los AEs, o podría deberse a un efecto sinérgico entre compuestos que se hallan en menor proporción, o a sinergias entre los compuestos principales y los de menor concentración, tal y como establece Carović-Stanko et al. (2010). Del mismo modo que no se conoce qué compuesto provoca la actividad antibacteriana, tampoco se conoce el mecanismo por el cual ésta se produce. En la literatura científica existen muchas teorías que tratan de explicarlo, entre ellas están: la rotura de la bicapa lipídica de la membrana celular de la bacteria, la interrupción de los sistemas enzimáticos, la coagulación del citoplasma celular, ataques sobre el material genético o daños sobre las proteínas de membrana que permiten el flujo de electrones al exterior (Oussalah et al., 2006; Viuda-Martos et al., 2011b; Xing et al., 2012). Los resultados obtenidos muestran que las características de pared de las bacterias (ser Gram-positivas o Gram-negativas) no han

condicionado la efectividad de los aceites sino que la sensibilidad a cada aceite es especie específica.

5.3.- Actividad antifúngica de los aceites esenciales sometidos a estudio

El objetivo de este trabajo, publicado en la revista "*Journal of Food Protection*" (2013, 76:1218-1225), fue la determinación de la actividad antifúngica de los AE obtenidos de *T. moroderi*, *T. piperella*, *S. angustifolia* y *S. chamaecyparissus* frente a mohos relacionados con el deterioro de alimentos, así como frente a levaduras. Para la determinación de la actividad antifúngica frente a los mohos se empleó el método de dilución en agar, mientras que para la determinación de la actividad antifúngica frente a las levaduras se empleó el método de microdilución.

La Tabla 7 muestra el porcentaje de inhibición del crecimiento del micelio (% ICM) provocado por los AEs de *T. moroderi*, *T. piperella*, *S. angustifolia* and *S. chamaecyparissus* frente a diversos mohos.

En el caso de *A. niger*, todos los AEs, a todas las concentraciones ensayadas, mostraron un reducción del crecimiento del micelio. El AE de *T. moroderi* fue el que provocó los mayores valores de ICM ($p<0,05$) cuando se aplicó a altas concentraciones (0,1 y 0,2 mL/100 mL). Por el contrario, el AE de *S. angustifolia* fue el que mostró los valores más bajos de ICM a todas las concentraciones analizadas. En lo que respecta a *A. flavus*, el AE de *S. chamaecyparissus* fue el que mostró los valores más altos ($p<0,05$) de ICM, cuando se usó a la máxima concentración (0,2 mL/100 mL), mientras que a concentraciones de 0,1 y 0,05 mL/100 mL fue el AE de *T. moroderi* el que obtuvo los mayores valores de ICM ($p<0,05$). El AE de *T. piperella*, a todas las concentraciones analizadas, fue el que mostró los valores más bajos de ICM para este moho. El AE de *S. angustifolia*, a las concentraciones de 0,1 and 0,2 mL/100 mL, fue el más efectivo ($p<0,05$) en reducir el crecimiento de *A. alternata*, *P. chrysogenum* y *M. racemosus*. El AE de *T. piperella* fue el que mostró los valores más bajos ($p<0,05$) de ICM para *A. alternata* y *P. chrysogenum*, a todas las concentraciones analizadas. Por lo que respecta a *M. circinelloides*, fue el AE de *S. chamaecyparissus* el que mostro los mayores valores de ICM ($p<0,05$), a las concentraciones más elevadas. Cuando se usaron concentraciones más bajas, fue el AE de *S. angustifolia* el que mostró los valores más bajos ($p<0,05$) de ICM.

Tabla 7. Porcentaje de inhibición del crecimiento del micelio (ICM) provocado por los aceites esenciales de *T. moroderi*, *T. piperella*, *S. angustifolia* y *S. chamaecyparissus*, frente a diversos mohos.

		% Inhibición del crecimiento del micelio (ICM)			
Mohos	C$^{\Psi}$	*T. moroderi*	*T. piperella*	*S. angustifolia*	*S. chamaecyparissus*
A. niger	0,025	6,42±0,12^{aA}	8,44±0,02^{bA}	4,06±0,15^{cA}	6,68±0,12^{aA}
	0,05	13,73±0,24^{aB}	14,33±0,01^{bB}	12,91±0,09^{cB}	14,50±0,06^{dB}
	0,1	29,01±0,15^{aC}	26,79±0,12^{bC}	22,10±0,11^{cC}	22,24±0,07^{cC}
	0,2	52,54±0,23^{aD}	44,94±0,05^{bD}	33,74±0,13^{cD}	42,82±0,12^{dD}
A. flavus	0,025	10,10±0,11^{aA}	3,94±0,07^{bA}	8,43±0,08^{cA}	7,06±0,11^{dA}
	0,05	19,89±0,23^{aB}	10,76±0,14^{bB}	13,61±0,10^{cB}	13,58±0,08^{cB}
	0,1	26,79±0,10^{aC}	16,21±0,09^{bC}	21,88±0,07^{cC}	23,26±0,14^{dC}
	0,2	34,84±0,11^{aD}	28,22±0,10^{bD}	29,54±0,09^{cD}	41,23±0,09^{dD}
A. alternata	0,025	12,83±0,06^{aA}	0,00±0,00^{bA}	14,47±0,01^{cA}	14,48±0,06^{cA}
	0,05	24,57±0,12^{aB}	15,80±0,06^{bB}	33,24±0,12^{cB}	23,96±0,07^{dB}
	0,1	39,27±0,14^{aC}	20,64±0,08^{bC}	50,29±0,12^{cC}	32,83±0,11^{dC}
	0,2	54,69±0,06^{aD}	29,43±0,09^{bD}	55,70±0,09^{cD}	43,96±0,14^{dD}
P. chrysogenum	0,025	13,84±0,05^{aA}	0,00±0,00^{bA}	10,51±0,12^{cA}	1,17±0,11^{dA}
	0,05	30,47±0,09^{aB}	5,56±0,11^{bB}	21,69±0,14^{cB}	7,24±0,15^{dB}
	0,1	51,57±0,11^{aC}	8,33±0,05^{bC}	60,56±0,05^{cC}	38,13±0,06^{dC}
	0,2	63,94±0,15^{aD}	28,33±0,13^{bD}	79,81±0,04^{cD}	53,94±0,01^{dD}
M. circinelloides	0,025	4,92±0,06^{aA}	7,01±0,03^{bA}	0,00±0,00^{cA}	1,60±0,20^{dA}
	0,05	13,98±0,07^{aB}	9,23±0,04^{bB}	1,12±0,01^{cB}	6,79±0,12^{dB}
	0,1	19,01±0,09^{aC}	29,14±0,14^{bC}	26,88±0,14^{cC}	33,59±0,09^{dC}
	0,2	22,57±0,13^{aD}	44,57±0,07^{bD}	42,81±0,12^{cD}	45,60±0,14^{dD}
M. racemosus	0,025	1,46±0,04^{aA}	3,58±0,04^{bA}	0,00±0,00^{cA}	0,00±0,00^{cA}
	0,05	17,54±0,12^{aB}	5,58±0,14^{bB}	12,61±0,08^{cB}	0,00±0,00^{dA}
	0,1	30,29±0,08^{aC}	18,28±0,14^{bC}	49,60±0,07^{cC}	14,93±0,18^{dB}
	0,2	40,02±0,09^{aD}	38,63±0,17^{bD}	62,24±0,11^{cD}	29,06±0,15^{dC}

$^{\Psi}$C: Concentración (mL/100 mL)
Para un mismo moho y una misma concentración: valores seguidos por la misma letra minúscula dentro de la misma fila no son significativamente diferentes (p>0,05) de acuerdo con el Test de Rangos Múltiples de Tukey.
Para un mismo moho y un mismo AE: valores seguidos por la misma letra mayúscula dentro de la misma columna no son significativamente diferentes (p>0,05) de acuerdo con el Test de Rangos Múltiples de Tukey.

El método de microdilución se empleó para determinar la actividad antifúngica de los AEs frente a diversas levaduras, y los resultados se muestran en la Tabla 8. Todos los AEs analizados presentaron efecto inhibidor del desarrollo de todas las cepas de levadura ensayadas. Fue el AE de *S. angustifolia* el que mostró la menor concentración mínima de inhibición (CMI) sobre las levaduras *R. mucilaginosa*, *S. cerevisiae*, *D. hansenii* y *P. carsoni*. Por otro lado el AE de *T. moroderi* fue el más eficaz frente a *Y. lipolytica* con un valor de CMI de 0,25 mL/100 mL. Sin embargo, este AE presentó los valores más altos de CMI para *C. zeylanoides* y *S. cerevisiae*.

Aligianis et al. (2001) propusieron una clasificación de la actividad antimicrobiana para productos vegetales, basada en los resultados de la CMI: compuestos vegetales con CMI ≤ 0,05 mL/100 ml, se clasificarían como inhibidores fuertes; compuestos vegetales con CMI entre 0,06 y 0,15 mL/100 mL, inhibidores moderados; compuestos vegetales con CMI ≥ 0,16 mL/100 mL, inhibidores débiles. Por lo tanto, basado en los resultados de la CMI de los ensayos de microdilución, los AEs analizados mostraron un pobre potencial como compuestos "antilevaduras", ya que todas las cepas de levaduras analizadas presentaron un valor de CMI > a 0,16 mL/100 mL.

Tabla 8. Valores de Concentración Mínima de Inhibición (CMI) provocado por los AEs de *T. moroderi*, *T. piperella*, *S. angustifolia* y *S. chamaecyparissus* frente a diversas levaduras

Levadura	Aceite esencial (µL/mL)			
	T. moroderi	*T. piperella*	*S. angustifolia*	*S. chamaecyparissus*
Y. lipolytica	2,5	5	5	5
C. zeylanoides	5	2,5	2,5	2,5
P. carsonni	5	5	2.5	5
S. cerevisiae	10	5	2,5	5
D. hansenii	2,5	2,5	1,25	2,5
R. mucilaginosa	5	5	2,5	5

Al igual que sucede con el mecanismo por el cual se produce la actividad antibacteriana, el mecanismo por el cual se produce la inhibición del crecimiento de los mohos y levaduras por la acción de los AEs tampoco es conocido con total detalle. Son

varias las publicaciones científicas que aportan datos para tratar de explicar dicho comportamiento. Así, Lucini et al. (2006) indicaron que la inhibición del crecimiento del micelio es causada por los monoterpenos presentes en los aceites esenciales. Estos componentes podrían aumentar la concentración de peróxidos lipídicos tales como radicales hidroxilo, alcoxilo y alkoperoxilo y así provocar la muerte celular. Para Sharma y Tripathi (2006), los AEs actuaría sobre las hifas del micelio, provocando la salida de los componentes del citoplasma, la pérdida de rigidez y la integridad de la pared celular de la hifa, lo que provocaría su colapso y como consecuencia la muerte del micelio.

5.4.- Actividad antioxidante de los aceites esenciales sometidos a estudio

El objetivo de este trabajo, publicado en la revista "*Journal of Food Protection*" (2013, 76:1218-1225), fue la evaluación de la actividad antioxidante de los AE obtenidos de *T. moroderi*, *T. piperella*, *S. angustifolia* y *S. chamaecyparissus*, así como su relación con el contenido en fenoles. La determinación de la actividad antioxidante *in vitro* se realizó siguiendo cuatro diferentes métodos analíticos, como son: el método del secuestro del radical 2,2'-difenil-1-picrilhidrazil (DPPH), el poder antioxidante de la reducción de hierro (FRAP), la determinación de la actividad antioxidante de las especies reactivas del ácido tiobarbitúrico (TBARS) y la capacidad quelante del ion ferroso (FIC).

La Figura 5 muestra el contenido en fenoles totales (FT) de los AEs obtenidos de *T. moroderi*, *T. piperella*, *S. angustifolia* y *S. chamaecyparissus*. El AE de *T. piperella* mostró los valores más elevados ($p<0,05$) de FT (57,43 mg equivalentes del ácido gálico (AGE)/g) de todas las muestras analizadas. Los AEs de *S. chamaecyparissus* y *S. angustifolia* mostraron un menor contenido en FT (21,59 y 38,48 mg de AGE/g, respectivamente), mientras que el AE de *T. moroderi* presentó los valores más bajos (9,33 mg de AGE/g).

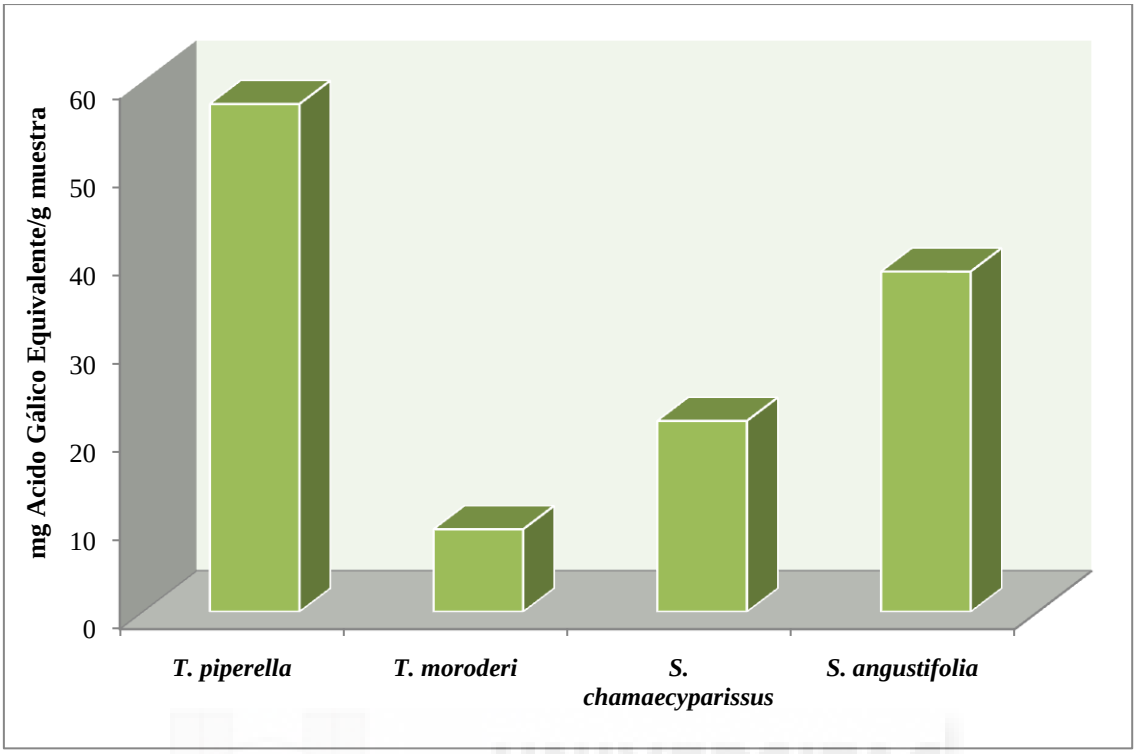

Figura 5. Contenido en fenoles totales de los aceites esenciales obtenidos de *T. moroderi, T. piperella, S. angustifolia* y *S. chamaecyparissus*.

Puesto que la actividad antioxidante en los alimentos está determinada por una mezcla de diferentes compuestos con actividad antioxidante, cada uno de los cuales presenta diferentes mecanismos de acción, e incluso se pueden producir interacciones sinérgicas entre ellos, se hace necesario la combinación de más de un método para determinar la capacidad antioxidante, *in vitro*, de cualquier producto con potencial actividad antioxidante (Pérez-Jiménez et al., 2008).

La Figura 6 muestra la actividad de secuestro de los radicales DPPH (determinada como porcentaje de inhibición de dichos radicales) por la acción de los AE obtenidos de *T. moroderi*, *T. piperella*, *S. angustifolia* y *S. chamaecyparissus*. Como se puede observar, el secuestro de radicales DPPH, para todos los AEs analizados, se produjo de forma concentración dependiente.

El AE de *T. piperella* fue el que mostró una mayor efectividad ($p<0,05$) en el secuestro de radicales DPPH, a todas las concentraciones analizadas, seguido por el AE de *S. angustifolia,* pero en esta ocasión solo a las concentraciones más elevadas (20, 10 y 5 mg/mL). A bajas concentraciones (<2,5 mg/mL) no se apreciaron diferencias

estadísticamente significativas (p>0,05) para los AEs de *T. moroderi*, *S. chamaecyparissus* y *S. angustifolia*.

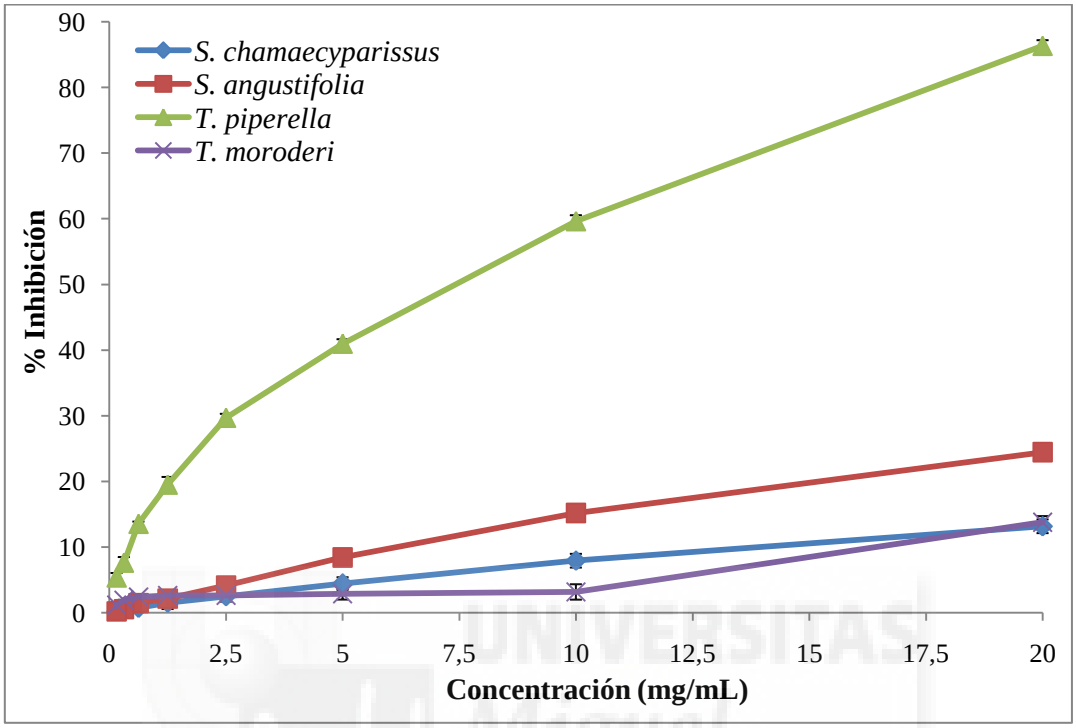

Figura 6. Secuestro de los radicales DPPH por la acción de los aceites esenciales (a diferentes concentraciones) de *T. moroderi*, *T. piperella*, *S. angustifolia* y *S. chamaecyparissus*.

Los valores de IC_{50} (concentración de AE que inhibe el 50% del radical DPPH) de los diferentes AEs siguieron el orden siguiente: AE de *T. piperella* (9,30 mg/mL) < AE de *S. angustifolia* (39,58 mg/mL) < AE de *S. chamaecyparissus* (75,45 mg/mL) < AE de *T. moroderi* (90,01 mg/mL).

La capacidad quelante del ion ferroso (Fe^{+2}) de los diferentes AEs obtenidos de *T. moroderi*, *T. piperella*, *S. angustifolia* y *S. chamaecyparissus*, se muestra en la Figura 7. El análisis de esta propiedad mostró que todos los AEs analizados fueron capaces de quelar el ion ferroso y dicha acción se produjo de forma dependiente de la concentración. En las concentraciones más elevadas (10 y 20 mg/mL) fueron los AEs de *S. chamaecyparissus* y *S. angustifolia* los que mostraron una mayor (p<0,05) capacidad quelante, no existiendo diferencias estadísticamente significativas entre ellos. El AE de *T. piperella*, a todas las concentraciones estudiadas, mostró los valores más bajos (p<0,05) en la quelación del ion ferroso.

La concentración más baja necesaria para obtener el 50% del efecto quelante (EC_{50}) la presentaron los diferentes AEs en el siguiente orden: AE de *S. chamaecyparissus* (3,94 mg/mL) < AE de *S. angustifolia* (5,99 mg/mL) < AE de *T. moroderi* (19,38 mg/mL) < AE de *T. piperella* (425,73 mg/mL).

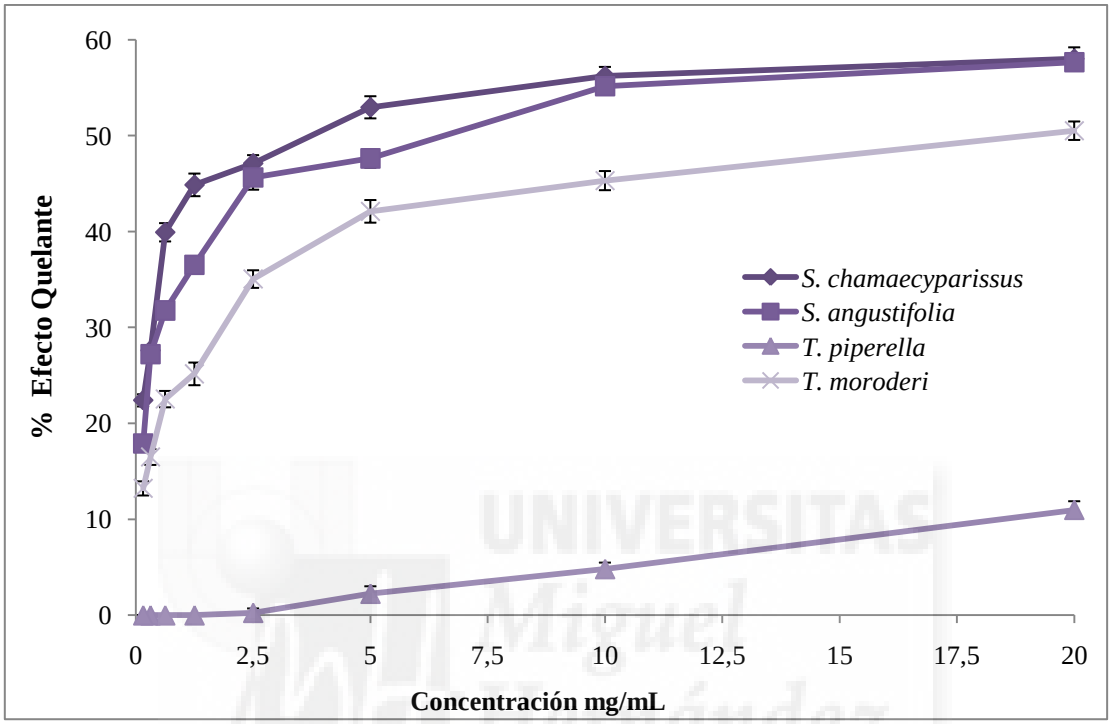

Figura 7. Capacidad quelante del ion ferroso (Fe^{+2}) de los aceites esenciales (a diferentes concentraciones) de *T. moroderi*, *T. piperella*, *S. angustifolia* y *S. chamaecyparissus*.

La Figura 8 muestra la capacidad de reducción del ion férrico (Fe^{+3}), en términos de concentración equivalente al Trolox (TEAC), de los AEs obtenidos de *T. moroderi*, *T. piperella*, *S. angustifolia* y *S. chamaecyparissus*.

Al igual que ocurría con los métodos anteriores, la capacidad de reducción del ion férrico, por parte de los AEs estudiados, se produjo de una forma dependiente de la concentración. El AE de *T. piperella*, a todas las concentraciones ensayadas, mostró los valores más elevados ($p<0,05$) en la capacidad de reducir el ion férrico en términos de concentración equivalente al Trolox, con valores comprendidos entre 0,1 y 2,64 mM/L Trolox. El AE de *T. moroderi* mostró los valores más bajos ($p<0,05$) en la capacidad de reducir el ion férrico si se compara con los otros AE estudiados.

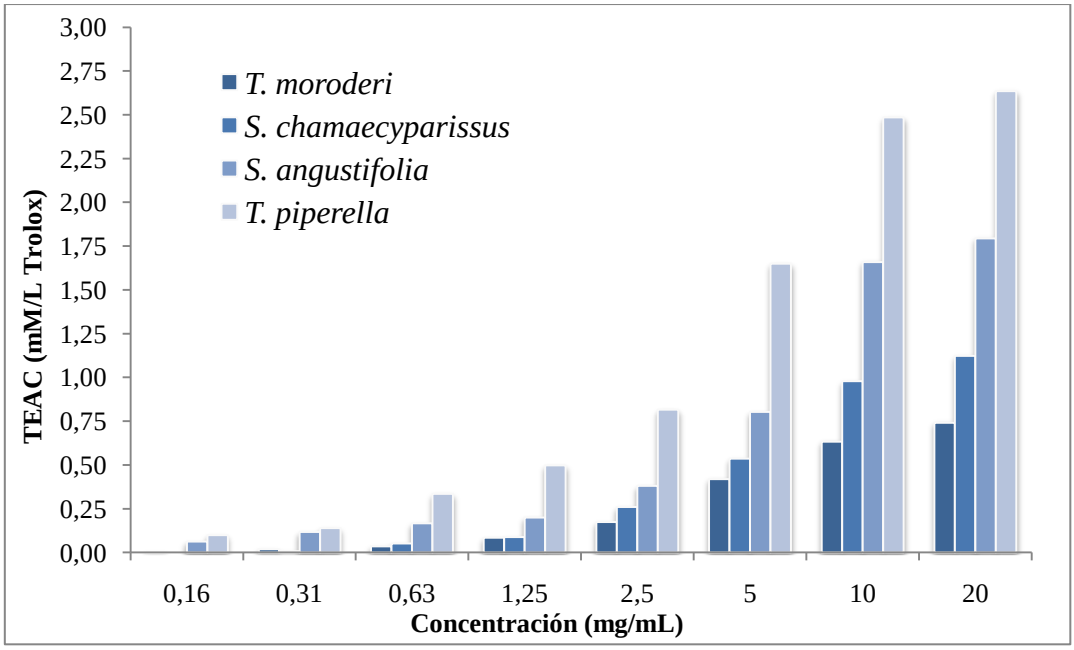

Figura 8. Capacidad de reducción del ion férrico (Fe^{+3}) debido a la acción de los AEs obtenidos de *T. moroderi*, *T. piperella*, *S. angustifolia* y *S. chamaecyparissus.*

La Figura 9 muestra la inhibición de la formación de especies reactivas del ácido tiobarbiturico (TBARs), el cual es un metabolito secundario de la oxidación lipídica. Todos los AEs analizados presentaron un alto porcentaje de inhibición de las TBARs y dicha inhibición se produjo de forma dependiente de la concentración.

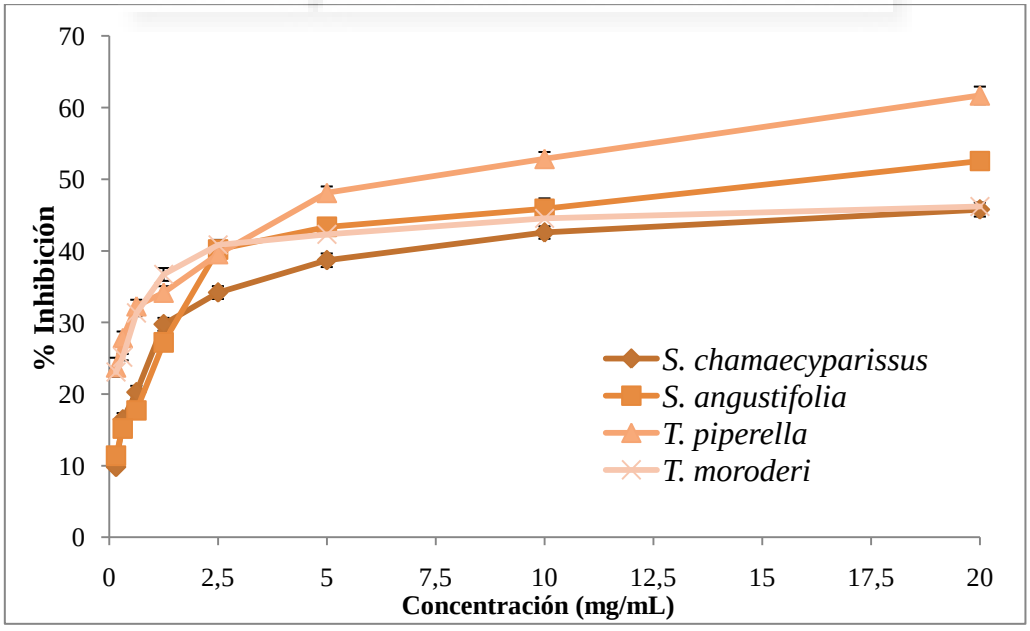

Figura 9. Capacidad de inhibición de la formación de las especies reactivas del ácido tiobarbitúrico (TBARs) debido a la acción de los aceites esenciales de *T. moroderi*, *T. piperella*, *S. angustifolia* y *S. chamaecyparissus.*

A todas las concentraciones ensayadas, fue el AE de *T. piperella* el que mostró los mayores valores ($p<0,05$) de inhibición de las TBARs. A altas concentraciones (5, 10 y 20 mg/mL) los AE de *S. chamaecyparissus* y *T. moroderi* mostraron los valores más bajos ($p<0,05$) de inhibición de las TBARs. Los valores de IC_{50} se obtuvieron en el siguiente orden: AE de *T. piperella* (6,30 mg/mL) < AE de *S. angustifolia* (12,25 mg/mL) < AE de *S. chamaecyparissus* (24,50 mg/mL) < AE de *T. moroderi* (26,46 mg/mL).

La actividad antioxidante de los AEs puede producirse siguiendo distintos mecanismos de acción: eliminación de radicales libres, capacidad de reducción de iones, quelación con metales de transición, donación de iones de hidrogeno, etc., (Mao et al., 2006). Esta actividad antioxidante podría relacionarse con el alto contenido de monoterpenos, sesquiterpenos y ácidos fenólicos presentes en los AEs, ya que está ampliamente demostrado la actividad antioxidante de estas sustancias (Edziri et al., 2010). Sin embargo, debe tenerse en cuenta que las propiedades de los AEs, tal como la actividad antioxidante, dependen no sólo de sus características estructurales, sino también de muchos otros factores como la concentración, la temperatura, la luz, el tipo de sustrato, el estado físico del sistema así como, la presencia de micro-componentes que pueden actuar como agentes pro-oxidantes o sinérgicos (Yanishlieva, 2001). Como se puede observar en la Tabla 9, excepto para el método donde se determina la capacidad de quelar metales (FIC), existe una relación directa entre el contenido en fenoles totales y la actividad antioxidante: a mayor contenido en fenoles totales mayor actividad antioxidante.

Tabla 9. Relación entre el contenido en fenoles totales y la actividad antioxidante.

	Capacidad Quelante de metales (FIC)	Poder reductor del hierro (FRAP)	Capacidad de donar H+ (DPPH)	Reducción de la peroxidación lipídica (TBARs)
T. piperella	*	****	****	****
	++++	++++	++++	++++
T. moroderi	**	*	**	**
	+	+	+	+
S. angustifolia	***	***	***	***
	+++	+++	+++	+++
S. chamaecyparissus	***	**	**	**
	++	++	++	++

Actividad antioxidante: * baja; ** media-baja; *** media-elevada; **** elevada

Contenido en FT: $^{+}$ bajo; $^{++}$ medio-bajo; $^{+++}$ medio-elevado; $^{++++}$ elevado

5.5.- Actividad antibacteriana de los films de quitosano adicionados con los aceites esenciales sometidos a estudio

El objetivo de estos trabajos, publicados en la revista "*Food Control*" (2013, 30:386-392) y en los *Proceedings de EUCHIS 2013 - International Conference of the European Chitin Society*, fue la determinación de la actividad antibacteriana de films de quitosano a los que se adicionó diferentes concentraciones de los AEs obtenidos de *T. moroderi*, *T. piperella*, *S. angustifolia* y *S. chamaecyparissus*. Dicha actividad se determinó mediante la técnica de difusión en disco frente a diversas bacterias relacionadas con el deterioro de alimentos o indicadoras de la presencia de patógenos.

En la Tabla 10 se muestra la actividad antibacteriana de los films de quitosano (CH) adicionados con AEs de *T. moroderi* (CH+TMAE) y *T. piperella* (CH+TPAE) a distintas concentraciones (0,5; 1 y 2%) frente a diversas bacterias. Los films formados únicamente por quitosano (CH) no presentaron ningún tipo de inhibición frente a ninguna de las bacterias analizadas. Estos resultados están en concordancia con los expuestos por Ojagh et al. (2010) y Wang et al. (2011) quienes informaron de que no existía ninguna inhibición significativa de los films de quitosano puro frente a diversas bacterias, y lo explicaron por el hecho de que el film de quitosano no difunde sobre los medios de cultivo formados por agar. En el presente estudio se puso de manifiesto que los films de quitosano depositados sobre los medios de cultivo no sufrían alteraciones, y por tanto el quitosano no se solubilizaba sobre el medio, no pudiendo de este modo ejercer efecto inhibitorio alguno fuera de la zona de contacto con el medio.

En lo que respecta a los films CH+TPAE, se puede observar que, a todas las concentraciones ensayadas, mostraron un efecto inhibidor sobre 3 de las 5 bacterias analizadas, produciéndose dicho efecto de una forma concentración dependiente. La bacteria más sensible a estos films fue *A. denitrificans* presentando halos de inhibición comprendidos entre 20 y 30 mm, no apreciándose diferencias ($p<0,05$) entre los tomillos ensayados. Para las bacterias *S. marcescens* y *A. faecalis*, los films CH+TPAE no mostraron ninguna actividad antibacteriana cuando se usó el AE a la concentración más baja (0,5%).

En el caso de los films CH+TMAE no se obtuvo ningún tipo de actividad antibacteriana, a ninguna de las concentraciones analizadas, frente a *S. marcescens*. Del

mismo modo para *L. innocua* y *A. hydrophila*, no se produjo ningún efecto inhibitorio a la concentración más baja (0,5%). Para *A. faecalis*, los halos de inhibición variaron entre 23 y 25 mm, sin diferencias significativas (p>0,05) entre las concentraciones del 1 y 2%. A la concentración más alta (2%) *A. denitrificans* mostró los mayores halos de inhibición.

Tabla 10. Actividad antibacteriana de los films de quitosano adicionados con aceites esenciales de *T. moroderi* y *T. piperella* a distintas concentraciones frente a diversas bacterias.

Films	Bacteria				
	S. marcescens	*A. hydrophila*	*A. faecalis*	*A. denitrificans*	*L. innocua*
CH	¥N.A.	N.A.	N.A.	N.A.	N.A.
CH+0,5% TPAE	N.A.	$12{,}00\pm0{,}00^{aA}$	N.A.	$20{,}00\pm0{,}00^{aA}$	$12{,}00\pm0{,}00^{aA}$
CH+1% TPAE	Ж$13{,}50\pm0{,}71^{aA}$	$17{,}00\pm1{,}41^{bBC}$	$13{,}50\pm0{,}71^{aA}$	$25{,}00\pm0{,}00^{bB}$	$15{,}00\pm0{,}00^{bB}$
CH+2% TPAE	$19{,}50\pm0{,}71^{bB}$	$18{,}50\pm0{,}71^{bBD}$	$20{,}50\pm0{,}71^{bB}$	$30{,}00\pm0{,}00^{cC}$	$19{,}00\pm1{,}41^{cC}$
CH+0,5% TMAE	N.A.	N.A.	$23{,}00\pm0{,}00^{aC}$	$20{,}00\pm0{,}00^{aA}$	N.A.
CH+1% TMAE	N.A.	$16{,}50\pm0{,}71^{aC}$	$24{,}50\pm0{,}71^{bD}$	$24{,}00\pm1{,}41^{bB}$	$13{,}00\pm0{,}00^{aD}$
CH+2% TMAE	N.A.	$19{,}50\pm0{,}71^{bD}$	$25{,}00\pm0{,}00^{bD}$	$29{,}50\pm0{,}71^{cC}$	$16{,}50\pm0{,}71^{bE}$

¥N.A.: No Activo
ЖDiámetro (media y desviación estándar) del halo de inhibición (mm) incluyendo el diámetro del film de 10 mm.
Para un mismo aceite esencial, valores seguidos por la misma letra minúscula dentro de la misma fila no son significativamente diferentes (p>0,05) de acuerdo con el Test de Rangos Múltiples de Tukey.
Para una misma bacteria, valores seguidos por la misma letra mayúscula dentro de la misma columna no son significativamente diferentes (p>0,05) de acuerdo con el Test de Rangos Múltiples de Tukey.

Los films de CH+TPAE fueron más eficaces (p<0,05) frente a *S. marcescens* y *L. innocua* que los films de CH+TMAE, mientras que no se encontraron diferencias estadísticas (p>0,05) entre los films CH+TPAE y CH+TMAE frente a *A. hydrophila* y *A. denitrificans*. Finalmente, para *A. faecalis*, los films de CH+TMAE mostraron mayores (p<0,05) halos de inhibición, a todas las concentraciones, que los films de CH+TPAE.

En la Tabla 11 se muestra la actividad antibacteriana de los films de quitosano adicionados con AEs de *S. angustifolia* (CH+SAAE) y *S. chamaecyparissus* (CH+SCAE) a distintas concentraciones (0,5 y 1%), frente a diversas bacterias. Como se observó anteriormente, tampoco en este caso se apreció ningún efecto inhibitorio de los films compuestos únicamente por quitosano puro. Del mismo modo no se apreció ningún efecto inhibidor de los films de CH+SAAE o de CH+SCAE, a ninguna de las

concentraciones ensayadas, sobre *L. innocua*. Los films de CH+SAAE fueron más efectivos ($p<0,05$) frente a *S. marcescens* y *A. faecalis*, a todas las concentraciones ensayadas, que los films de CH+SCAE.

Tabla 11. Actividad antibacteriana de los films de quitosano adicionados con aceites esenciales de *S. angustifolia y S. chamaecyparissus* a distintas concentraciones, frente a diversas bacterias.

Films	Bacteria		
	S. marcescens	*L. innocua*	*A. faecalis*
CH	¥N.A.	N.A.	N.A.
CH+0.5%SCAE	N.A.	N.A.	14,72±0,17^{D}
CH+1%SCAE	14,18±0,21^{bB}	N.A.	18,70±0,11^{aB}
CH+0.5%SAAE	12,84±0,12^{bC}	N.A.	17,85±0,15^{aC}
CH+1%SAAE	18,22±0,24^{bA}	N.A.	22,20±0,23^{aA}

¥N.A.: No Activo

ЖDiámetro (media y desviación estándar) del halo de inhibición (mm) incluyendo el diámetro del film de 10 mm.

Para un mismo aceite esencial, valores seguidos por la misma letra minúscula dentro de la misma fila no son significativamente diferentes ($p>0,05$) de acuerdo con el Test de Rangos Múltiples de Tukey.

Para una misma bacteria, valores seguidos por la misma letra mayúscula dentro de la misma columna no son significativamente diferentes ($p>0,05$) de acuerdo con el Test de Rangos Múltiples de Tukey.

Este es el primer estudio donde se analiza la actividad antibacteriana de films de quitosano a los que se han incorporado AEs de *T. moroderi*, *T. piperella*, *S. angustifolia* o *S. chamaecyparissus*. Sin embargo, sí que hay referencias sobre la actividad antibacteriana de films de quitosano adicionados con AEs procedentes de otras hierbas aromáticas y especias. Hosseini et al. (2009) observaron que la incorporación de AEs de tomillo, clavo o canela a films de quitosano provocaba la inhibición del crecimiento de bacterias como *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enteritidis* o *Pseudomonas aeruginosa*. En otro estudio Sánchez-González et al., (2011) analizaron la actividad antibacteriana de films de quitosano adicionados con AEs de bergamota, limón o árbol del té, concluyendo que dichos films presentaban un significativo efecto bacteriostático frente a bacterias como *Escherichia coli*, *L. monocytogenes* o *S. aureus*.

Las propiedades antibacterianas de los films de quitosano adicionados con AEs podrían estar relacionadas con la presencia, en los films, de compuestos bioactivos tales como ácidos fenólicos, terpenoides o sesquiterpenoides procedentes de los AE. El mecanismo de acción por el cual los AEs muestran actividad antibacteriana ya ha sido discutido en el apartado 5.2 de esta Memoria.

5.6.- Actividad antioxidante de films de quitosano adicionados con los aceites esenciales sometidos a estudio

El objetivo de estos trabajos, publicados en la revista "*Food Control*" (2013, 30:386-392) y en los *Proceedings de EUCHIS 2013 - International Conference of the European Chitin Society*, fue la determinación de la actividad antioxidante de films de quitosano a los que se adicionó distintas concentraciones de AEs obtenidos de *T. moroderi*, *T. piperella*, *S. angustifolia* y *S. chamaecyparissus* mediante el empleo de tres diferentes métodos analíticos: el método del secuestro del radical 2,2'-difenil-1-picrilhidrazil (DPPH), el poder antioxidante de la reducción de hierro (FRAP), y la capacidad quelante del ion ferroso (FIC). Además se determinó el contenido en fenoles totales de los diferentes films para estudiar su posible correlación con la actividad antioxidante. La Figura 10 muestra el contenido en fenoles totales (FT) de los films de quitosano adicionados, a distintas concentraciones, con AEs obtenidos de *T. moroderi* y *T. piperella*.

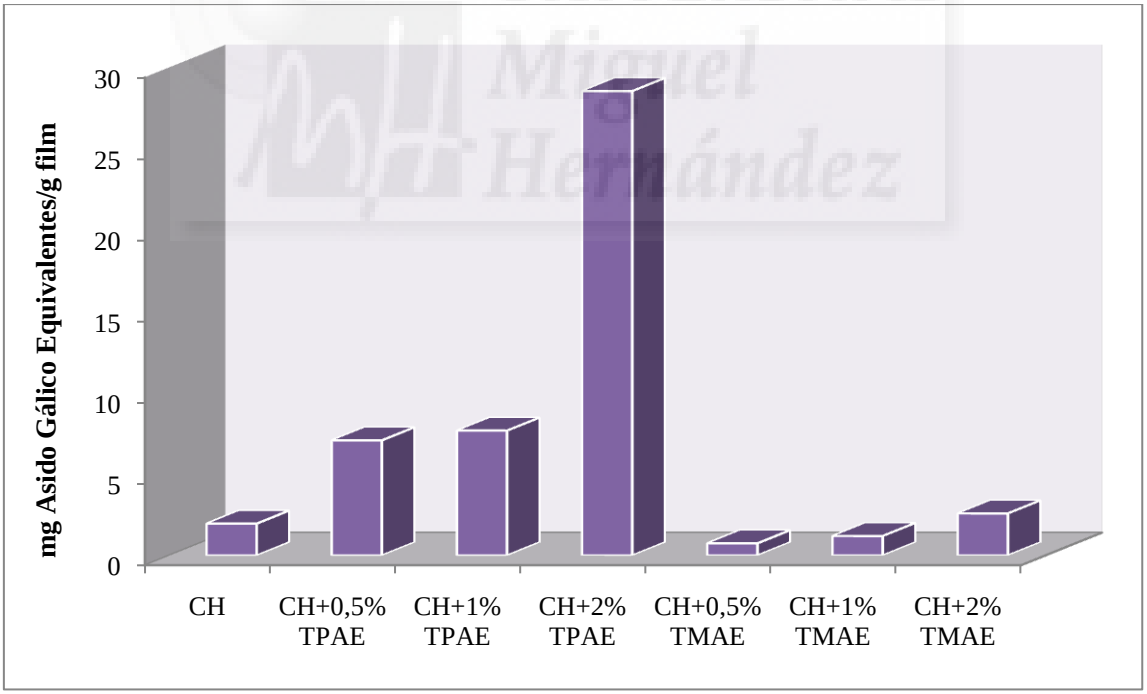

Figura 10. Contenido en fenoles totales (mg equivalentes de ácido gálico/g de film) de los films de quitosano adicionados, a distintas concentraciones, con aceites esenciales obtenidos de *T. moroderi* (TMAE) y *T. piperella* (TPAE).

Los films de quitosano puro (CH) presentaron un contenido en FT de 1,95 mg de equivalentes de ácido gálico /g de film. Estos valores se encuentran en concordancia con

los obtenidos por Siripatrawan y Harte (2010) y Moradi et al. (2012). Dichos autores lo atribuyen a la formación de cromógenos producida, posiblemente, por la reacción del reactivo Folin Ciocalteu con sustancias reductoras de origen no fenólico y que se podrían detectar espectrofotométricamente. Tanto para los films de CH+TPAE como para los films de CH+TMAE, la incorporación del AE provocó un aumento en el contenido de FT y esto ocurrió de una forma dependiente de la concentración. Cabe destacar que los films de CH+TPAE mostraron un contenido en FT, a todas las concentraciones analizadas, superior ($p<0,05$) que los films de CH+TMAE. Como se vió en el apartado 5.4, el AE de *T. piperella*, presentaba un mayor contenido en fenoles totales que el AE de *T. moroderi*.

La Figura 11 muestra el contenido en fenoles totales de los films de quitosano adicionados, a distintas concentraciones, con AEs de *S. angustifolia y S. chamaecyparissus.*

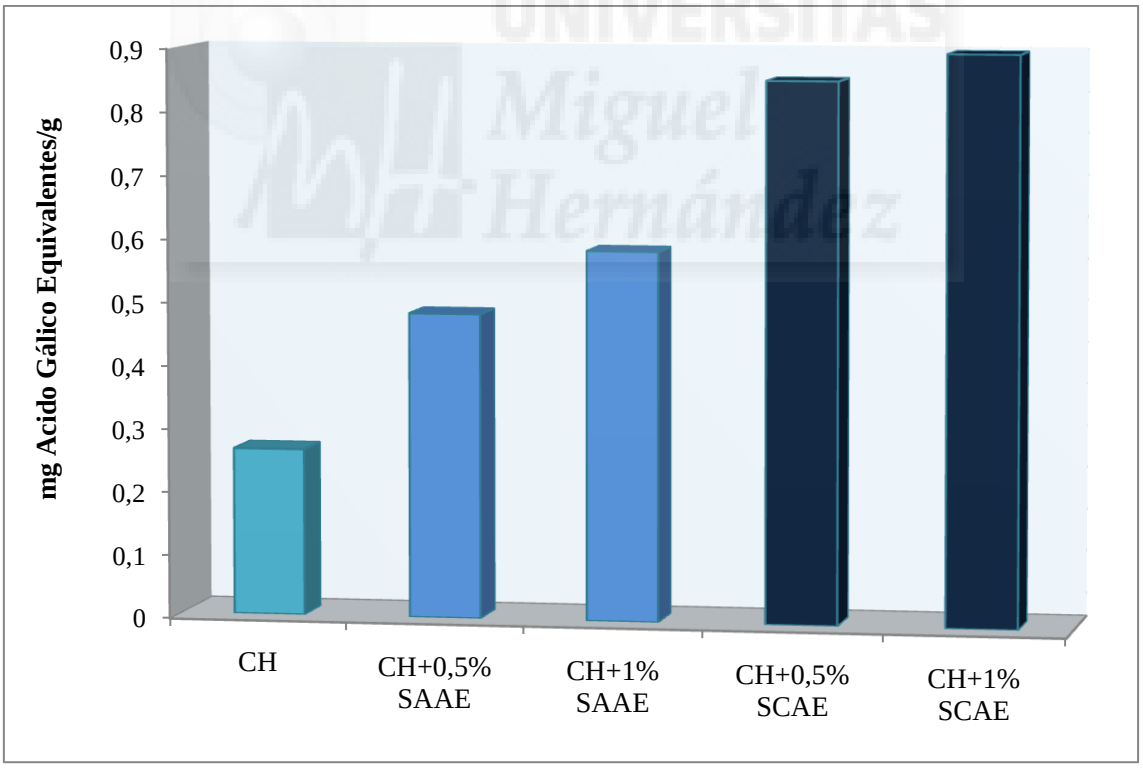

Figura 11. Contenido en Fenoles Totales (FT) de los films de quitosano adicionados, a distintas concentraciones, con aceites esenciales obtenidos de *S. angustifolia* (SAAE) y *S. chamaecyparissus* (SCAE).

Los films elaborados con quitosano puro (CH) mostraron un contenido de FT de aproximadamente 0,26 mg de equivalentes del ácido gálico/g de film. Como se ha mencionado anteriormente, este hecho estaría explicado por la producción de cromógenos. Después de la incorporación de los AE, como era de esperar, el contenido en FT de los films de CH aumentó. Dicho aumento se produjo de forma dependiente de la concentración ($p<0,05$) para ambos AEs. Los films de CH+SCAE mostraron un contenido en FT, a todas las concentraciones analizadas, superior ($p<0,05$) que los films de CH+SAAE. Es importante destacar que, pese a que el contenido en FT del AE de *S. angustifolia* es mayor que el del AE *de S. chamaecyparissus* en los films elaborados con estos aceites ocurre al contrario. Los films elaborados con AE de *S. chamaecyparissus* presentan mayor contenido en FT que los films adicionados con AE de *S. angustifolia*. Esto podría deberse a que se haya producido una mayor evaporación de este tipo de compuestos durante la elaboración del film.

Para determinar la actividad antioxidante, *in vitro*, de los films de quitosano adicionados, a distintas concentraciones, con AE de *T. moroderi*, *T. piperella*, *S. angustifolia* y *S. chamaecyparissus* se emplearon tres métodos distintos, como son: DPPH, FRAP y FIC.

La Figura 12 muestra la actividad de secuestro de los radicales DPPH por la acción de los films de quitosano adicionados con AEs obtenidos de *T. moroderi* o *T. piperella*.

Los films de quitosano puro sin la adición de ningún tipo de AE mostraron una leve actividad secuestrante de radicales DPPH (3 µg equivalentes del trolox (TE)/g film). Como era de esperar, la adición de AE a los films provocó un aumento significativo en la capacidad de secuestro de radicales libres DPPH, produciéndose esta capacidad secuestrante de forma dependiente de la concentración. Los films de CH+TPAE mostraron unos valores comprendidos entre 690 y 1090 µg TE/g film ($p<0,05$). En el caso de los films de CH+TMAE, los valores de secuestro de radicales libres oscilaron entre 420 y 630 µg TE/g film, produciéndose también la actividad antioxidante de forma dependiente de la concentración.

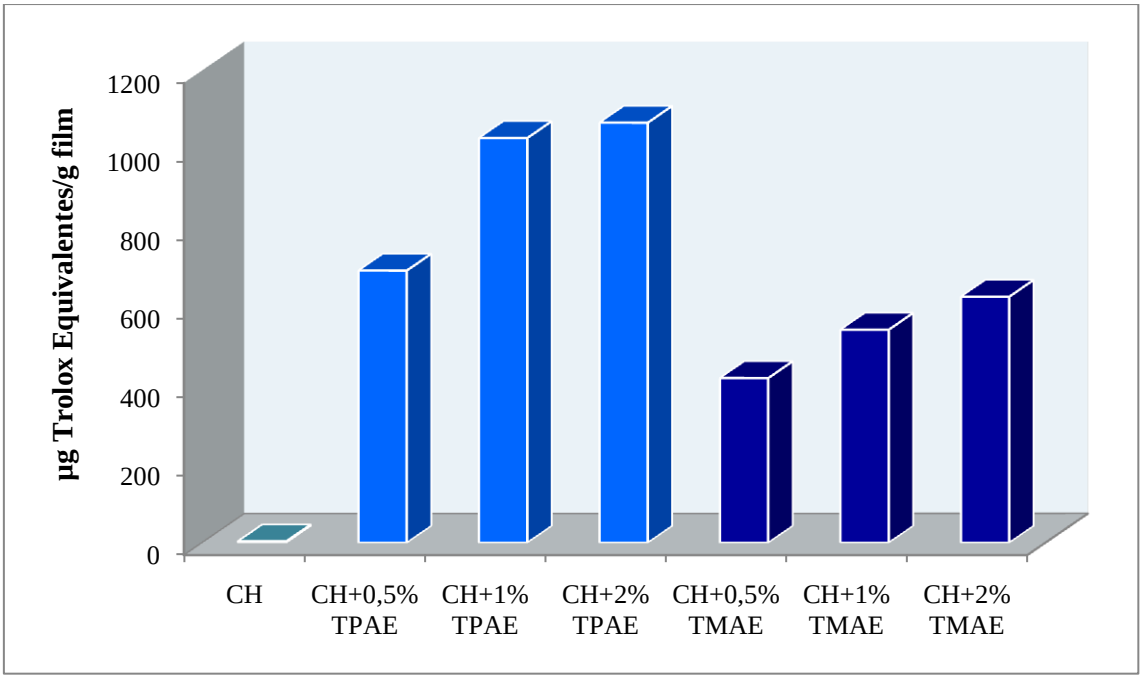

Figura 12. Secuestro de los radicales DPPH de los films de quitosano (CH) adicionados con aceites esenciales de *T. moroderi* (TMAE) o *T. piperella* (TPAE), a diferentes concentraciones.

La Figura 13 muestra la actividad de secuestro de los radicales DPPH por la acción de los films de quitosano adicionados con AEs obtenidos de *S. angustifolia* y *S. chamaecyparissus*.

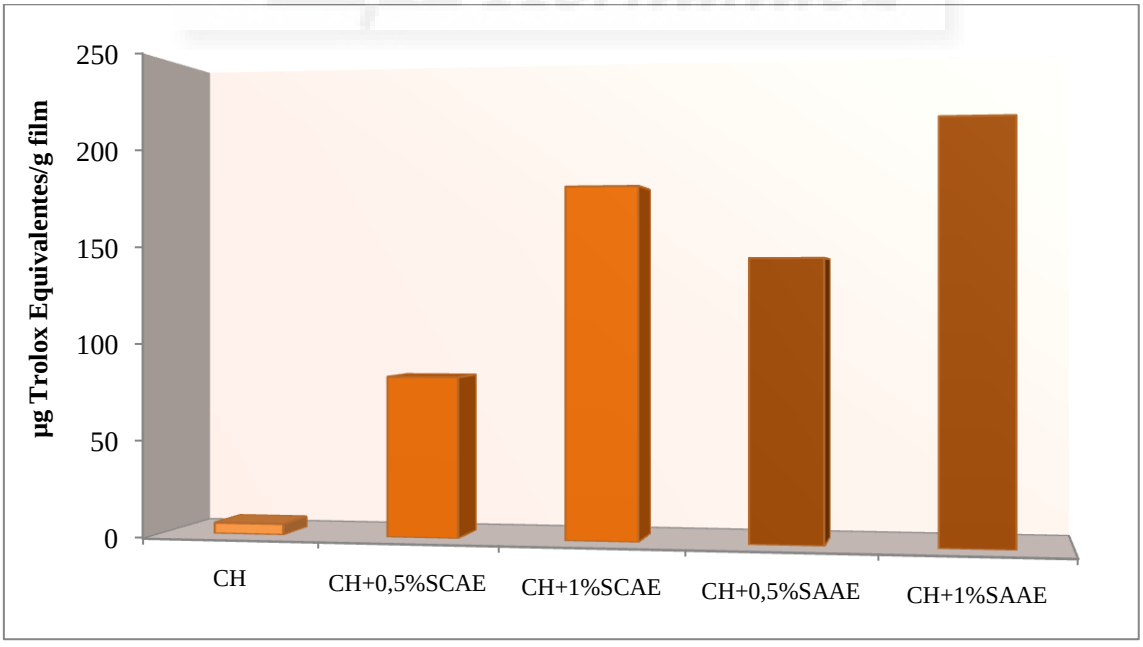

Figura 13. Secuestro de los radicales DPPH por acción de los films de quitosano adicionados con aceites esenciales de *S. angustifolia* (SAAE) y *S. chamaecyparissus* (SCAE), a diferentes concentraciones

El film de quitosano puro mostró un ligera actividad secuestrante de radicales DPPH, muy similar a la reportada anteriormente. En el caso de los films CH+SCAE, los valores de actividad secuestrante de radicales DPPH fueron 0,33 y 0,72 mM TE/g film ($p<0,05$), para las concentraciones de 0,5 y 1%, respectivamente. Los films CH+SAAE, a todas las concentraciones ensayadas, mostraron una actividad secuestrante de radicales DPPH superior ($p<0,05$) que los films de CH+SCAE, obteniendo unos valores de 0,57 y 0,85 mM TE/g film para las concentraciones de 0,5 y 1%, respectivamente.

La Figura 14 muestra la capacidad de reducción del ion férrico (Fe^{+3}) de los films de quitosano adicionados con AEs obtenidos de *T. moroderi y T. piperella*.

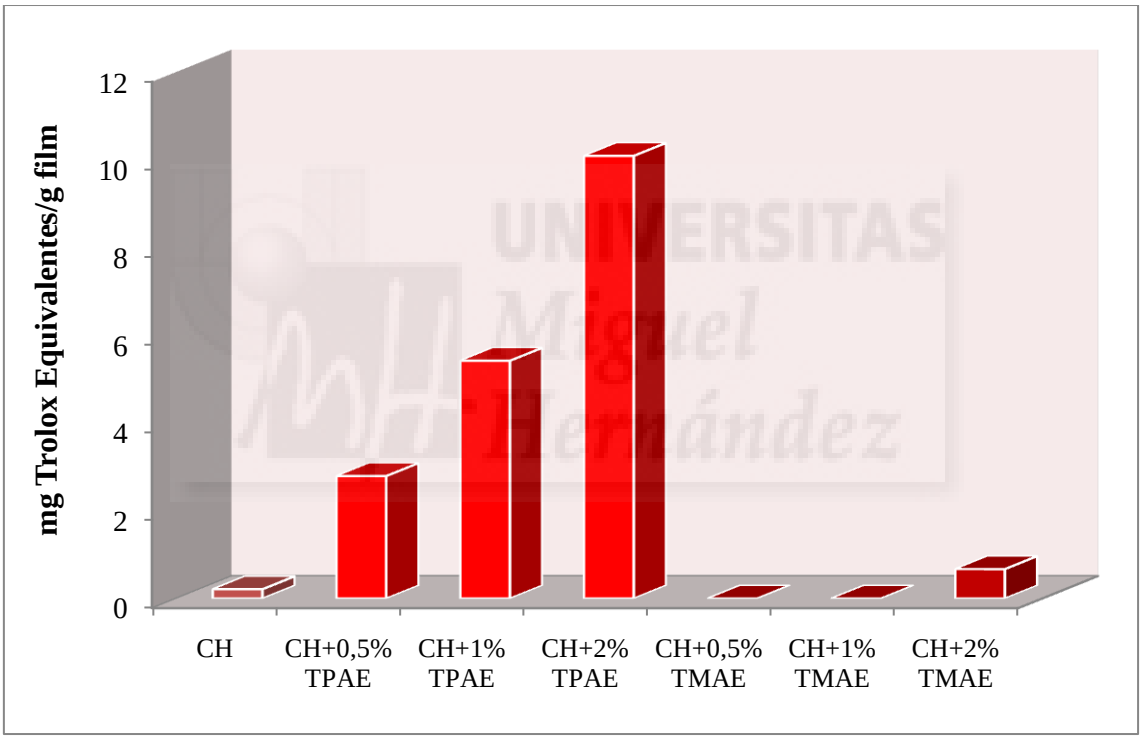

Figura 14. Capacidad de reducción del ion férrico (Fe^{+3}) debido a la acción de los films de quitosano adicionados con aceites esenciales obtenidos de *T. moroderi, T. piperella,* a diferentes concentraciones.

Los films de CH puro mostraron una leve capacidad de reducción del ion Fe^{+3} (0,21 mg TE/g film). El análisis de los films CH+TPAE mostró un amplio rango de actividad reductora con valores comprendidos entre 2,79 y 10,09 mg TE/g film ($p<0,05$). Como en el caso del ensayo DPPH, la actividad reductora del ion Fe^{+3} sucede de una forma dependiente de la concentración. Con respecto a los films CH+TMAE, a bajas concentraciones (0,5 y 1%) no se apreció ningún tipo de actividad reductora,

mientras que a la concentración más elevada (2%) mostró una leve capacidad de reducción (0,67 mg TE/g film). A todas las concentraciones ensayadas, los films CH+TPAE mostraron una actividad de reducción del ion Fe^{+3} superior ($p<0,05$) a la de los films CH+TMAE.

La Figura 15 muestra la capacidad de reducción del ion férrico (Fe^{+3}) de los films de quitosano adicionados con AEs obtenidos de *S. angustifolia* y *S. chamaecyparissus*. Como se aprecia en dicha figura la capacidad de reducción del ion F^{+3} aumentó por la incorporación de los AE de *S. angustifolia y S. chamaecyparissus*, a los films de CH y esta reducción se produjo de forma dependiente de la concentración.

En el caso de los films CH+SCAE los valores de reducción del ion Fe^{+3} ($p<0,05$) fueron de 1,89 y 3,05 mM TE/g film, para las concentraciones de 0,5 y 1%, respectivamente. Los films CH+SAAE mostraron una mayor actividad de reducción del ion Fe^{+3} ($p<0,05$), a todas las concentraciones ensayadas, que los films CH+SCAE, obteniendo unos valores de 3,09 y 4,23 mM TE/g film para las concentraciones de 0,5 y 1%, respectivamente.

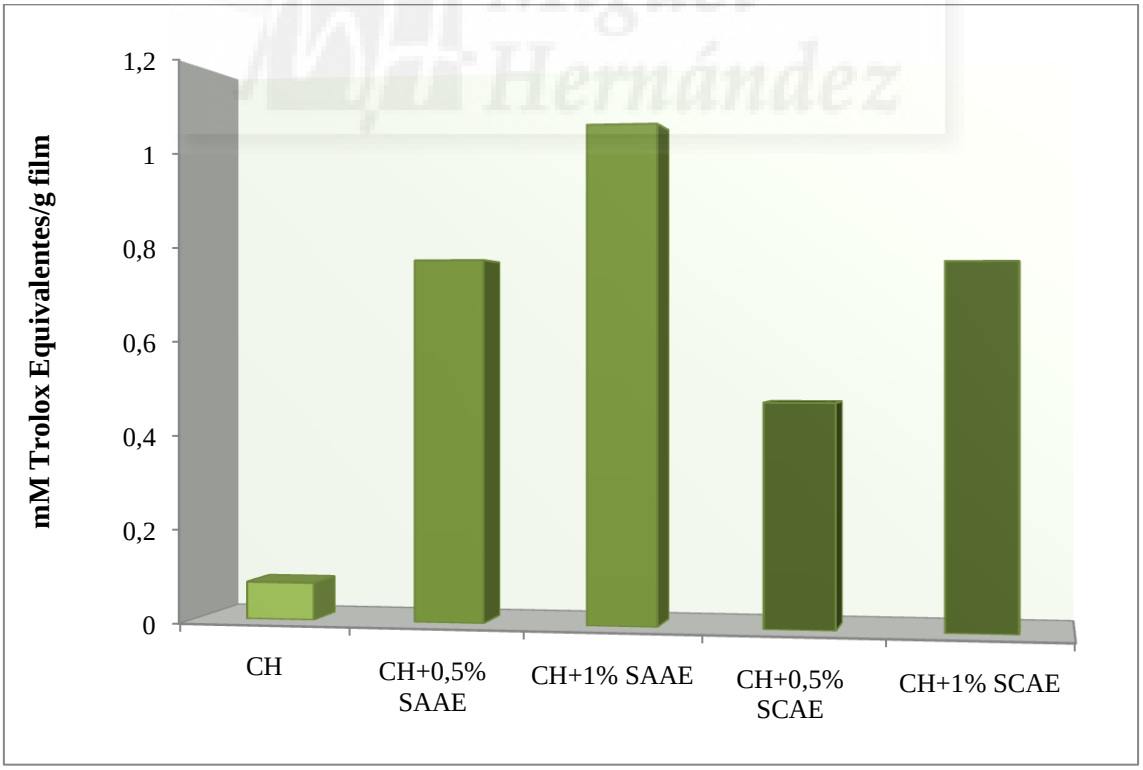

Figura 15. Capacidad de reducción del ion férrico (Fe^{+3}) de los films de quitosano adicionados con aceites esenciales de *S. angustifolia* y *S. chamaecyparissus*, a diferentes concentraciones.

La Figura 16 muestra la capacidad quelante del ion ferroso (Fe^{+2}) de los films de quitosano adicionados con AEs obtenidos de *T. moroderi*, *T. piperella.*

Como se aprecia en la Figura 15 los films CH+TPAE, a todas las concentraciones analizadas, fueron capaces de quelar el ion ferroso y esto ocurrió de una forma dependiente de la concentración ($p<0,05$). Del mismo modo, los films CH+TMAE mostraron una alta capacidad para quelar dicho ion ferroso y como en el caso de los films CH+TPAE, esto ocurrió de una forma dependiente de la concentración ($p<0,05$).

A todas las concentraciones analizadas (excepto al 0,5%) los films CH+TMAE fueron mejores agentes quelantes del ion ferroso ($p<0,05$) que los films CH+TPAE. En términos generales se puede decir que los films de CH adicionados con AE de *T. moroderi* y *T. piperella* mostraron un considerable capacidad quelante de metales.

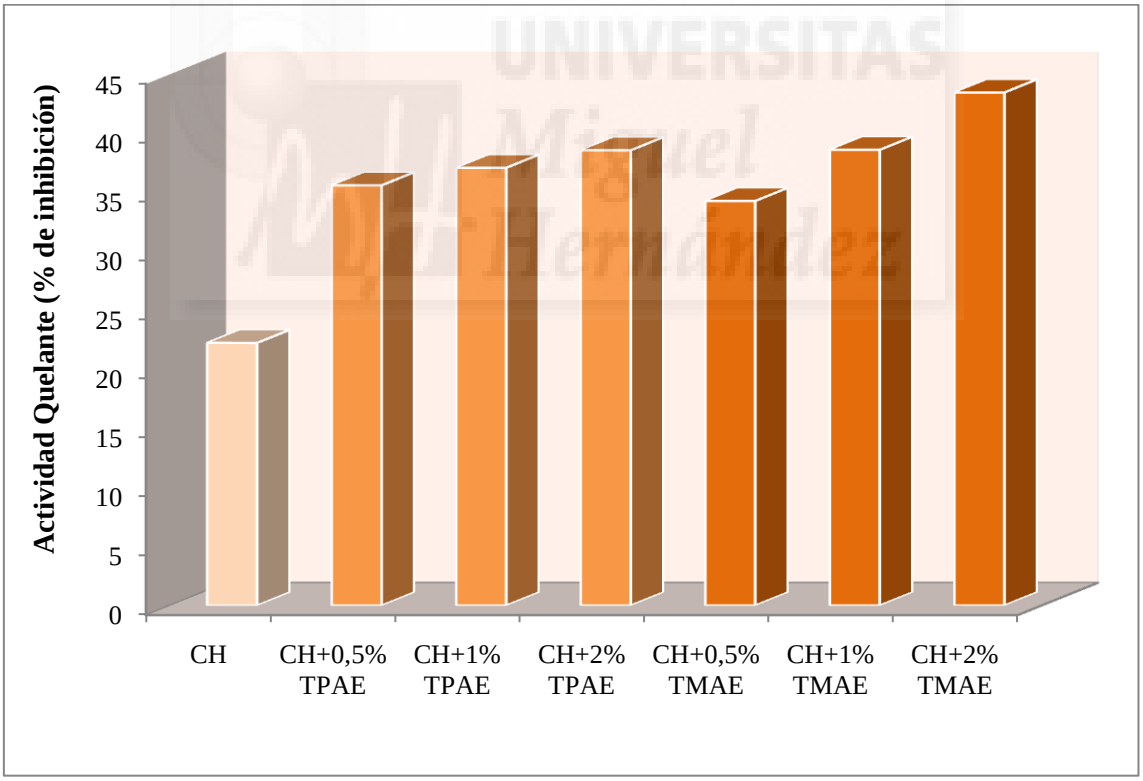

Figura 16. Capacidad quelante del ion ferroso (Fe^{+2}) de los films de quitosano adicionados con aceites esenciales de *T. moroderi*, *T. piperella*, a diferentes concentraciones.

5.7.- Aplicación de los films de quitosano adicionados con AEs de *T. moroderi*, *T. piperella* sobre un producto cárnico cocido.

El objetivo de este trabajo, que se encuentra en revisión en la revista *Meat Science*, fue determinar el efecto que presentaban los films de quitosano adicionados con AEs de *T. moroderi* y *T. piperella* (a concentraciones del 1 y al 2%) sobre la seguridad (propiedades antioxidantes y antibacterianas) y sobre la vida útil de un producto cárnico tipo Jamón cocido loncheado

5.7.1.- Oxidación lipídica

En la Figura 17 se observa la evolución de la oxidación lipídica, analizada con el Test del ácido 2-Tiobarbiturico, de las muestra de jamón cocido en las que se emplearon, como separadores entre lonchas, films de quitosano adicionados con AEs de *T. moroderi* y *T. piperella*.

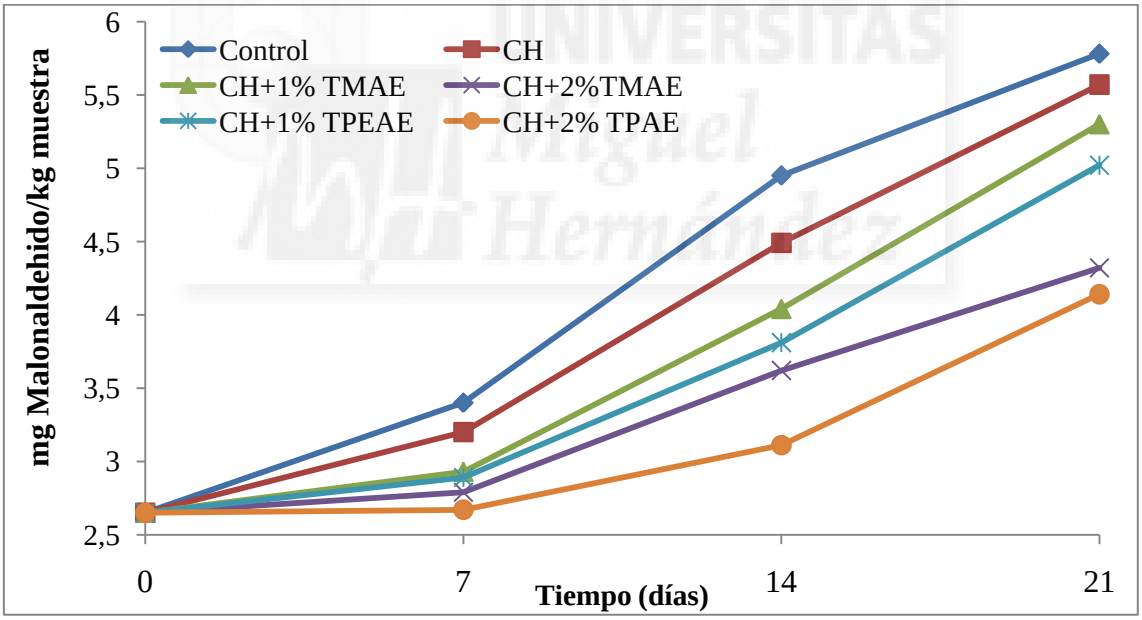

Figura 17. Evolución de la oxidación lipídica de las muestra de jamón cocido en las que se emplearon como separadores entre lonchas films de quitosano adicionados con aceites esenciales de *T. moroderi* y *T. piperella*.

Al comienzo del análisis (día cero) todas las muestras presentaron un valor de 2,65 mg MA/kg de muestra. A los 7 días, las muestras en las que se empleó CH+2%TMAE y CH+2%TPAE son las que presentaron los valores más bajos ($p<0,05$), alcanzándose reducciones del grado de oxidación con respecto al control, de 17,94 y

21,47%, respectivamente, existiendo diferencias significativas ($p<0,05$) entre ellas. Para este mismo día la muestra en la que solo se empleó quitosano el grado de reducción de la oxidación lipídica fue solo del 5,88%. Entre las muestras en las que se empleó CH+1%TMAE y CH+1%TPAE no se apreciaban diferencias ($p>0,05$).

A los 14 días, nuevamente las muestras en las que se empleó CH+2%TMAE y CH+2%TPAE son las que presentaronn los valores más bajos ($p<0,05$), con reducciones del grado de oxidación con respecto al control de 26,86 y 37,17%, respectivamente. Al final del ensayo (21 días), la muestra control mostraba unos valores de 5,78 mg MA/kg de muestra, siendo las muestras en las que se empleó CH+2%TMAE y CH+2%TPAE las que presentaron los valores más bajos ($p<0,05$), con reducciones del grado de oxidación respecto al control de 25,25 y 28,37% respectivamente, y con diferencias estadísticamente significativas ($p<0,05$) ente ellas.

El descenso en el grado de oxidación puede deberse, probablemente, a una liberación muy pronunciada, que se produce fundamentalmente durante los primeros días de almacenamiento, de los compuestos bioactivos presentes en los films de quitosano y que proceden de los AEs, tal y como proponen Oussalah et al. (2007). Para Moradi et al. (2011), la absorción, por parte del film, de agua presente en el producto cárnico, puede causar que éste se hinche y que las tasas de migración cambien continuamente debido a un proceso de relajación, dependiente del tiempo, provocado por el propio hinchamiento.

5.7.2.- pH

En la Figura 18 se observa la evolución del pH de las muestra de jamón cocido en las que se emplearon, como separadores de lonchas, films de quitosano adicionados con AEs de *T. moroderi* y *T. piperella.*

En la muestra control (no contenía ningún tipo de separador) se produjo un leve descenso del valor del pH con el paso del tiempo, partiendo de valores medios de 6,24 y alcanzando un valor final 6,12 ($p<0,05$). En las muestras, en las que se emplearon los films de quitosano y los films de quitosano adicionado con AEs de *T. moroderi* y *T. piperella* se produjo un descenso más pronunciado de este parámetro.

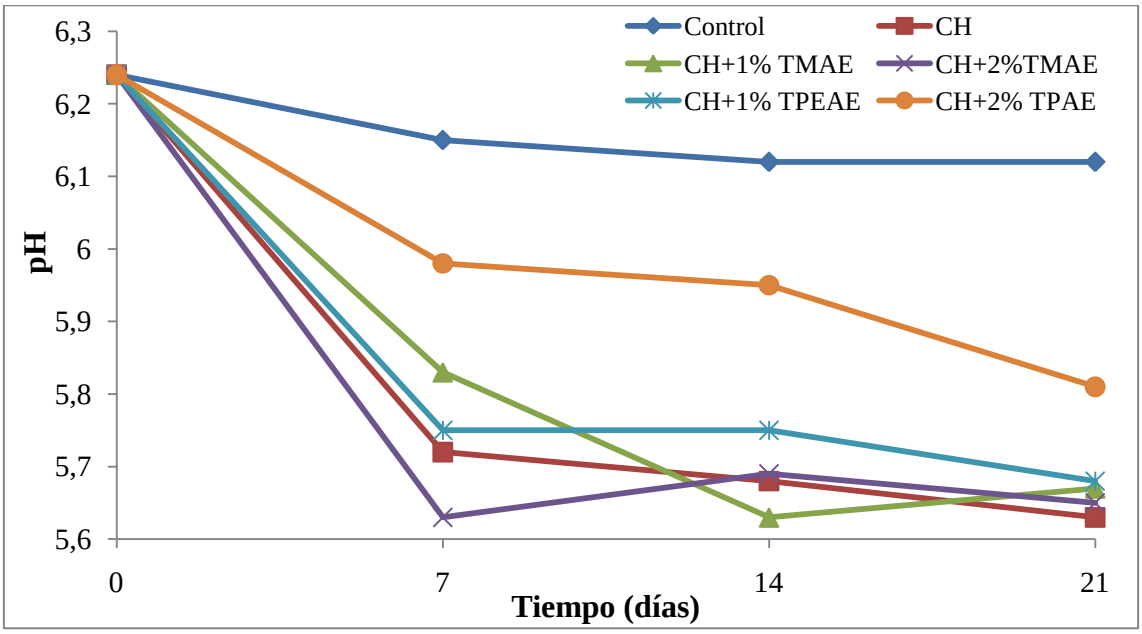

Figura 18. Evolución del pH de las muestra de jamón cocido en las que se emplearon, como separadores entre lonchas, films de quitosano adicionados con aceites esenciales de *T. moroderi* y *T. piperella.*

A los 7 días de almacenamiento, la muestra en la que se empleó CH+2%TMAE mostró el valor más bajo ($p<0,05$) de pH, con un reducción, con respecto al control, de aproximadamente 0,6 unidades. Al final del ensayo (día 21), como se ha mencionado anteriormente, la muestra control presentó el valor más elevado de pH (6,12) seguida por la muestra ($p<0,05$) en la que se empleó CH+2%TPAE (pH 5,81). Para el resto de las muestras, con valores de pH comprendidos entre 5,63 y 5,68, no se apreciaron diferencias estadísticamente significativas ($p>0,05$) entre ellas; aunque sí existían entre éstas y el control ($p<0,05$) y entre éstas y la muestra ($p<0,05$) con CH+2%TPAE. Como veremos a continuación, este descenso de pH se explicaría por el aumento en las poblaciones de bacterias ácido lácticas que se observó en dichas muestras.

5.7.3.- Análisis microbiológico

En las Figura 19 y 20 se observa la evolución de los recuentos de bacterias aerobias mesófilas y bacterias acido lácticas, respectivamente, de las muestra de jamón cocido, almacenadas a 4ºC durante 21 días, en las que se emplearon, como separadores entre lonchas, films de quitosano o films de quitosano adicionados con AEs de *T. moroderi* y *T. piperella.*

Las poblaciones de bacterias aerobias mesófilas y de bacterias ácido lácticas aumentaron gradualmente durante el almacenamiento a 4ºC, aunque la liberación de los compuestos bioactivos presentes en los films de quitosano influenció de forma significativa el grado en el que estos microorganismos se desarrollaron.

Cabe destacar que no se apreció crecimiento ni de enterobacterias ni de mohos y levaduras en ninguna de las muestras tratadas con films de quitosano adicionados con AEs de *T. moroderi* y *T. piperella*. Como se ha discutido anteriormente, los mecanismos de acción por los cuales estos compuestos bioactivos, fundamentalmente compuestos fenólicos con grupos hidroxilo, podrían ejercer ejercen cierto efecto antibacteriano serian (i) interfiriendo con los fosfolípidos presentes en la bicapa lipídica de la membrana celular, lo cual provoca un aumento en la permeabilidad de esta y una pérdida de los constituyentes celulares (ii) alternado sistemas enzimáticos y/o (iii) inactivando o destruyendo material genético (Corbo et al., 2008).

En cuanto a los recuentos de bacterias aerobias mesófilas (Figura 19), se puede observar como a los 7 días de almacenamiento, las muestras en las que se empleó CH+2%TMAE y CH+2%TPAE presentaron los valores más bajos ($p<0,05$) de crecimiento microbiano, con reducciones de los recuentos, para estas bacterias, de 2,57 y 2,61 ciclos logarítmicos, con respecto al control, no apreciándose diferencias ($p>0,05$) entre ambas. Las muestras en las que solo se empleó quitosano presentaron una reducción ($p<0,05$) de los recuentos de 0,62 ciclos logarítmicos respecto al control, evidenciando un cierta actividad antibacteriana del quitosano.

Al final del almacenamiento, día 21, las muestras en las que se empleó CH+2%TPAE presentaron los recuentos más bajos ($p<0,05$), alcanzándose una reducción de 0,87 ciclos logarítmicos respecto al control. Entre las muestras en las que se empleó CH+1%TMAE, CH+2%TMAE y CH+1%TPAE no se apreciaron diferencias significativas ($p>0,05$) aunque sí entre éstas y el control ($p<0,05$), con reducciones de los recuentos de 0,34; 0,53 y 0,37 ciclos logarítmicos, respectivamente.

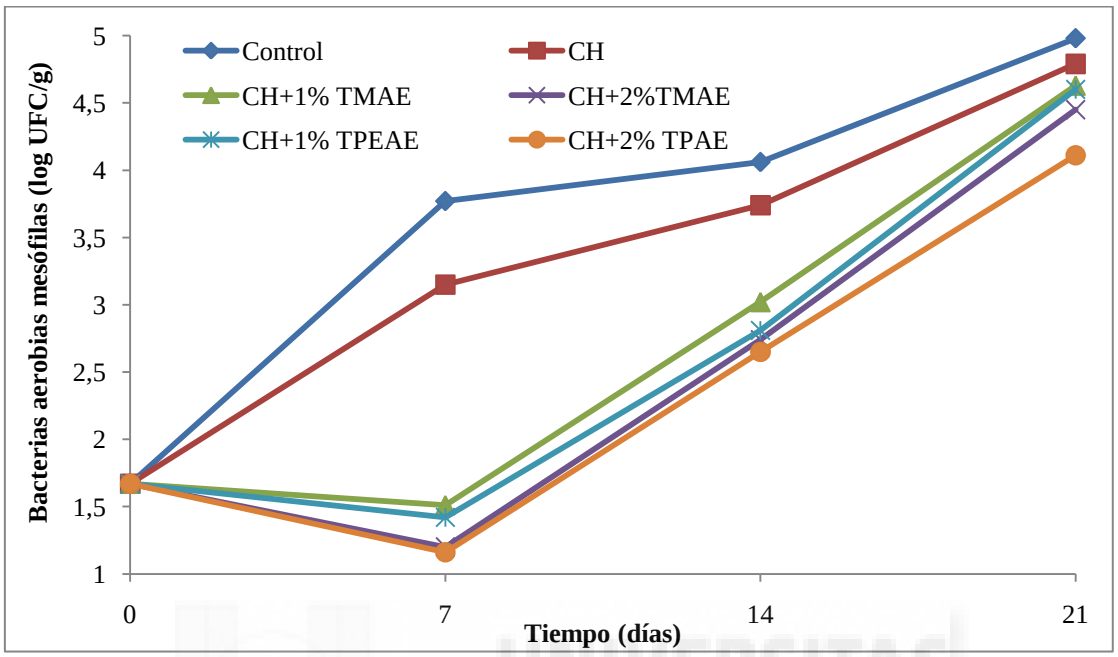

Figura 19. Evolución de los recuentos de bacterias aerobias mesófilas de las muestra de jamón cocido en las que se emplearon, como separadores entre lonchas, films de quitosano adicionados con aceites esenciales de *T. moroderi* y *T. piperella.*

En el caso de las bacterias ácido lácticas (Figura 20), a los 7 días de almacenamiento, las muestras en las que se empleó CH+2%TPAE mostraron los recuentos más bajos ($p<0,05$), con una reducción, respecto al control, de 2,16 ciclos logarítmicos.

Entre las muestras en las que se empleó CH+1%TMAE, CH+2%TMAE y CH+1%TPAE no se apreciaron diferencias significativas ($p>0,05$) aunque sí entre éstas y el control ($p<0,05$) con reducciones de los recuentos de 1,89; 1,98 y 1,93 ciclos logarítmicos, respectivamente. Al final del ensayo, no se apreciaron diferencias ($p>0,05$) entre el control y el grupo en el que se emplearon solo films de quitosano. De nuevo, el empleo de CH+2%TPAE dio lugar a los recuentos más bajos ($p<0,05$) seguido por las muestras con CH+2%TMAE, alcanzándose reducciones con respecto al control de 1,14 y 0,84 ciclos logarítmicos, respectivamente. Entre las muestras en las que se empleó CH+1%TMAE y CH+1%TPAE no se apreciaron diferencias significativas ($p>0,05$) aunque sí ($p<0,05$) de ellas frente al control.

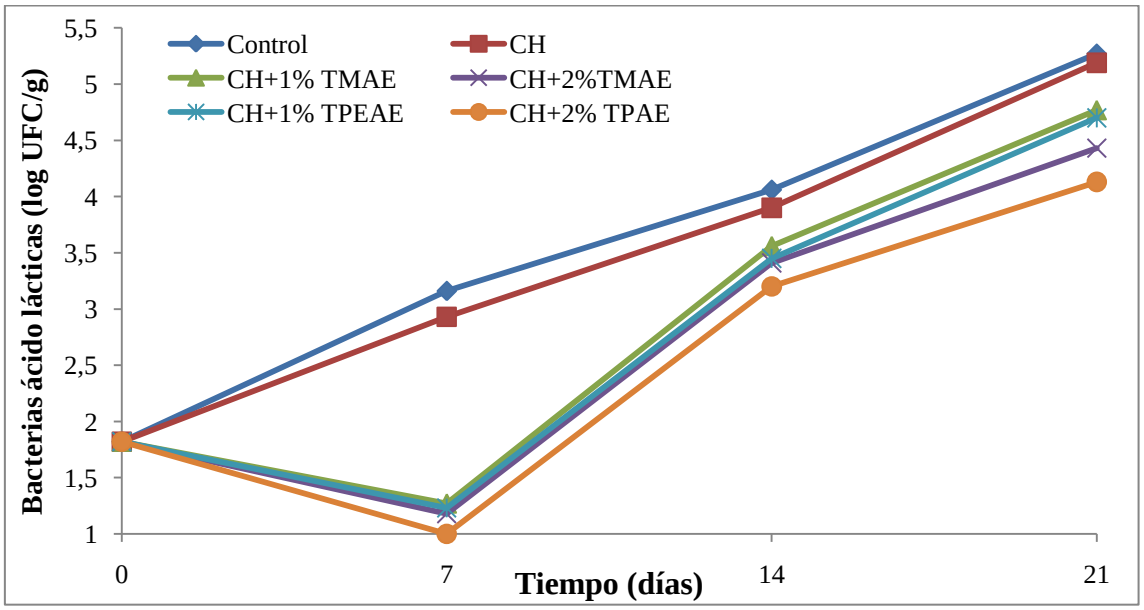

Figura 20. Evolución de los recuentos de bacterias ácido lácticas de las muestra de jamón cocido en las que se emplearon, como separadores de lonchas, films de quitosano adicionados con aceites esenciales de *T. moroderi* y *T. piperella.*

5.7.4.- Liberación de compuestos bioactivos del film al producto cárnico.

La Figura 21 muestra una estimación de la liberación de los compuestos bioactivos desde los films de quitosano adicionados con AEs de *T. moroderi* y *T. piperella* (que se emplearon como separadores de lonchas) a las muestra de jamón cocido, durante su almacenamiento a 4ºC durante 21 días.

Entre los días 0 y 7, para todas las muestras estudiadas (CH+1%TMAE, CH+2%TMAE, CH+1%TPAE y CH+2%TPAE), se produjo una liberación muy pronunciada de compuestos bioactivos desde el film al producto cárnico, siendo esta liberación más pronunciada en las muestras que contenían mayor concentración de AE (2%); se liberaron 7,27 y 8,34 mg AGE/g film para las muestras CH+2%TMAE y CH+2%TPAE, respectivamente, mientras que para las muestras CH+1%TMAE y CH+1% TPAE la liberación fue de 0,87 y 1,46 mg AGE/g film. Sin embargo, a partir del día 7, la liberación de los compuestos bioactivos fue más gradual, hasta llegar al final del ensayo (día 21), con valores comprendidos entre 0,13 y 0,49 mg AGE/g film para todas las muestras, a excepción de la muestra de CH+2%TPAE.

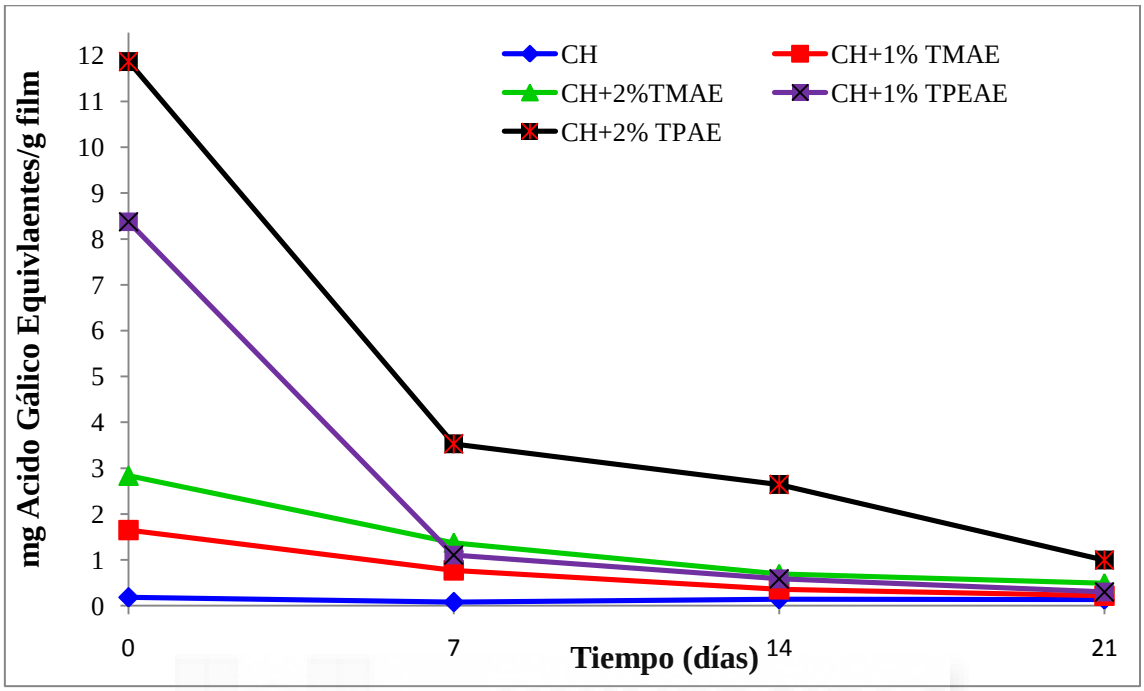

Figura 21. Liberación de los compuestos bioactivos desde los films de quitosano adicionados con aceites esenciales de *T. moroderi* y *T. piperella* (que se emplearon como separadores de lonchas) a las muestra de jamón cocido, durante su almacenamiento a 4ºC durante 21 días.

La migración de los compuestos bioactivos con propiedades antimicrobianas desde los films al producto cárnico, depende de diversos factores, entre los que se encuentran la composición de dicho producto cárnico, interacciones electrostáticas entre el agente antibacteriano y la cadena del polímero, en este caso el quitosano, ósmosis iónica, cambios estructurales en la cadena del polímero provocada por la presencia de los compuestos bioactivos, además de por la variación de las condiciones ambientales (Tª de almacenamiento) o del producto (pH, *a*w), etc. (Cagri et al., 2004). El tipo y la polaridad de las sustancias con propiedades antibacterianas también podrían afectar a la difusión, desde el polímero al producto cárnico (Moradi et al., 2011).

5.7.5.- Color

Los valores de las coordenadas de color CIELAB (L*, a* y b*) de las muestra de jamón cocido, almacenadas a 4ºC durante 21 días, en las que se emplearon como separadores de lonchas, films de quitosano o films de quitosano adicionados con AEs de *T. moroderi* y *T. Piperella*, se muestran en la Tabla 12.

En referencia a la coordenada de luminosidad L*, en las medidas realizadas durante los días 0 y 7, no se apreciaronn diferencias estadísticamente significativas (p>0,05) entre las muestras control y aquellas en la que se utilizaron films de quitosano o films de quitosano adicionado con AE de *T. piperella* y *T. moroderi*.

Tabla 12. Valores de las coordenadas de color (L*, a* y b*) de las muestra de jamón cocido, almacenadas a 4ºC durante 21 días, en las que se emplearon, como separadores de lonchas, films de quitosano o films de quitosano adicionados con aceites esenciales de *T. moroderi* y *T. piperella*

	Muestra	Tiempo (Días)			
		0	7	14	21
L*	Control	60,43±0,53^{aA}	57,92±0,66^{bA}	55,76±0,18^{cA}	58,95±0,56^{bA}
	CH	60,43±0,53^{aA}	60,62±1,93^{aA}	59,65±1,79^{aB}	59,53±0,64^{aA}
	CH+1%TMAE	60,43±0,53^{aA}	60,62±1,17^{aA}	58,28±0,76^{bB}	59,47±0,58^{aA}
	CH+2%TMAE	60,43±0,53^{aA}	57,18±2,38^{aA}	59,80±1,03^{aB}	59,53±1,09^{aA}
	CH+1%TPAE	60,43±0,53^{aA}	60,72±2,95^{abcA}	58,86±0,67^{bB}	62,99±0,39^{cB}
	CH+2%TPAE	60,43±0,53^{aA}	58,18±1,03^{bA}	59,45±0,59^{abB}	59,66±1,09^{abA}
a*	Control	10,08±0,43^{aA}	9,58±1,66^{abA}	10,15±0,56^{aA}	8,89±0,68^{bA}
	CH	10,08±0,43^{aA}	9,19±2,40^{abA}	9,52±0,14^{aA}	8,54±0,37^{bA}
	CH+1%TMAE	10,08±0,43^{aA}	8,99±2,89^{abA}	10,25±0,54^{aA}	8,91±0,31^{bA}
	CH+2%TMAE	10,08±0,43^{aA}	10,18±1,14^{aA}	9,68±0,23^{aA}	8,77±0,19^{bA}
	CH+1%TPAE	10,08±0,43^{aA}	9,24±1,93^{aA}	9,66±0,23^{aA}	5,06±0,30^{bB}
	CH+2%TPAE	10,08±0,43^{aA}	8,99±3,25^{abA}	8,71±0,96^{abA}	8,43±0,99^{bA}
b*	Control	8,37±0,11^{aA}	8,74±1,39^{aA}	8,81±0,46^{aA}	8,17±0,66^{aA}
	CH	8,37±0,11^{aA}	7,78±2,93^{aA}	8,69±0,35^{aA}	7,90±0,75^{aA}
	CH+1%TMAE	8,37±0,11^{aA}	8,54±2,29^{aA}	8,09±0,47^{aA}	7,98±0,40^{aA}
	CH+2%TMAE	8,37±0,11^{aA}	9,74±2,44^{abA}	8,15±0,48^{aA}	10,05±0,82^{bB}
	CH+1%TPAE	8,37±0,11^{aA}	8,19±1,31^{abA}	7,72±2,36^{abA}	11,37±0,93^{cB}
	CH+2%TPAE	8,37±0,11^{aA}	12,26±2,08^{bB}	8,36±0,22^{aA}	7,37±0,63^{cA}

Para una misma coordenada, valores seguidos por la misma letra minúscula dentro de la misma fila no son significativamente diferentes (p>0,05) de acuerdo con el Test de Rangos Múltiples de Tukey.
Para una misma coordenada,, valores seguidos por la misma letra mayúscula dentro de la misma columna no son significativamente diferentes (p>0,05) de acuerdo con el Test de Rangos Múltiples de Tukey.

A los 14 días, la muestra control es la que presentaba los valores más bajos de L* (p<0,05), no apreciándose diferencias significativas entre las muestras en las que se empleó quitosano o quitosano adicionado con AE de *T. piperella* y *T. moroderi*. Sin embargo, al final del ensayo (día 21) todas las muestras mostraban unos valores de luminosidad muy similares, no existiendo diferencias estadísticamente significativas (p>0.05) entre ellas, a excepción de la muestra CH+1%TPAE que mostraba valores de L* (p<0,05) superiores al resto. El tiempo de almacenamiento afectó significativamente (p<0,05) a la L* de la muestra control, provocando un leve descenso con respecto a la

medida inicial (tiempo 0). También afectó a la muestra CH+1%TPAE, pero en sentido contrario, pues mostraba valores superiores de L* al final, que los obtenidos al inicio del ensayo. En el resto de las muestras, el tiempo de almacenamiento, no provocó ($p>0,05$) ningún efecto sobre esta coordenada (L*). Diversos autores indican que los valores de luminosidad (L*) en la carne y los productos cárnicos está relacionada con el contenido en agua de la superficie del producto, los intercambios de vapor de agua entre el medio y el producto o las modificaciones que se producen en los diferentes estados de los hemopigmentos del producto (Fernández-López, et al., 2000).

En cuanto a la coordenada rojo-verde (a*), el empleo de los films de quitosano no provocó ningún cambio significativo ($p<0,05$) con respecto al control, a excepción de la muestra CH+1%TPAE que mostró los valores más bajos de a* ($p>0,05$) al final del ensayo. Sin embargo, el tiempo de almacenamiento sí influyó de forma significativa sobre esta coordenada. Al final del ensayo, todas las muestras mostraban unos valores de a* inferiores ($p<0,05$) a los obtenidos al inicio del estudio. Este descenso en los valores de la coordenada rojo-verde podrían estar relacionados con procesos oxidativos, tal y como reportaron Fernández-López et al. (2006b). Estos autores indicaron que el descenso en la coordenada a* está relacionado con la oxidación de los lípidos y de los hemopigmentos, y como puede verse en la Figura 17 la oxidación aumentó a lo largo del almacenamiento.

Para la coordenada amarillo-verde (b*), la utilización de los films de quitosano no tubo ningún efecto significativo ($p>0,05$) para esta coordenada, a excepción de las muestras CH+2%TPAE y CH+1%TMAE donde se produjo un aumento de b*, no apreciándose diferencias significativas entre ellas ($p<0,05$). De igual modo, el tiempo de almacenamiento tampoco provocó cambios ($p>0,05$) en esta coordenada (b*) para las muestras control, las muestras en las que se empleó quitosano (CH) o las muestras en la que se empleó CH+1%TMAE. En contraste, las muestras en las que se utilizó CH+1%TPAE, CH+2%TPAE y CH+2%TMAE sí se vieron afectadas significativamente ($p<0,05$) por el tiempo de almacenamiento. El comportamiento de la coordenada b* depende en gran medida de la matriz del alimento. Según Cofrades et al. (2004) cambios en el pH, actividad de agua y grado de oxidación del producto, influirán, de forma significativa, sobre esta coordenada.

CONCLUSIONES

6.-CONCLUSIONES

1. Los aceites esenciales obtenidos de las cuatro hierbas aromáticas analizadas (*Thymus piperella*, *Thymus moroderi*, *Sideritis angustifolia* y *Santolina chamaecyparissus*) muestran una gran variedad de compuestos en su composición variando éstos, desde los 48 hasta los 77. Entre los distintos grupos funcionales podemos encontrar fundamentalmente terpenos (dihidro-aromadendreno) fenoles monoterpenicos (carvacrol) y éteres monoterpenicos (1,8-cineol).

2. Los aceites esenciales ensayados muestran una moderada actividad antibacteriana, siendo efectivos, al máximo volumen ensayado (40 µL), sobre 5 de las 11 bacterias analizadas. Para estos volúmenes, el aceite esencial de *Thymus piperella* presenta el mayor efecto antibacteriano, mientras que el aceite esencial de *Santolina chamaecyparissus* es el menos efectivo.

3. Cuando se analiza el efecto de la concentracion, solo el aceite esencial de *Thymus piperella*, muestra efecto inhibidor del crecimiento bacteriano, sobre las cepas sensibles, a todos las concentraciones analizadas. A bajas concentraciones (4 y 2 µL) los aceites esenciales de *Thymus moroderi*, *Sideritis angustifolia* y *Santolina chamaecyparissus* no ejercen ningún efecto inhibidor del desarrollo bacteriano.

4. Todos los aceites esenciales analizados, a todas las concentraciones ensayadas, presentan actividad antifúngica, tanto frente a hongos como frente a levaduras. El aceite esencial de *Sideritis angustifolia*, a la máxima concentración ensayada, presenta los mayores valores de inhibición del crecimiento del micelio frente a tres de los seis hongos analizados, seguido por el aceite esencial de *Santolina chamaecyparissus* en dos de los seis hongos analizados. En el caso de las levaduras, el aceite esencial de *Sideritis angustifolia* mostró la menor concentración mínima de inhibición sobre 4 de las 6 levaduras analizadas.

5. Todos los aceites esenciales analizados presentan un alto contenido en fenoles totales, así como una importante actividad antioxidante, con todos los métodos

utilizados. El aceite esencial de *Thymus piperella* muestra los mayores valores de actividad antioxidante en tres de los cuatro métodos empleados (capacidad de donar H^+, capacidad de reducir el ion férrico y la capacidad de inhibición de la peroxidación lipídica); sin embargo, muestra los valores más bajos en la capacidad de quelar metales.

6. Para todos los aceites esenciales analizados y todos los métodos antioxidantes ensayados, excepto la capacidad quelante de metales, hay una importante correlación entre el contenido en fenoles totales y la actividad antioxidante.

7. La adición de los aceites esenciales sometidos a estudio a films de quitosano, aumenta sus propiedades antibacterianas. Los films de quitosano adicionados con aceite esencial de *Thymus piperella* son los que muestran una mayor actividad antibacteriana.

8. La adición de los aceites esenciales sometidos a estudio a los films de quitosano, mejora, considerablemente, sus propiedades antioxidantes. Nuevamente los films de quitosano a los que se adicionó aceite esencial de *Thymus piperella* son los que muestran una mayor actividad antioxidante (en los tres métodos ensayados).

9. La utilización de los films de quitosano adicionados con aceite esencial de *Thymus piperella* o *Thymus moroderi* como separadores de lonchas de un producto cárnico cocido tipo jamón cocido, reduce los valores de oxidación lipídica de dicho producto. Este efecto es más acentuado en las muestras en las que se utiliza el aceite esencial de *Thymus piperella*.

10. El empleo de films de quitosano adicionados con aceite esencial de *Thymus piperella* o *Thymus moroderi*, como separadores de lonchas de un producto cárnico cocido tipo jamón cocido, reduce los recuentos de bacterias aerobias mesófilas y bacterias ácido lácticas en el producto cárnico. Tampoco se observó en ningún caso crecimiento de mohos, levaduras y enterobacterias.

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7.-BIBLIOGRAFÍA

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ABSTRACT

The aim of this work was to determine the following characteristics of the essentials oils (EOs) obtained from two *Thymus* endemic species such as *Thymus moroderi* and *Thymus piperella*: (i) their chemical composition and (ii) their effect on the growth of several bacteria related to food spoilage, such as *Listeria innocua*, *Serratia marcenscens*, *Pseudomonas fragi*, *Pseudomonas fluorescens*, *Aeromonas hydrophila*, *Shewanella putrefaciens*, *Achromobacter denitrificans*, *Enterobacter amnigenus*, *Enterobacter gergoviae*, *Alcaligenes faecalis* and *Leuconostoc carnosum*. The EOs were chemically analysed and identified by GC and GC–MS, while the agar disc diffusion method was used to determine their antibacterial activities. The concentration effect was also determined.

The major constituents of *T. moroderi* EO were camphor (26.74%), 1,8-cineol (24.99%) and myrcene (5.63%). In the *T. piperella* EO the main constituents were carvacrol (31.92%), *para*-cymene (16.18%) and γ-terpinene (10.11%). *T. piperella* EO was much more effective than *T. moroderi* EO, and had a much greater effect on the Gram-positive bacteria than on the Gram-negative bacteria.

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1. Introduction

Thymus L. (Labiatae) consists of about 215 species of herbaceous perennials and sub-shrubs (Nickavar, Mojab, & Dolat-Abadi, 2005). Species of *Thymus* are well adapted to the hot and dry climate of the Mediterranean region and are widespread in the arid parts of the Iberian Peninsula (Horwath, Grayer, Keith-Lucas, & Simmonds, 2008). "Thyme", with a long list of pharmacological and aromatic properties, is commonly used in the herbalist sector. *Thymus* is used as spice in several foods. In addition, it is one of the most widely used genuses in folk medicine, where it is popular for its stimulatory action on all organism functions (Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez, 2011) and also for the antimicrobial activity of its essential oil. Growing wild in the Mediterranean environment, there are several ecotypes of thyme, which differ in their morphological characteristics (Tedone, D'andrea, & Marzi, 2001) and in the composition of their essential oils (Corticchiato, Tomi, Bernardin, & Casanova, 1998), although all are characterized by a strong and penetrating odour and sometimes a very pronounced balsamic and spicy flavour. Two species found in the *Thymus* genus are *Thymus piperella* and *Thymus moroderi*. The first of these is an endemic eastern Iberian taxon, extending over approximately 800 km^2 of the province of Valencia. *T. moroderi*, which is closely related to common thyme (*Thymus vulgaris* L.), is also endemic to southeast of Spain. The genus *Thymus* is characterized by its high content of essential oils (EOs), which leads to it being regarded as an aromatic herb with potential health benefits.

In general terms, EOs are bioactive compounds formed of a mixture of many substances; they have a complicated chemical composition and are obtained from the secondary metabolism of plants (Delamare, Moschen-Pistorello, Artico, Atti-Serafini, & Echeverrigaray, 2007). They are simple lipids without fatty acids and are composed of volatile substances, most of which derived from three main classes of compound: terpenoids, phenylpropanoids/benzenoids and fatty acid derivates (Vainstein, Lewinsohn, Pichersky, & Weiss, 2001). Among the numerous properties of these EOs are their antioxidant, digestion-stimulating, hypolipidemic, antiinflammatory, anticarcinogenic and antibacterial activities (Viuda-Martos et al., 2011).

The antimicrobial substances used in the food industry include chemical compounds (added or naturally present in foods) for two main reasons: (i) to control natural deterioration processes (food preservation) and (ii) to prevent or limit the growth of

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microorganisms, including pathogenic strains (Tajkarimi, Ibrahim, & Cliver, 2010). However, the industry is increasingly being pressed by consumers to reduce the synthetic chemical compounds used to inhibit microbial growth (Deba, Xuan, Yasuda, & Tawata, 2008). Partly as a consequence, aromatic herbs or spices, especially their derivates such as EOs, are gaining in interest for their potential as preservatives and as decontamination agents, since such substances have been recognised as safe (GRAS) and are widely accepted by consumers (Burt, 2004). However, a balance must be struck between their maximum efficacy and the organoleptic properties of the foods to which they are added (Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez, 2008). In this respect, EOs have been incorporated in various food matrixes to determine their microbial properties. For example, Karabagias, Badeka, and Kontominas (2011) studied the effect on the shelf life of lamb added with thyme EO and packed in a modified atmosphere packaging, and observed a reducing effect on bacteria like *Pseudomonas* spp., lactic acid bacteria, *Brochothrix thermosphacta* and Enterobacteriaceae spp. Similarly, Viuda-Martos, Ruiz-Navajas, Fernández-López, and Pérez-Álvarez (2009) applied thyme EO to a cooked meat product and observed a reduction in the growth of lactic acid bacteria and Total Aerobic Counts. Ruiz-Navajas et al. (2011) analysed the antimicrobial activity of the EOs of two thyme species on a meat extract and cooked meat products, observing the inhibition of growth of bacteria such as *Listeria innocua* and *Aeromonas hydrophila*, both of which are related with food spoilage.

In contrast to the numerous studies on the essential oils of *Thymus* (De Lisi, Tedone, Montesano, Sarli, & Negro, 2011; Sarikurkcu et al., 2010; Zouari et al., 2011) there are only limited data on the composition and antimicrobial activities of *T. moroderi* and *T. piperella*. Thus, the aim of this study was to determine the chemical composition and antibacterial properties of the EOs of two aromatic endemic herbs, *T. moroderi* and *T. piperella*, both endemic to the southeast of Spain.

2. Materials and methods

2.1. Plant materials

T. moroderi and *T. piperella* were collected during their flowering period. Identification of the plant material was made by Prof. Dra. Concepcion Obon de Castro, Biology Department of Miguel Hernandez University (Spain).

2.2. Extraction of the essential oils

The EOs of *T. moroderi* and *T. piperella* were extracted from whole plant (stems, leaves and flowers) by hydro-distillation using a Clevenger-type apparatus for 3 h. The oily layer obtained on top of the aqueous distillate was separated and dried with anhydrous sodium sulphate (0.5 g). The extracted EOs was kept in sealed air-tight glass vials and covered with aluminium foil at 4 °C until further analysis. The respective yields of the essential oils were *T. moroderi* 1.90% and *T. piperella* 1.66%.

2.3. Gas chromatography analysis (GC)

The EOs were analysed using a Shimadzu GC-17A equipped with FID detector and a TRACSIL Meta X5 column (Teknokroma S. Coop. C. Ltd, Barcelona, Spain), 30 m × 0.25 mm i.d., 0.25 μm film thickness. Injector and detector temperatures were set at 250 and 300 °C, respectively. The oven temperature was kept at 40 °C for 5 min; raised to 200 °C at 3.0 °C/min and held for 1 min; raised to 280 °C at 15 °C/min and held for 10 min. Helium was used as carrier gas, at a flow rate of 1 mL/min. Diluted samples (1/10 cyclohexane, v/v) of 0.2 μL were injected manually in the split mode (split ratio 1/44). Quantitative data were obtained electronically from FID area data without using correction factors. All the tests were performed in triplicate.

2.4. Gas chromatography–mass spectrometry analysis (GC–MS)

The volatile compounds were isolated, identified and quantified on a Shimadzu GC-17A gas chromatograph (Shimadzu Corporation, Kyoto, Japan), coupled to a Shimadzu mass spectrometer detector (GC–MS QP-5050A). The GC–MS system was equipped with the same column used in the GC analysis and with the same temperature programme. Analyses were carried out using helium as carrier gas at a flow rate of 1 mL/min, at a split ratio of 1:10 Diluted samples (1/10 cyclohexane, v/v) of 0.2 μL of the extracts were injected. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of 45–450 *m/z*. Most of the compounds were identified using two different analytical methods: (a) KI, Kováts Indexes in reference to n-alkanes (C_8–C_{32}) (NIST, 2010); and (b) mass spectra (authentic chemicals and Wiley spectral library collection). Identification was considered tentative when based on mass spectral data alone.

2.5. Microbial strains

The essentials oils were individually tested against *L. innocua* CECT 910, *Serratia marcenscens* CECT 854, *Pseudomonas fragi* CEPT 446, *Pseudomonas fluorescens* CECT 844, *A. hydrophila* CECT 5734, *Shewanella putrefaciens* CECT 5346, *Achromobacter denitrificans* CECT 449, *Enterobacter amnigenus* CECT 4078, *Enterobacter gergoviae* CECT 587, *Alcaligenes faecalis* CECT 145, *Leuconostoc carnosum* CECT 4024. These species were supplied by the Spanish Type Culture Collection (CECT) of the University of Valencia. These bacteria were chosen due to they are commonly associated with refrigerated foods: as indicator of pathogenic bacteria or as spoilage bacteria.

2.6. Agar disc diffusion method

The agar disc diffusion method described by Tepe, Daferera, Sokmen, Sokmen, and Polissiou (2005) with some modifications was used to determine the antibacterial capacity of the essential oils. Briefly, a suspension (0.1 mL of 10^6 cfu/mL) of each microorganism was spread on the solid medium plates. Nutrient Agar II (Oxoid, Basingstoke, Hampshire, England) in the case of *S. marcenscens*, *P. fragi*, *P. fluorescens*, *A. hydrophila*, *S. putrefaciens*; Nutrient Agar I (Oxoid, Basingstoke, Hampshire, England) in the case of *E. gergoviae*, *E. amnigenus*, *A. faecalis*, *A. denitrificans*; de Mann Rogosa Sharpe (MRS) agar (Sharlau, Barcelona, Spain) for *L. carnosum* and Brain Heart Infusion agar (Sharlab, Sharlab SL, Barcelona, Spain) for *L. innocua*. Filter paper discs, 9 mm in diameter (Schlinder & Schuell, Dassel, Germany), were impregnated with 40 μL of the oil and placed on the inoculated plates; these plates were incubated at 37 °C for 48 h in the case of *E. gergoviae*, *E. amnigenus*, 37 °C for 24 h in the case of *A. faecalis* and *L. innocua*; at 26 °C for 48 h in the case of *P. fragi*, *P. fluorescens*, *S. putrefaciens*, *L. carnosum* and 26 °C for 24 h for *S. marcenscens*, *A. hydrophila*, *A. denitrificans*. The diameters of the inhibition zones were measured in millimetres. All tests were performed in triplicate.

2.7. Determination of concentration effect

The concentration effect (CE) was studied to ascertain which doses of essential oil had an inhibitory effect on bacterial growth in

the disc diffusion assay. The culture techniques used were those described in the previous paragraph (Agar disc diffusion method), but adding 40, 20, 10, 4 and 2 μL of essential oil which meant doses of 100%, 50%, 25%, 10% and 5% of the initial volume (Viuda-Martos, Fernández-López, & Pérez-Álvarez, 2005). All tests were performed in triplicate.

2.8. Statistical analysis

Conventional statistical methods were used to calculate means and standard deviations of three simultaneous assays carried out with the different methods. Statistical analysis (ANOVA) was applied to the data to determine differences ($P < 0.05$). To discover whether there were significant differences between the levels of the main factor, contrasts between means were made using a Tukey test (Afifi & Azen, 1979). For the antibacterial activity, an ANOVA was applied, with the following factors: doses (five levels; 40, 20, 10, 4 and 2 μL) for each essential oil. The statistical analyses were made using Statgraphics 5.1 for Windows.

3. Results and discussions

3.1. Chemical composition

The chemical composition of *T. moroderi* and *T. piperella* essential oil was investigated using both GC and GC–MS techniques. The percentages and the retention indexes of the main oil components are listed in Table 1 in the order of their elution on the TRACSIL Meta X5 column. In the case of *T. moroderi* essential oil, 51 compounds were identified as representing 92.1% of the oil. The main components were camphor (26.74%), 1.8-cineol (24.99%), myrcene (5.63%) and α-pinene (4.35%). Forty-eight compounds were identified for *T. piperella*, representing 90.5% of the essential oil. The major component was carvacrol (31.92%) followed by *para*-cymene (16.18%), γ-terpinene (10.11%) and, to a lesser extent, 4-terpineol (7.29%). Marti et al. (2007) reported that the major constituents of *T. piperella* essential oil were *para*-cymene (31.70%), carvacrol (14.00%) and γ-terpinene (11.30%), while Boira and Blanquer (1998) investigated the essential oil composition of three subspecies of *T. piperella,* reporting that there are three chemotypes: chemotype A composed of *para*-cymene, carvacrol and γ-terpinnene, chemotype B composed of *para*-cymene and thymol, and chemotype C composed of *para*-cymene and carvacrol. The EO of *T. piperella* analysed in this study may be include in chemotype C.

Table 1
Constituents of *T. moroderi* and *T. piperella* and their relative percentages of total chromatogram area, Kováts index and retention time.

Compounds	Id[a]	Kováts indexes		Essential oils	
		KI	Lit[b]	*T. moroderi* (% area)	*T. piperella* (% area)
Alpha pinene	KI,W	940	939	4.35	–
Camphene	KI,W	960	953	8.8	–
Sabinene	KI,W	979	976	1.14	–
Myrcene	KI,W	989	991	5.63	–
Alpha-terpinene	KI,W	1023	1018	–	2.69
Para-cymene	KI,W,St	1032	1027	–	16.18
l-Limonene	KI,W,St	1035	1036	1.93	1.54
1,8-Cineole	KI,W,St	1042	1039	24.99	–
Gamma, terpinene	KI,W	1064	1062	–	10.11
l-Linalool	KI,W	1102	1098	1.31	2.84
Cis-sabinene hydrate	KI,W	1111	1097	–	2.02
Camphor	KI,W	1163	1143	26.74	–
1-Borneol	KI,W	1186	1165	3.62	–
Terpineol-4	KI,W	1192	1189	2.36	7.29
Alpha-terpineol	KI,W	1205	1207	2.62	1.14
Thymol	KI,W,St	1294	1290	–	5.19
Carvacrol	KI,W,St	1307	1299	–	31.92
Beta-elemene	KI,W	1399	1393	1.06	–
Trans-caryophyllene	KI,W	1436	1430	–	6.09
Bicyclogermacrene	KI,W	1511	1494	1.86	–
Viridiflorol	KI,W	1595	1590	2.08	–
Caryophyllene oxide	KI,W	1602	1606	–	1.58

[a] "KI,W" means that identification was based on Kováts indexes and comparison with Wiley library. "KI" compounds tentatively identified based on Kováts indexes, "W" compounds tentatively identified (only Wiley library was used for identification).
[b] NIST database.

This is the first report to analyse the chemical composition of *T. moroderi* and no information on its composition has been reported previously. However, the chemical composition of the EO obtained from plants of genus *Thymus* has been widely investigated. Thus, Bagamboula, Uyttendaele, and Debevere (2003) reported that no major differences were observed between commercial thyme essential oil and the essential oil obtained by steam distillation of finely ground dried thyme leaves, and only linalool and γ-terpinene were found in higher concentrations in the commercial essential oil. In another study the same authors (Bagamboula, Uyttendaele, & Debevere, 2004) investigated the EO of thyme, identifying 28 compounds, which represented 92.6% of the total oil, the major constituents being thymol (32.23%), γ-terpinene (21.19%) and *para*-cymene (20.27%). Napoli, Curcuruto, and Ruberto (2010) reported on the composition of the essential oils of the thirty samples of thyme collected in Italy. In total, 46 components were fully identified, covering more than 96% of the total composition. Pinto et al. (2006) analysed the composition of the EO of *Thymus pulegioides* from Portugal. In this case, the oil was characterized by high amounts of thymol (26.0%), and carvacrol (21.0%) and its biogenetic precursors, γ-terpinene (8.8%) and *para*-cymene (7.8%).

The compositional data shows that carvacrol is by far the main compound in almost all samples. It is accepted that the terpenes thymol, *para*-cymene and carvacrol are the major volatile components of thyme. Some studies have reported that thyme essential oil possesses a high level of the phenolic precursors, *para*-cymene and γ-terpinene, probably due to the early flowering time of the plant (Saez, 1998). In other published results, Lee, Umano, Shibamoto, and Lee (2005) identified 43 monoterpenes, 16 sesquiterpenes, 14 aromatic compounds, 7 alcohols, 3 aldehydes, 4 ketones and esters, and 3 acids in a thyme extract. Phenolic compounds were the aroma principles in this chemotype of thyme. Quantitatively, the most important compounds were thymol (72%) and isothymol (carvacrol) (5.7%), which constituted almost three-quarters of the total volatiles quantified. This great variability and diversity observed in the chemical composition of the essential oils of *Thymus* species and subspecies can be attributed to climatic and soil variations, the stage of the vegetative cycle, seasonal variations, etc. (Cosentino et al., 1999). Tzakou, Verykokidou, Rousis, and Chinou (1998) reported that in some cases two different varieties may provide the same essential oil yield and quality, even though the plants are morphologically different.

3.2. Antibacterial activity

The antibacterial activity of the essential oils of *T. moroderi* and *T. piperella* was assayed *in vitro* following the diffusion in agar disc method using eleven bacteria associated with food spoilage. Table 2 shows the microbial growth inhibition achieved by each of the EOs assayed. As can be seen, *T. piperella* essential oil had an inhibitory effect on 5 of the 11 bacteria assayed, with inhibition halos that ranged from 16.00 mm for *A. denitrificans* to 45.00 mm for *A. hydrophila,* and no antibacterial activity against *P. fragi*, *P. fluorescens*, *S. putrefaciens*, *E. gergoviae*, *Enterobacter anmigenus* and *L. carnosum.*

Table 2
Antimicrobial activity of *T. moroderi* and *T. Piperella* essential oils using disc diffusion method.

Essential oils	Diameter (mean and SD) of inhibition zone (mm) including disc diameter of 9 mm										
	S. marcenscens	*P. fragi*	*P. fluorescens*	*A. hydrophila*	*S. putrefaciens*	*E. gergoviae*	*E. amnigenus*	*A. faecalis*	*A. denitrificans*	*L. carnosum*	*L. innocua*
T. moroderi	23.90 ± 0.10^{a}	N.A.	N.A.	20.50 ± 0.70^{a}	N.A.	N.A.	N.A.	24.00 ± 1.40^{a}	N.A.	N.A.	15.50 ± 0.70^{a}
T. piperella	24.50 ± 0.70^{a}	N.A.	N.A.	45.50 ± 3.50^{b}	N.A.	N.A.	N.A.	45.50 ± 0.70^{b}	16.00 ± 0.00	N.A.	27.30 ± 1.20^{b}

For the same bacteria, values followed by different letters within the same column are significantly different ($P < 0.05$) according to Turkey's multiple range test.
N.A., non-active.

The *T. moroderi* essential oil had an inhibitory effect on 4 of the bacteria, with inhibition halos from 15 mm (*L. innocua*) to 24.00 mm (*A. faecalis*). This EO showed no activity against *P. fragi*, *P. fluorescens*, *S. putrefaciens*, *E. gergoviae*, *E. anmigenus*, *A. denitrificans* and *L. carnosum*. This is the first study to analyse the antibacterial activity of the EOs from *T. moroderi* and *T. piperella.* However, as in the case of the chemical composition, the antibacterial activity of several other thyme varieties has been studied.

De Martino et al. (2009) investigated the antibacterial activity of essential oils of the aerial parts of *Thymus longicaulis* and *T. pulegioides* collected in Italy. At a concentration of 10 mg/mL both EOs showed substantial antibacterial activity against Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus*, *Streptococcus faecalis*, *B. subtilis*, *Bacillus cereus*, *Proteus mirabilis*, *Enterobacter coli*, *Salmonella typhi Ty2* and *Pseudomonas aeruginosa*, with inhibition zones that ranged between 9 and 18 mm for *T. longicaulis* and between 11 and 20 mm for *T. pulegioides*. Cetin, Cakmakci, and Cakmakci (2011) investigated the antibacterial activity of *Thymus sipyleus* subsp. *sipyleus* var. *rosulans* EO from Turkey. The EO showed the highest inhibitory effects on *Pseudomonas pseudoalkaligenes* (59 mm) and *S. aureus* (56 mm), followed by *B. subtilis, P. aeruginosa*, *S. pyogenes*, and *Pseudomonas vulgaris* (nearly to the same extent) and a lower inhibitory effect on *Enterobacter cloacae*. Viuda-Martos et al. (2008) analysed the antimicrobial activity of the essential oil from *T. vulgaris* cultivated in Spain at a volume of 40 μL, and obtained inhibition zones of 21.60, 28.57, 53.85, 21.61, 24.05 and 23.64 mm for *Staphylococcus xylosus*, *Staphylococcus carnosus*, *Lactobacillus sakei*, *Lactobacillus curvatus*, *E. gergoviae* and *Enterobacter Amnigenus*, respectively.

The antimicrobial activity of the EOs from aromatic herbs and spices results from a small number of terpenes and phenolic compounds (Dorman & Deans, 2000). As mentioned above, the principal components of *T. piperella* EO are carvacrol and *para*-cymene, and those of *T. moroderi* EO are 1,8-cineole and camphor. Bagamboula et al. (2003) mentioned that carvacrol at concentrations of 0.5% and 1% shows antibacterial activity against *Shigella sonnei* and *Shigella flexneri*. Similarly, Rattachaikunsopo and Phumkhachrom (2010) suggested that the antimicrobial effect of *T. moroderi* EO may be partly associated with the presence of camphor and 1,8-cineole since both compounds have been documented as having antibacterial activity (Mourey & Canillac, 2002; Viljoen et al., 2003).

In the present study, it was observed that *T. moroderi* EO contained neither carvacrol nor *para*-cymene, the major components being 1,8-cineole and camphor. It has been shown that carvacrol is a stronger inhibitor than camphor and 1,8-cineole, which may explain the lower antimicrobial activity of *T. moroderi* EO compared with *T. piperella* EO.

Considering the large number of different groups of chemical compounds present in EOs, it is most likely that their antibacterial activity is not attributable to one specific mechanism but that there are several targets in the cell (Burt, 2004). Different modes of action have been suggested: for example, EOs may act by causing structural and functional damage to the bacterial cell membrane (Goni et al., 2009). The leakage of ions and other cell contents may also occur (Carson, Hammer, & Riley, 2002). Davidson (2001) reported that the exact cause–effect relation for the mode of action of phenolic compounds, such as thymol, eugenol and carvacrol, has not been determined, but that they may inactivate essential enzymes, react with the cell membrane or disturb genetic material.

The apparent antimicrobial efficacy of plant-origin antimicrobials depends on factors such as the method of extracting EOs from plant material, the volume of inoculum, growth phase, the culture medium used, and intrinsic or extrinsic properties of the food such as pH, fat, protein, water content, antioxidants, preservatives,

Table 3
Antimicrobial activity of *T. moroderi* and *T. Piperella* essential oils using disc diffusion method.

Essential oils	Doses (μL)	Diameter (mean and SD) of inhibition zone (mm) including disc diameter of 9 mm									
		S. marcenscens	*P. fragi*	*P. fluorescens*	*A. hydrophila*	*S. putrefaciens*	*E. gergoviae*	*E. amnigenus*	*A. faecalis*	*A. denitrificans*	*L. carnosum*	*L. innocua*
T. moroderi	2	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	4	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	10	11.30 ± 0.10^{a}	N.A.	N.A.	12.50 ± 0.70^{a}	N.A.	N.A.	N.A.	11.50 ± 0.70^{a}	N.A.	N.A.	10.90 ± 0.10^{a}
	20	14.90 ± 0.10^{b}	N.A.	N.A.	16.00 ± 1.40^{b}	N.A.	N.A.	N.A.	13.50 ± 0.00^{b}	N.A.	N.A.	12.20 ± 0.10^{b}
	40	23.90 ± 0.10^{c}	N.A.	N.A.	20.50 ± 0.70^{c}	N.A.	N.A.	N.A.	24.00 ± 1.40^{c}	N.A.	N.A.	15.50 ± 0.70^{c}
T. Piperella	2	13.80 ± 0.40^{a}	N.A.	N.A.	32.00 ± 0.00^{a}	N.A.	N.A.	N.A.	21.50 ± 0.70^{a}	11.00 ± 0.00^{a}	N.A.	15.80 ± 0.30^{a}
	4	15.70 ± 0.60^{b}	N.A.	N.A.	35.50 ± 2.10^{b}	N.A.	N.A.	N.A.	27.50 ± 2.10^{b}	12.00 ± 0.00^{b}	N.A.	17.80 ± 0.40^{b}
	10	19.60 ± 0.60^{c}	N.A.	N.A.	38.50 ± 2.10^{b}	N.A.	N.A.	N.A.	30.05 ± 2.10^{c}	13.00 ± 0.00^{c}	N.A.	21.50 ± 0.70^{c}
	20	22.80 ± 0.70^{d}	N.A.	N.A.	43.00 ± 2.80^{c}	N.A.	N.A.	N.A.	35.00 ± 0.70^{d}	14.50 ± 0.70^{d}	N.A.	23.30 ± 1.10^{d}
	40	24.50 ± 0.70^{e}	N.A.	N.A	45.50 ± 3.50^{c}	N.A	N.A.	N.A.	45.50 ± 0.70^{e}	16.00 ± 0.00^{e}	N.A.	27.30 ± 1.20^{e}

For the same essential oil, values followed by different letters within the same column are significantly different ($P < 0.05$) according to Tukey's multiple range test.
N.A., non-active.

incubation time/temperature, packaging procedure, and the physical structure of food (Brandi, Amagliani, Schiavano, De Santi, & Sisti, 2006; Burt, 2004; Lis-Balchin, Steyrl, & Krenn, 2003; Lopez-Malo, Palou, & Alzamora, 2005).

The type of bacterium also has an influence on the effectiveness of EOs. Generally, Gram-negative bacteria are less sensitive to the antimicrobials (Gilles, Zhao, An, & Agboola, 2010). The difference in susceptibility could be due to the lipopolysaccharide outer membrane of this group, which restricts diffusion of hydrophobic compounds. However, this does not mean that Gram-positive bacteria are always more susceptible (Burt, 2004). Gram-negative bacteria are usually more resistant to the plant-origin antimicrobials (which may even have no effect), than Gram-positive bacteria (Rameshkumar, George, & Shiburaj, 2007; Stefanello et al., 2008). On the other hand, Sokmen et al. (2004) affirmed that the essential oils of spices show no selectivity as regards the cell walls of bacteria.

3.3. Concentration effect

Table 3 shows the concentration effect (CE) of the EOs from *T. moroderi* and *T. piperella* on the eleven bacterial strains studied. In the case of *T. moroderi* EO, no antibacterial effect was observed at any concentration on *S. putrefaciens*, *P. fragi*, *P. fluorescens*, *E. gergoviae*, *E. amnigenus*, *L. carnosum* and *A. denitrificans*. Neither 4 nor 2 μL had any effect on discs impregnated with *S. marcenscens* but concentrations of 40, 20 and 10 μL did have an effect, with statistically significant differences between them ($P < 0.05$). The same behaviour was seen with *A. hydrophila*, *A. faecalis* and *L. innocua*.

As regards *T. piperella* EO, all the concentrations assayed produced inhibition halos with *S. marcenscens*, *A. denitrificans*, *A. faecalis*, *A. hydrophila* and *L. innocua.* There were statistically significant differences for the concentrations and strains ($P < 0.05$) except for *A. hydrophila*, when no differences ($P < 0.05$) existed between the discs impregnated with 10 and 4 μL or between 40 and 20 μL, as occurred with *T. moroderi* EO. The EO from *T. piperella* was not active at any concentration against *S. putrefaciens*, *P. fragi*, *P. fluorescens*, *E. gergoviae*, *E. amnigenus* and *L. innocua*. Of note was the fact that *T. piperella* EO, when it had an effect, produced greater inhibition halos than *T. moroderi* EO.

The antibacterial activity of both EOs was dose-dependent. However, it should be borne in mind that when these substances are used as antibacterials in food their efficiency diminishes when they are added to complex matrixes and their organoleptic impact could be noticeable. There are also questions of safety and potential toxicity that should not be forgotten.

4. Conclusions

The essential oils from *T. moroderi* and *T. piperella* are a source of important bioactive compounds with antibacterial properties which merit further study for possible application in the food industry, where they may be considered as natural preservatives to replace the synthetic preservatives of which consumers are increasingly distrustful. However, the use of these essential oils may be limited by changes to the organoleptic properties of foods that contain them and so the most suitable essential oil must be chosen for each type of food. In-depth studies are required, since the absence of norms to test and guarantee the quality of essential oils is a major problem faced by the food industry when they are used as a basic ingredient.

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Título: Chemical characterization and antibacterial activity of two aromatic herbs (*Santolina chamaecyparissus and Sideritis angustifolia*) widely used in the folk medicine

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CHEMICAL CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF TWO AROMATIC HERBS (*SANTOLINA CHAMAECYPARISSUS AND SIDERITIS ANGUSTIFOLIA*) WIDELY USED IN THE FOLK MEDICINE

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ABSTRACT

The aim of this work was characterize the essentials oils (EOs) obtained from *Santolina chamaecyparissus* and *Sideritis angustifolia* determining (1) its chemical composition and (2) its effectiveness on the growth of some bacteria related to food spoilage, such as *Listeria innocua*, *Serratia marcescens*, *Pseudomonas fragi*, *Pseudomonas fluorescens*, *Aeromonas hydrophila*, *Shewanella putrefaciens*, *Alcaligenes faecalis*, *Achromobacter denitrificans*, *Enterobacter amnigenus*, *Enterobacter gergoviae* and *Leuconostoc carnosum*. The EOs were chemically analyzed and identified by gas chromatography and gas chromatography/mass spectrometry, while the agar disc diffusion method was used to determine their antibacterial activities. The concentration effect was also determined. In *S. chamaecyparissus*, the major constituents were artemisia ketone (27.19%), dihydroaromadendrene (18.21%) and β-phellandrene (7.49%), while in *S. angustifolia* EO, the major constituent is α-pinene (12.71%). Another important compound was β-phellandrene (11.97%). The agar disc diffusion method indicated that *S. chamaecyparissus* EO showed the highest antibacterial activity against the *L. innocua* and *A. hydrophila*, with inhibition zones of 12.70 and 16.50 mm, respectively. *S. angustifolia* EO showed the highest antibacterial activity against *A. faecalis*, *S. marcescens* and *A. denitrificans*. *S. chamaecyparissus* and *S. angustifolia* EOs can be used as antibacterial agents.

PRACTICAL APPLICATIONS

The research on plant-based natural bioactive compounds to control the foodborne and spoilage pathogens has attained special interest in the recent years. The use of essential oils of *Santolina chamaecyparissus* and *Sideritis angustifolia* as antibacterial agents will be suitable for applications on the food industry as natural preservatives or flavoring to control foodborne pathogens. Another important reason for their suitability is their natural origin, which consumers find comforting.

INTRODUCTION

Many of the valuable organoleptic and sensory properties of food products diminish with time. This may be attributed to a large number of factors, including the action of oxygen, light and temperature that provoke the oxidation process. However, the most limiting factor of food shelf life is the growth of microorganisms, whether they are molds, bacteria or yeasts (Viuda-Martos *et al.* 2008). In addition, food processors, food safety researchers and regulatory agencies have been increasingly concerned with the growing number of foodborne illness outbreaks caused by some pathogens

and/or their enterotoxins (Oroojalian *et al*. 2010). At present, therefore, a wide variety of chemical preservatives are used throughout the food industry to prevent the growth of food-spoiling bacteria (Davidson 2001).

During the recent decades and mostly as a result of their diversity, versatility and safety in comparison with the synthetic materials, natural products of plant, animal and microorganism origins have attained special interest. The exploration of naturally occurring antimicrobial for food preservation receives increasing attention owing to the consumer awareness of natural food products and a growing concern of microbial resistance toward conventional preservatives (Sharififar *et al*. 2007).

Many spices and aromatic plants that contain essential oils (EOs) are within the major plant categories in the Mediterranean region. These plants have the ability to synthesize, accumulate and emit volatile compounds that may act as aroma and flavor molecules (Hassiotis and Lazari 2010). These mixtures of volatile compounds (mainly mono- and sesquiterpenoids, benzoids, phenylpropanoids, etc.) exert different biological actions on humans, animals and other plants. The antimicrobial activity of EOs was recognized long ago; these components of spices and aromatic herbs are gaining interest for their potential as preservatives and as decontamination agents, since such substances have been generally recognized as safe (GRAS) and are widely accepted by consumers (Burt 2004). Thus, the ability of plant EOs to protect foods against pathogenic and spoilage microorganisms has been reported by several researchers (Viuda-Martos *et al*. 2009, 2011; Ahmadi *et al*. 2010; Tenore *et al*. 2011). However, in order to achieve effective antimicrobial activity in direct food applications, high concentrations of EOs are generally needed, which might impact inappropriate flavors and odors of the product (Seydim and Sarikus 2006). The specific advantage of EOs appears to be in synergistic effects of their compounds, as evidenced in greater activity when applied as natural EOs compared with reported effects of the individual active substances (Zivanovic *et al*. 2005).

The genus *Santolina*, belonging to the family Asteraceae, is represented by more than 10 species widely distributed in the Mediterranean area (Derbesy *et al*. 1989). Among *Santolina* species, *Santolina viridis* W. (south of France and north of Spain), *Santolina pectinata* Lag. (Iberian Peninsula) and *Santolina chamaecyparissus* L. (which grows wild around the Mediterranean basin) are the most widespread. *S. chamaecyparissus* is a hardy aromatic, dwarf evergreen shrub native to the West and Central Mediterranean area (growing wild, e.g., in Spain, Italy, Tunisia and Morocco, and being naturalized in parts of Britain). It is often grown in gardens for its attractive wooly silver-gay leaves born on woody stems and for its yellow flowers. *S. chamaecyparissus* is widely used in Mediterranean folk medicine. The flowers are used for their analgesic, anti-inflamatory, antiseptic, antispasmodic, bactericidal, fungicidal, digestive and vulnerary properties, and are used in phytotherapy for different kinds of dermatitis (El-Sahhar *et al*. 2011).

The genus *Sideritis*, belonging to the family Lamiaceae, subfamily Lamioideae, comprises at least 150 species. Plants of this genus, mainly *Sideritis angustifolia*, have been widely used in folk medicine as anti-inflammatory, anti-ulcer, cytostatic, antimicrobial, vulnerary, astringent, flu vaccine and stimulant circulatory agents (Palomino *et al*. 1996). Furthermore, EOs from *Sideritis* species have been used for many therapeutic purposes, such as lung disinfectants, diuretics, stomachics and neurorelaxings (Senatore 2000).

To our best knowledge, no comprehensive and comparative work has been carried out on the chemical composition as well as antimicrobial activity with respect to *S. chamaecyparissus* and *S. angustifolia* EOs. Therefore, the aims of this work were to (1) determine the chemical composition and (2) the effectiveness of the essentials oils obtained from *S. chamaecyparissus* and *S. angustifolia* on the growth of some bacteria related to food spoilage to determine if these EOs could be used as natural food preservatives.

MATERIALS AND METHODS

Plant Materials

S. chamaecyparissus and *S. angustifolia* were collected during their flowering period. Identification of the plant material was made by Prof. Dr. Concepcion Obon de Castro, Applied Biology Department of Miguel Hernandez University (Spain).

Extraction of the EOs

The EOs of *S. chamaecyparissus* and *S. angustifolia* were extracted from whole plant (stems, leaves and flowers) by hydro-distillation using a Clevenger-type apparatus for 3 h. The oily layer obtained on top of the aqueous distillate was separated and dried with anhydrous sodium sulfate (0.5 g). The extracted EOs were kept in sealed air-tight glass vials and covered with aluminum foil at 4C until further analysis. The respective yields of the EOs were $1.00 \pm 0.05\%$ (*S. chamaecyparissus*) and $0.90 \pm 0.08\%$ (*S. angustifolia*).

Gas Chromatography (GC) Analysis

The EOs were analyzed using a Shimadzu GC-17A (Shimadzu Corporation, Kyoto, Japan) equipped with flame ionization detector (FID) and a TRACSIL Meta X5 column (Teknokroma S. Coop. C. Ltd., Barcelona, Spain), 60 m × 0.25 mm i.d., 0.25-μm film thickness. Injector and detector temperatures were set at 250 and 300C, respectively. The

oven temperature was kept at 40C for 5 min, raised to 200C at 3.0C/min and held for 1 min, raised to 280C at 15C/min and held for 10 min. Helium was used as the carrier gas, at a flow rate of 1 mL/min. Diluted samples (1/10 cyclohexane, v/v) of 0.2 μL were injected manually in the split mode (split ratio 1/44). Quantitative data were obtained electronically from FID area data without using correction factors. All tests were performed in triplicate.

Gas Chromatography/Mass Spectrometry (GC-MS) Analysis

The volatile compounds were isolated, identified and quantified on the Shimadzu GC-17A gas chromatograph (Shimadzu Corporation), coupled to a Shimadzu mass spectrometer detector (GC-MS QP-5050A). The GC-MS system was equipped with the same column used in the GC analysis and with the same temperature program. Analyses were carried out using helium as the carrier gas at a flow rate of 1 mL/min, at a split ratio of 1:10. Diluted samples (1/10 cyclohexane, v/v) of 0.2 μL of the extracts were injected. Mass spectra were obtained by electron ionization at 70 eV using a spectral range of 45–450 *m/z*. Most of the compounds were identified using two different analytical methods: (1) Kováts indices (KI) in reference to *n*-alkanes (C_8–C_{32}) (NIST 2010) and (2) mass spectra (authentic chemicals and Wiley spectral library collection). Identification was considered tentative when based on mass spectral data alone.

Microbial Strains

The essentials oils were individually tested against *Listeria innocua* (CECT 910), *Serratia marcescens* (CECT 854), *Pseudomonas fragi* (CEPT 446), *Pseudomonas fluorescens* (CECT 844), *Aeromonas hydrophila* (CECT 5734), *Shewanella putrefaciens* (CECT 5346), *Achromobacter denitrificans* (CECT 449), *Enterobacter amnigenus* (CECT 4078), *Enterobacter gergoviae* (CECT 587), *Alcaligenes faecalis* (CECT 145) and *Leuconostoc carnosum* (CECT 4024). These species were supplied by the Spanish Type Culture Collection (CECT) of the University of Valencia. These bacteria were chosen based on their activity as spoilage bacteria from refrigerated foods (mainly meat and meat products), or as indicators or models of food pathogenic bacteria.

Agar Disc Diffusion Method

The agar disc diffusion method described by Tepe *et al.* (2005), with some modifications, was used to determine the antibacterial capacity of the EOs. Briefly, a suspension (0.1 mL of 10^6 cfu/mL) of each microorganism was spread on the solid medium plates: nutrient agar II (Oxoid, Basingstoke, Hampshire, England) in the case of *S. marcescens*, *P. fragi*, *P. fluorescens*, *A. hydrophila* and *S. putrefaciens*; nutrient agar I (Oxoid) in the case of *E. gergoviae*, *E. amnigenus*, *A. faecalis* and *A. denitrificans*; de Mann Rogosa Sharpe agar (Sharlau, Barcelona, Spain) for *L. carnosum* and brain–heart infusion agar (Sharlab, Barcelona, Spain) for *L. innocua*. Filter paper discs, 9 mm in diameter (Schlinder & Schuell, Dassel, Germany), were impregnated with 40 μL of the oil and placed on the inoculated plates; these plates were incubated at 37C for 48 h in the case of *E. gergoviae* and *E. amnigenus*; at 37C for 24 h in the case of *A. faecalis* and *L. innocua*; at 26C for 48 h in the case of *P. fragi*, *P. fluorescens*, *S. putrefaciens* and *L. carnosum*; and at 26C for 24 h for *S. marcescens*, *A. hydrophila* and *A. denitrificans*. The diameters of the inhibition zones were measured in millimeters. All tests were performed in triplicate.

Determination of Concentration Effect (CE)

The CE was studied to ascertain which concentrations of EO had an inhibitory effect on the bacterial growth in the disc diffusion assay. The culture techniques used were those described in the previous paragraph (agar disc diffusion method), but adding 40, 20, 10, 4 and 2 μL of EO, which meant concentrations of 100, 50, 25, 10 and 5% of the initial volume (Viuda-Martos *et al.* 2005). All tests were performed in triplicate.

Statistical Analysis

Conventional statistical methods were used to calculate means and standard deviations of three simultaneous assays carried out with the different methods. Statistical analysis of variance (ANOVA) was applied to the data to determine differences ($P < 0.05$). To discover whether there were significant differences between the levels of the main factor, contrasts between means were made using a Tukey's test (Afifi and Azen 1979). For the antibacterial activity, an ANOVA was applied, with the following factors: concentration (five levels: 40, 20, 10, 4 and 2 μL) for each EO. The statistical analyses were made using STATGRAPHICS 5.1 for Windows (Statistical Graphics Corp., Rockville, MD).

RESULTS AND DISCUSSION

Chemical Composition

The chemical composition of *S. chamaecyparissus* and *S. angustifolia* EOs was investigated using both GC and GC-MS techniques (Table 1). In the case of *S. chamaecyparissus* EO, 58 compounds were identified as representing 90.1% of the oil. The main components were artemisia ketone (27.19%), dihydroaromadendrene (18.21%), β-phellandrene (7.49%)

TABLE 1. THE MAIN CONSTITUENTS OF *SIDERITIS ANGUSTIFOLIA* AND *SANTOLINA CHAMAECYPARISSUS* ESSENTIAL OILS AND THEIR RELATIVE PERCENTAGES OF TOTAL CHROMATOGRAM AREA, KOVÁTS INDEX AND RETENTION TIME

		Kováts indexes		Essential oils	
Compounds	ID*	KI	Lit†	*S. angustifolia* (% area)	*S. chamaecyparissus* (% area)
α-Pinene	KI,W	940	939	12.71	–
Sabinene	KI,W	982	976	–	2.4
Myrcene	KI,W	992	991	–	6.94
β-Phellandrene	KI,W	1033	1031	11.97	7.49
1,8-Cineole	KI,W	1041	1039	7.41	–
Artemisia ketone	KI,W	1061	1062	–	27.19
Hotrienol	KI,W	1101	1101	5.33	–
Camphor	KI,W	1145	1143	–	3.88
Terpinen-4-ol	KI,W	1191	1189	2.46	–
trans-Caryophyllene	KI,W	1435	1430	6.33	–
α-Zingiberene	KI,W	1493	1495	–	2.29
α-Curcumene	KI,W	1499	1498	–	2.74
Bicyclogermacrene	KI,W	1501	1494	5.11	–
δ-Cadinene	KI,W	1530	1534	3.45	–
Isocitronellen	KI,W	1565	1563	5.83	–
Spathulenol	KI,W	1595	1586	–	2.27
α-Cadinol	KI,W	1663	1653	3.88	–
Dihydroaromadendrene	KI,W	1669	1665	–	18.21

* "KI, W" means that identification was based on Kováts indexes and comparison with Wiley library. "KI" compounds tentatively identified based on Kováts indexes. "W" compounds tentatively identified (only Wiley library was used for identification).
† NIST database.

and myrcene (6.94%). These results were very similar to that reported by Demirci *et al.* (2000). They found that the main components identified in the EO from plants gathered in Turkey included artemisia ketone (38.1%), camphor (11.7%), β-phellandrene (9.2%), α-bisabolol (6.6%), myrcene (4.3%), yomogi alcohol (1.5%) and artemisia alcohol (1.5%). In the same way, Pérez-Alonso and Velasco-Negueruela (1992) analyzed samples from *S. chamaecyparissus* subspecies, both wild (Spanish insular and peninsular) and cultivated (Spanish and British). They found that cultivated plants showed a preponderance of artemisia ketone (27.8–35.6%) and δ-cadinol (4.8–23.6%), whereas camphor (42.9%) and cubenol (17.3%) were predominated in the insular plant-derived oils. Haggag *et al.* (2000) mentioned that the volatile oil of *S. chamaecyparissus* fresh arial parts was found to contain 74 identified components: artemisia ketone (35.49%) was the major one. Grosso *et al.* (2009) investigated the EO obtained from *S. chamaecyparissus* L. flower heads and reported that the main components were 1,8-cineole (25–30%), camphor (7–9%), borneol (7–8%) and terpinen-4-ol (6–7%). Villar *et al.* (1986) analyzed the EO of *S. chamaecyparissus* ssp. *squarrosa* from Valencia (Spain). They showed that the main components were camphor (25%), alloaromadendrene (19%), *p*-cymene + 1,8-cineole (10%) and α-muurolene (7%). Ahuja *et al.* (2005) analyzed the EO isolated from foliage *S. chamaecyparissus*. They found that the major constituents were myrcene, limonene, (E)-linalool, (Z)-ocimene and caryophyllene oxide.

As regards *S. angustifolia*, 77 compounds were indentified, representing the 94.6% of the oil. The major constituent was α-pinene (12.71%). Other important compounds were β-phellandrene (11.97%), 1,8-cineole (7.41%) and *trans*-caryophyllene (6.33%). This is the first report to analyze the chemical composition of *S. angustifolia* and no information on its composition has been reported previously. However, the chemical composition of the EO obtained from plants of genus *Sideritis* has been widely investigated. Thus, Formisano *et al.* (2010) analyzed the chemical composition of the EOs of *Sideritis italica* flowers and leaves. These authors identified 43 compounds in the EO from flowers and 29 in the EO from leaves. Kaur-15-ene (20.0%) was recognized as the main constituent of the EO from flowers, together with β-cubebene (12.1%), β-pinene (8.5%), (Z)-nuciferol (6.5%) and tricyclene (4.5%). In the EO from leaves, *p*-methoxyacetophenone (26.0%) prevailed, as did hexadecanoic acid (21.3%), followed by nonanal (4.8%), α-bisabolol (4.5%) and β-cubebene (4.7%). Aboutabl *et al.* (2009) investigated the aerial parts of *Sideritis taurica* Stephan ex Willd grown in Egypt. These authors reported that monoterpene hydrocarbons were mainly β-pinene (17.7%) and α-pinene (12.8%). The main sesquit-

erpene hydrocarbons were aromadendrene (3.5%) and α-cubebene (2.2%). The main oxygenated monoterpenes were α-terpineol (2%) and *trans*-pinocarveol (1.4%), while the major oxygenated sesquiterpenes were α-cadinol (12.2%), curcumenol (3.5%) and α-bisabolol (7.4 and 2.9%).

This great variability and diversity observed in the chemical composition of the EOs of the same aromatic herb species and subspecies can be attributed to several factors, including local climatic and environmental conditions, season, geographical location, geology, postharvest drying and storage, availability of water, height above sea level, presence of fungal diseases and insects, part of the plant and the method used to obtain the EO (Viuda-Martos *et al.* 2007, 2008).

Antibacterial Activity

The *in vitro* antibacterial activities of *S. chamaecyparissus* and *S. angustifolia* EOs against 11 bacteria associated with food spoilage and their activity potentials was quantitatively assessed, following the agar disc diffusion method, for the presence or absence of inhibition zones (Table 2).

As can be seen, *S. chamaecyparissus* EO had an inhibitory effect on 5 of the 11 bacteria assayed, with inhibition halos that ranged from 11.50 mm for *A. denitrificans* to 16.50 mm for *A. hydrophila* and *A. faecalis*. However, no antibacterial activity was found against *P. fragi*, *P. fluorescens*, *S. putrefaciens*, *E. gergoviae*, *E. anmigenus* and *L. carnosum*. In the same way as the *S. chamaecyparissus, S. angustifolia* EO had an inhibitory effect on five of the bacteria, with inhibition halos from 11 mm (*A. hydrophila*) to 18.00 mm (*A. faecalis*). This EO showed no antibacterial activity against *P. fragi*, *P. fluorescens*, *S. putrefaciens*, *E. gergoviae*, *E. anmigenus* and *L. carnosum*, the same as reported for *S. chamaecyparissus*. The agar disc diffusion method indicated that *S. chamaecyparissus* EO showed the highest ($P < 0.05$) antibacterial activity against *A. faecalis*, *A. hydrophila* and *S. marcescens*, while *S. angustifolia* EO showed the highest antibacterial ($P < 0.05$) activity against *A. faecalis*, *S. marcescens* and *A. denitrificans*.

This is the first study to analyze the antibacterial activity of the EOs from *S. chamaecyparissus* and *S. angustifolia*. There are no studies where the antibacterial activity of EOs obtained from plants of *Santolina* genus has been determined. However, as in the case of the chemical composition, the antibacterial activity of several other *Sideritis* has been studied. Thus, Kiliç *et al.* (2003) reported that *Sideritis athoa*, *Sideritis trojana*, *Sideritis dichotoma*, *Sideritis spilyea* and *Sideritis argyrea* EOs were active against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Enterococcus faecalis*. Basile *et al.* (2006) investigated the antibacterial activity of *Sideritis*

TABLE 2. ANTIMICROBIAL ACTIVITY OF *SANTOLINA CHAMAECYPARISSUS* AND *SIDERITIS ANGUSTIFOLIA* ESSENTIAL OILS USING DISC DIFFUSION METHOD

	Diameter (mean and SD) of inhibition zone (mm) including disc diameter of 9 mm									
Essential oils	*Serratia marcescens*	*Pseudomonas fragi*	*Pseudomonas fluorescens*	*Aeromonas hydrophila*	*Shewanella putrefaciens*	*Enterobacter gergoviae*	*Enterobacter amnigenus*	*Alcaligenes faecalis*	*Achromobacter denitrificans*	*Leuconostoc camosum*	*Listeria innocua*
S. chamaecyparissus	13.50 ± 0.07a	NA	NA	16.50 ± 0.07a	NA	NA	NA	16.50 ± 0.07a	11.50 ± 0.07a	NA	12.70 ± 0.02a
S. angustifolia	14.80 ± 0.04b	NA	NA	11.00 ± 0.00b	NA	NA	NA	18.00 ± 0.70b	12.50 ± 0.07b	NA	12.10 ± 0.01b

For the same bacteria, values followed by different letters within the same column are significantly different ($P < 0.05$) according to Tukey's multiple range test.
NA, non-active; SD, standard deviation.

italic (Miller) EO against nine gram-positive and gram-negative bacterial strains. These authors found that at concentrations between 3.9 and 250 μg/mL, the EOs showed a significant antibacterial effect against *P. aeruginosa* (minimum inhibitory concentrations [MICs] = 3.9 and 7.8 μg/mL for flower heads and leaves, respectively), *Proteus mirabilis* (MICs = 15.6 and 7.8 μg/mL), *Salmonella typhi* (MIC = 7.8 μg/mL) and *Proteus vulgaris* (MIC = 15.6 μg/mL). Kirimer *et al*. (2008) tested the antimicrobial activity of *Sideritis perfoliata* and *Sideritis trojana* EOs against *E. coli*, methicillin-resistant *S. aureus*, *Enterobacter aerogenes*, *Salmonella typhimurium*, *Bacillus cereus*, *Staphylococcus epidermidis* and *Candida albicans*. These authors indicated that *E. coli*, methicillin-resistant *S. aureus*, *E. aerogenes*, *B. cereus* and *C. albicans* were inhibited by *S. trojana* EO with MIC values between 125 and 250 μg/mL, but the oil showed strong inhibitory effects against *S. epidermidis* with a MIC value of 62.5 μg/mL. *S. perfoliata* EO, on the other hand, was less active against the test microorganisms except for *C. albicans*. Köse *et al*. (2010) analyzed the antibacterial activity of two varieties of *Sideritis erythrantha* endemic in Turkey. They showed that *S. erythrantha* var. *cedretorum* EO was as effective as antibiotic against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *E. faecalis*, ampicillin-resistant *Haemophilus influenzae* and vancomycin-sensitive *E. faecalis*. Similarly, *S. erythrantha* var. *erythrantha* EO was also as effective as antibiotic against *E. faecalis* and ampicillin-resistant *H. influenzae*.

The composition, structure as well as functional groups of the EO play an important role in determining their antimicrobial activity (Holly and Patel 2005). Considering the large number of different groups of chemical compounds present in EOs, it is most likely that their antibacterial activity is not attributable to one specific mechanism, but that there are several targets in the cell (Burt 2004). Different modes of action have been suggested. Thus, the EOs affect microbial cells by various antimicrobial mechanisms, including attacking the phospholipid bilayer of the cell membrane, disrupting enzyme systems, compromising the genetic material of bacteria and forming fatty acid hydroperoxidase caused by oxygenation of unsaturated fatty acids (Burt *et al*. 2007; Arques *et al*. 2008). The leakage of ions and other cell contents may also occur (Carson *et al*. 2002). Thus, the EO components can provoke an increase in K^+ and often cytoplasmic content effluxes from cells in response to antimicrobial challenge. These effects may develop as result of (1) membrane depolarization by altered ion transport or through changes in membrane structure; (2) inhibition of energy (ATP) generation by interference with glucose uptake; or (3) inhibition of enzymes involved in oxidative or substrate level phosphorylation (Skocibusic *et al*. 2006). In addition, the EOs can coagulate the cytoplasm and damage lipids and proteins. Their mechanism of action would be similar to other phenolics, i.e., the disturbance of the proton motive force, electron flow, active transport and coagulation of cell contents (Burt 2004).

The apparent antimicrobial efficacy of plant-origin antimicrobials depends on factors such as the method of extracting EOs from plant material, the volume of inoculum, growth phase, the culture medium used, and intrinsic or extrinsic properties of the food such as pH, fat, protein, water content, antioxidants, preservatives, incubation time/temperature, packaging procedure and the physical structure of food (Burt 2004; Lopez-Malo *et al*. 2005; Brandi *et al*. 2006).

The type of bacterium also has an influence on the effectiveness of EOs. Generally, thanks to their extra protective outer membrane, gram-negative bacteria are usually considerably more resistant to antibacterial agents than their gram-positive counterparts (Saier 2009). This outer membrane may restrict the diffusion of hydrophobic compounds through its lipopolysaccharide covering. Gram-positive bacteria allow direct contact of the EOs' hydrophobic constituents with the phospholipid bilayer of the cell membrane, where they bring about their effect, either causing or increasing ion permeability and leakage of vital intracellular constituents, or impairment of bacterial enzyme systems (Sandri *et al*. 2007). However, this does not mean that gram-positive bacteria are always more susceptible (Burt 2004). In this way, Sokmen *et al*. (2004) affirmed that the EOs of spices show no selectivity as regards the cell walls of bacteria. In this regard, the EOs obtained from *S. chamaecyparissus* and *S. angustifolia* showed similar antibacterial activity in disc diffusion test on gram-positive and gram-negative bacteria.

Concentration Effect

Table 3 shows the CE of the EOs from *S. chamaecyparissus* and *S. angustifolia* on the 11 bacterial strains studied. None of the two EOs showed an inhibitory capacity at all the concentrations; as can be seen, the inhibitory effect increased with increasing concentrations. In other words, their inhibitory effect was related to the concentration used. In the case of *S. chamaecyparissus* EO, no antibacterial effect was observed at any concentration on *S. putrefaciens*, *P. fragi*, *P. fluorescens*, *E. gergoviae*, *E. amnigenus* and *L. carnosum*. Neither of discs impregnated with 4 nor 2 μL had any effect against *A. faecalis*, but concentrations of 40, 20 and 10 μL had an inhibitory effect, with statistically significant differences between them ($P < 0.05$). For *S. marcescens, A. denitrificans* and *L. innocua*, no antibacterial effect was observed at any concentration, except 40 μL. *A. hydrophila* showed antibacterial activity at concentrations of 40 and 20 μL, with significant differences between them ($P < 0.05$).

TABLE 3. CONCENTRATION EFFECT OF *SANTOLINA CHAMAECYPARISSUS* AND *SIDERITIS ANGUSTIFOLIA* ESSENTIAL OILS USING DISC DIFFUSION METHOD

		Diameter (mean and SD) of inhibition zone (mm) including disc diameter of 9 mm									
Essential oils	Volume (μL)	*Serratia marcescens*	*Pseudomonas fragi*	*Pseudomonas fluorescens*	*Aeromonas hydrophila*	*Shewanella putrefaciens*	*Enterobacter gergoviae*	*Enterobacter amnigenus*	*Alcaligenes faecalis*	*Achromobacter denitrificans*	*Leuconostoc carnosum*	*Listeria innocua*
S. chamaecyparissus	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	10	NA	NA	NA	NA	NA	NA	NA	11.50 ± 0.70c	NA	NA	NA
	20	NA	NA	NA	10.00 ± 0.00b	NA	NA.	NA	14.00 ± 0.00b	NA	NA	NA
	40	13.50 ± 0.07	NA	NA	16.50 ± 0.07a	NA	NA	NA	16.50 ± 0.07a	11.50 ± 0.07	NA	12.70 ± 0.02
S. angustifolia	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	10	NA	NA	NA	NA	NA	NA	NA	12.00 ± 0.00c	NA	NA	NA
	20	NA	NA	NA	NA	NA	NA	NA	14.00 ± 0.00b	NA	NA	NA
	40	14.80 ± 0.04	NA	NA	11.00 ± 0.00	NA	NA	NA	18.00 ± 0.00a	12.50 ± 0.07	NA	12.10 ± 0.01

For the same essential oil, values followed by different letters within the same column are significantly different ($P < 0.05$) according to Tukey's multiple range test.
NA, non-active; SD, standard deviation.

As regards *S. angustifolia* EO, discs added with 4 and 2 μL had no any effect against *A. faecalis*, but at concentrations of 40, 20 and 10 μL, it had an inhibitory effect, with statistically significant differences between them ($P < 0.05$). For *S. marcescens, A. hydrophila, A. denitrificans* and *L. innocua*, no antibacterial effect was observed at any concentration, except 40 μL. As occurred with *S. chamaecyparissus* EO, the EO from *S. angustifolia* was not active at any concentration against *S. putrefaciens*, *P. fragi*, *P. fluorescens*, *E. gergoviae*, *E. amnigenus* and *L. innocua*.

CONCLUSIONS

The EOs obtained from two aromatic herbs used in the folk medicine such as *Santolina chamaecyparissus* and *Sideritis angustifolia* showed a moderate antibacterial activity against several indicators of pathogenic or spoilage bacteria in foods (mainly meat and meat products). More in-depth studies are required for the possible application of these EOs in the food industry as natural preservatives, as results of *in vitro* investigations revealed that EOs are not applicable to food conditions because antimicrobials in foods may be hampered by effective dosages, interference by food constituents or other food-grade compounds, unsuitable water activity, incompatible pH or processing regiments. In addition, the absence of norms to test and guarantee the quality of EOs is a major problem faced by the food industry when they are used as a basic ingredient. Combination of EOs from different plants should be studied. The possible combination with other widely consumed EOs with demonstrated antibacterial activity against other bacteria may allow the development of mixtures of EOs to be used as effective natural preservatives for several refrigerated foods.

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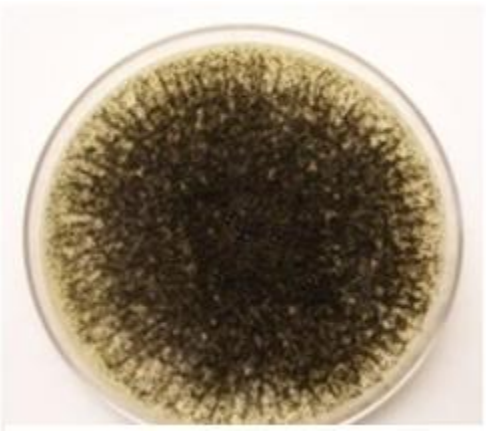

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In Vitro Antioxidant and Antifungal Properties of Essential Oils Obtained from Aromatic Herbs Endemic to the Southeast of Spain

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ABSTRACT

The aim of this work was to determine (i) the antioxidant capacity of *Thymus moroderi, Thymus piperella, Santolina chamaecyparissus,* and *Sideritis angustifolia* essential oils (EOs) by means of four different antioxidant tests (the 2,2′-diphenyl-1-picrylhydrazyl radical scavenging method, the ferrous ion–chelating ability assay, the ferric reducing antioxidant power test, and the thiobarbituric acid reactive species test) and (ii) the antifungal activity against molds and yeast of these EOs by the agar dilution and the microdilution methods. *T. piperella* EO showed the lowest 50% inhibitory concentration by the 2,2′-diphenyl-1-picrylhydrazyl radical scavenging method (9.30 mg/ml) and by the thiobarbituric acid reactive species test (6.30 mg/ml) and the highest value by the ferric reducing antioxidant power test (2.64 Trolox equivalent antioxidant capacity), while *S. chamaecyparissus* showed the lowest 50% inhibitory concentration in the ferrous ion–chelating ability assay (3.94 mg/ml). All EOs had a substantial inhibitory effect on all assayed yeast strains. *S. angustifolia* EO had the lowest MICs (2.5 μl/ml) for the yeasts *Saccharomyces cerevisiae, Debaryomyces hansenii, Rhodotorula mucilaginosa,* and *Pichia carsonii*. As regards antifungal activity, *S. angustifolia* EO at high concentrations was the most effective EO in reducing the growth of *Alternaria alternata, Penicillium chrysogenum,* and *Mucor racemosus,* while *S. chamaecyparissus* was the best inhibitor of the molds *Aspergillus flavus* and *Mucor circinelloides*. The results obtained in this study suggest the possibility of using these essential oils as natural antioxidant food preservatives.

Lipid oxidation and microbiological degradation occurring in food products are the major concerns in food technology.

They are responsible for rancid odors and flavors of the products, with a consequent decrease in nutritional quality and safety caused by the formation of secondary, potentially toxic compounds *(49)*. In addition, illnesses caused by consumption of foods contaminated with pathogens have a great economic and public health impact worldwide *(18)*.

In the food industry, the rate of auto-oxidation is reduced by freezing, refrigeration, packaging under inert gas, and vacuum packaging *(10)*. When these methods are neither economic nor practical from nutritional and technological points of view, it is highly desirable to control oxidation by addition of antioxidants, inhibitory substances that do not reduce food quality *(20)*. As regards the control of microorganisms, a range of different chemical and synthetic compounds have been used as antibacterial and antifungal agents to inhibit microbial food spoilage. Antimicrobial substances used in the food industry include chemical substances (added or already present naturally in foods), which are used in the food industry for two main reasons: (i) to control natural spoilage processes (food preservation) and (ii) to prevent or control the growth of microorganisms, including pathogenic microorganisms (food safety) *(39)*. Yeasts, one group of microorganisms, are best known for their positive contributions to society, particularly through their activities in food fermentation. Yeasts also cause spoilage, although this effect is limited to a narrow range of products, especially those of low pH or high sugar concentration, where competition from bacterial growth is restricted *(16)*. The role played by molds in food spoilage not only involves mycotoxin production but also has very important adulterating effects on sensorial properties *(15)*.

At present, consumer demands for less use of synthetic food preservatives have increased throughout the world. So, the replacement of traditional food preservation agents and synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene (BHT) by natural vegetables and plants materials have caused great interest in nutrition research *(3)*.

Herbs and spices and their derivatives such as essential oils (EOs) have been known since ancient times and used in the preparation of foodstuffs to enhance their flavor and organoleptic properties. Nowadays, they have great potential in a growing nutrition industry because many plant-derived

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phytochemical preparations possess dual functionality in preventing lipid oxidation and microbial spoilage, the main causes of food quality deterioration *(33).* Thus, there are numerous studies in which the antioxidant and antimicrobial activities of herbs, spices, and especially the EOs have been determined *(3, 43, 45–47, 50).*

The genus *Thymus* is a member of the Lamiaceae family. It is characterized by a high content of bioactive compounds such as phenolic acids, flavonoids, and terpenes present in EOs, which leads to its being regarded as an aromatic herb with potential health benefits. Two species found in the genus *Thymus* are *Thymus piperella* and *Thymus moroderi,* both endemic to the southeast of Spain *(30).* The genus *Santolina,* family Asteraceae, is widely distributed in the Mediterranean area. *Santolina chamaecyparissus* is widely used in Mediterranean folk medicine; the flowers are used for their analgesic, anti-inflammatory, antiseptic, and antispasmodic properties *(13).* The genus *Sideritis,* family Lamiaceae, subfamily Lamioideae, comprises at least 150 species. Plants of this genus, mainly *Sideritis angustifolia,* have been widely used in folk medicine as anti-inflammatory, antiulcer, cytostatic, and stimulant circulatory agents *(28).*

According to our best knowledge, no report of the antifungal and antioxidant activities of *T. moroderi, T. piperella, S. chamaecyparissus,* and *S. angustifolia* EOs exists. Thus, the aim of this work was to determine (i) the antioxidant capacity of *T. moroderi, T. piperella, S. chamaecyparissus,* and *S. angustifolia* EOs by means of four different antioxidant tests (the 2,2′-diphenyl-1-picrylhydrazyl radical scavenging method [DPPH], the ferrous ion–chelating ability assay [FIC], the ferric reducing antioxidant power [FRAP] test, and the thiobarbituric acid reactive species test [TBARs]) and (ii) the antifungal activity (against several molds and yeasts) of these EOs by the agar dilution method and the microdilution method, to investigate whether these EOs could be used as natural food ingredients and represent a useful alternative for the food industry to reduce the quantity of synthetic additives used in their attempt to satisfy the demands of consumers.

MATERIALS AND METHODS

Plant materials. *T. moroderi, T. piperella, S. chamaecyparissus,* and *S. angustifolia* were collected in the province of Valencia (southeast of Spain) during their flowering period. Identification of the plant material was made by Prof. Dr. Concepcion Obon de Castro, Applied Biology Department of Miguel Hernandez University (Spain).

Extraction of the EOs. The EOs of *T. moroderi, T. piperella, S. chamaecyparissus,* and *S. angustifolia* were extracted from 500 g of whole plant (stems, leaves, and flowers) by hydrodistillation using a Clevenger-type apparatus for 3 h. The respective yields of the EOs were as follows: *T. moroderi,* 1.90%; *T. piperella,* 1.66%; *S. chamaecyparissus,* 1.00%; and *S. angustifolia,* 0.90%. The oily layer obtained on top of the aqueous distillate was separated and dried with 0.5 g of anhydrous sodium sulfate (Panreac Quimica, Barcelona, Spain). The extracted EOs were kept in sealed air-tight glass vials and covered with aluminum foil at 4°C until further analysis. There were three replicates for each EO.

TPC. The total phenol content (TPC) was determined using the Folin-Ciocalteu's reagent *(35).* The results were expressed in milligrams of gallic acid equivalents (GAE) per gram of EO as the mean of three replicates.

Antioxidant activity assays. (i) Determination of antioxidant activity using the DPPH radical scavenging method. The antioxidant activities of different concentrations (0.15 to 20 mg/ml) of EO samples were measured in terms of hydrogen-donating or radical-scavenging ability, using the stable radical DPPH *(8).* BHT (0.02 to 2.5 mg/ml) was used as a positive control. The results were expressed as the mean percent inhibition of three replicates.

(ii) FRAP. The FRAP of different concentrations (0.15 to 20 mg/ml) of EO samples was determined by using the potassium ferricyanide–ferric chloride method *(27).* The FRAP of the samples was estimated in terms of Trolox equivalent antioxidant capacity (TEAC) in millimolar Trolox per liter as the mean of three replicates.

(iii) TBARS. The method of Daker et al. *(10)* was used to determine the TBARS, a secondary product of lipid peroxidation. To measure the percentage of lipid peroxidation inhibition, different concentrations (0.15 to 20 mg/ml) of EO samples were used. BHT (0.002 to 0.31 mg/ml) was used as a positive control. The results were expressed as the mean percent inhibition of three replicates.

(iv) FIC assay. The FIC of different concentrations (0.15 to 20 mg/ml) of EO samples was measured by inhibiting the formation of Fe^{2+}–ferrozine complex after treatment of test material with Fe^{2+}, following the method of Carter *(9).* EDTA (0.01 to 1.25 mg/ml) was used as a positive control. The results were expressed as the mean percent chelating effect of three replicates.

Microbial strains. The EOs were individually tested against yeasts (*Yarrowia lipolytica* CECT 1240, *Saccharomyces cerevisiae* CECT 1383, *Candida zeylanoides* CECT 10048, *Debaryomyces hansenii* CEPT 11369, *Rhodotorula mucilaginosa* CECT 10011, and *Pichia carsonii* CECT 10227) and molds (*Aspergillus niger* CECT 2091, *Aspergillus flavus* CECT 2685, *Mucor racemosus* CECT 2670, *Mucor circinelloides* CECT 20765, *Penicillium chrysogenum* CECT 2784, and *Alternaria alternata* CECT 20560). These species were supplied by the Spanish Type Culture Collection of the University of Valencia.

Antifungal activity assays. (i) Microdilution method. The antifungal activity against yeast species was determined based on a colorimetric broth microdilution method proposed by Abate et al. *(1),* with some modification. The yeast strains *Y. lipolytica, S. cerevisiae, C. zeylanoides, R. mucilaginosa*, *D. hansenii,* and *P. carsonii* were cultured 24 h at 26°C in Litmus Milk (LM) broth and diluted in sterile LM broth to a final level of 10^6 CFU/ml (adjusted to a turbidity of 0.5 McFarland standard). EOs were dissolved in dimethyl sulfoxide to reach a final concentration of 40 μl/ml. Serial twofold dilutions were made in a concentration ranging from 0.04 to 40 μl/ml in sterile test tubes containing Mueller-Hinton broth. The 96-well microplates (Iwaki, Japan) were prepared by dispensing 90 μl of Mueller-Hinton broth and 10 μl of the yeast inocula into each well. A 100-μl aliquot from each EO initially prepared was added into the first wells. Then, 100 μl from their serial dilutions was transferred into 11 consecutive wells. The last row, containing 190 μl of Mueller-Hinton broth without the compound and 10 μl of the inoculum, was used as negative control.

The final volume in each well was 200 μl. Contents of each well were mixed on a plate shaker at 150 rpm for 2 min prior to incubation for 24 h at 26°C. After incubation, 25 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Life Science), dissolved in dimethyl sulfoxide (0.8 mg/ml) was added to each of the wells and incubated for 1 h in order to allow the viable microorganisms to metabolize the yellow MTT dye into formazan (purple crystals). The MIC was considered as the concentration of the first well that did not undergo color change (from yellow to purple) and was confirmed by plating 10-μl samples from clear wells onto Mueller-Hinton agar medium. The procedure was repeated three times for each microorganism.

(ii) Agar dilution method. The food spoilage molds were tested by the agar dilution method *(17)* with some modifications. The EOs tested were added to the culture medium (potato dextrose agar, Oxoid, Basingstoke, Hampshire, England) at a temperature of 40 to 45°C, and the mixtures were then poured into petri dishes (9-cm diameter). Concentrations of 0.2, 0.1, 0.05, and 0.025 ml/100 ml of medium were tested for *T. moroderi, T. piperella, S. chamaecyparissus,* and *S. angustifolia* EOs. The molds were inoculated as soon as the medium had solidified. A disc (9 mm in diameter, Schlinder & Schuell, Dassel, Germany) of mycelium material, taken from the edge of 5-day-old fungi cultures, was placed at the center of each petri dish. The petri dish with the inoculum was then incubated at 26°C. The incubation was stopped when the mycelium mass of control petri dishes had almost filled the petri dish (6 days). The diameter of the growth mass was determined by averaging the radial growth of the mycelial mass in two orthogonal directions. The efficacy of treatment was evaluated each day during 9 days by measuring the diameter of the fungus colony. The values were expressed in diameter millimeters per day. All tests were performed in triplicate.

The antifungal activity was calculated in terms of percentage of mycelial inhibition growth (MIG), using the following equation: $\%\text{MIG} = [(D_C - D_T)/D_C] \times 100$, where D_C is the average diameter of the mycelial colonies of the control on day 8 and D_T is the average diameter of the mycelial colonies of treated sets on day 6. Each test was carried out in triplicate.

Statistical analysis. Conventional statistical methods were used to calculate means and standard deviations of three simultaneous assays carried out with the different methods. Data collected for antioxidant properties were analyzed by two-way analysis of variance to test the effects of two fixed factors: concentration (levels, 0.15 to 20 mg/ml) and EO (levels for *T. piperella, T. moroderi, S. angustifolia,* and *S. chamaecyparissus*). Data collected for antifungal properties were analyzed by two-way analysis of variance to test the effects of two fixed factors: concentration (levels, 0.025, 0.05, 0.1, and 0.2 ml/100 ml) and EO (levels for *T. piperella, T. moroderi, S. angustifolia,* and *S. chamaecyparissus*). The Tukey post hoc test was applied for comparisons of means; differences were considered significant at P values of <0.05. Statistical analysis and comparisons among means were carried out using the statistical package Statgraphics 5.1 for Windows.

RESULTS AND DISCUSSION

The chemical composition of the essential oils used in this work was previously determined by Ruiz-Navajas et al. *(30, 31)*. In the *T. moroderi* EO, the main components were camphor (26.74%), 1,8-cineol (24.99%), myrcene (5.63%), and α-pinene (4.35%). In *T. piperella* EO, the predominant compounds were carvacrol (31.92%), *para*-cymene (16.18%), γ-terpinene (10.11%), and α-terpineol (7.29%). In *S. chamaecyparissus,* the major constituents were artemisia ketone (27.19%), dihydroaromadendrene (18.21%), and β-phellandrene (7.49%), while in *S. angustifolia* EO the major constituent was α-pinene (12.71%). Other important compounds in *S. angustifolia* EO were β-phellandrene (11.97%), 1,8-cineol (7.41%), and *trans*-caryophyllene (6.33%).

TABLE 1. *Total phenol content (TPC) of* T. moroderi, T. piperella, S. chamaecyparissus, *and* S. angustifolia *EOs*

EO from:	TPC (mg of GAE/g of EO)
T. piperella	57.43 ± 0.38
T. moroderi	9.33 ± 0.53
S. chamaecyparissus	21.59 ± 0.39
S. angustifolia	38.48 ± 0.39

TPCs. The TPCs of *T. moroderi, T. piperella, S. chamaecyparissus,* and *S. angustifolia* EOs are presented in Table 1. *T. piperella* EO showed the highest content of total phenols (57.43 mg of GAE/g). *S. chamaecyparissus* and *S. angustifolia* EOs were seen to be a less rich source of total phenols (21.59 and 38.48 mg of GAE/g, respectively), while *T. moroderi* EO showed the lowest amount of total phenols. The concentration and type of phenolic substances in a plant depend on several factors, such as differences in varieties, ripeness, season, environmental factors, soil type and climate, genetic factors, and processing and extraction methods. The TPC could be used as an important indicator of the antibacterial and antioxidant capacities, since phenolic compounds are considered to have a high redox potential allowing them to act as radical scavengers or hydrogen donors *(44)*.

Antioxidant activity. The antioxidant potential of different plant extracts and pure compounds can be measured using numerous in vitro assays. Each of these tests is based on one feature of the antioxidant activity, such as the ability to scavenge free radicals, the inhibition of lipid peroxidation, or chelation of the transition metal ions *(33)*. Thus, the antioxidant activity assessment may require a combination of different methods because there are substantial differences in sample preparation, extraction of antioxidants, the selection of endpoints, and the way in which the results are expressed, even for the same method *(47)*. In this study, four different methods were used to determine the antioxidant activities of EOs.

The DPPH radical is one of the most commonly used substrates for fast evaluation of antioxidant activity because of its stability (in radical form) and the simplicity of the assay. Figure 1 shows the DPPH radical-scavenging activity of *T. moroderi, T. piperella, S. chamaecyparissus,* and *S. angustifolia*. It can be seen that EOs present different scavenging capacities. A concentration-dependent scavenging activity was found for all the EOs studied. *T. piperella* EO showed the highest ($P < 0.05$) DPPH radical-scavenging activity at all concentrations assayed, followed

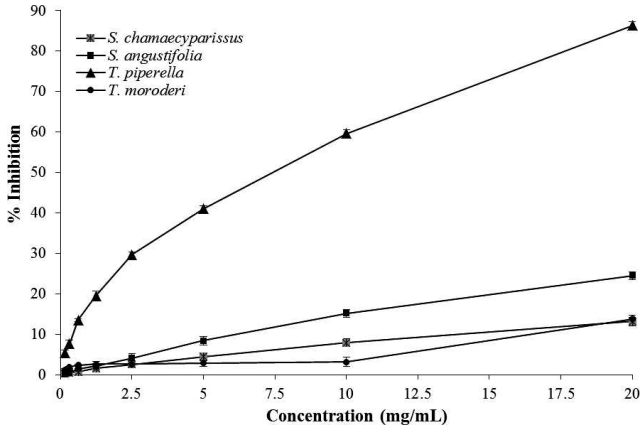

FIGURE 1. *Antioxidant activity of* T. piperella, T. moroderi, S. chamaecyparissus, *and* S. angustifolia *EOs at different concentrations (0.16 to 20 mg/ml) by the DPPH assay.*

by *S. angustifolia* EO at high concentrations (5, 10, and 20 mg/ml). At low concentrations (<2.5 mg/ml), no statistically significant differences ($P > 0.05$) were found for the *T. moroderi, S. chamaecyparissus,* and *S. angustifolia* EOs. BHT showed the highest ($P < 0.05$) radical activity with a 50% inhibitory concentration (IC_{50}; the concentration that inhibits 50% of the DPPH radical) value of 0.49 mg/ml. The IC_{50}s were in the following order: *T. piperella* EO < *S. angustifolia* EO < *S. chamaecyparissus* EO < *T. moroderi,* with a value of 9.30 for *T. piperella* EO. The extrapolated IC_{50} values obtained for *S. angustifolia, S. chamaecyparissus,* and *T. moroderi* were 39.6, 75.5, and 90.0 mg/ml, respectively.

Metal ions may catalyze lipid peroxidation, which can lead to both free radical generation and lipid peroxide production *(22)*. The Fe^{2+} chelating capacity of different aromatic herbs EOs is shown in Figure 2. The analysis of this property showed that all the EOs studied were capable of chelating Fe^{2+} and did so in a concentration-dependent manner. At high concentrations (10 and 20 mg/ml), *S. chamaecyparissus* and *S. angustifolia* EOs showed the highest ($P < 0.05$) chelating capacity with no statistically significant differences ($P > 0.05$) between them. *T.*

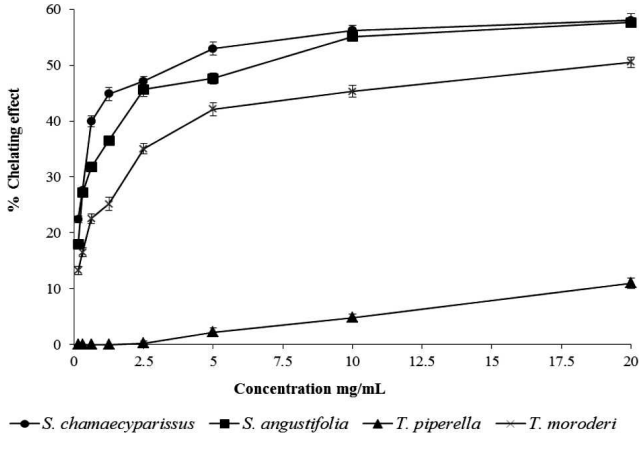

FIGURE 2. *Antioxidant activity of* T. piperella, T. moroderi, S. chamaecyparissus, *and* S. angustifolia *EOs at different concentrations (0.16 to 20 mg/ml) by the FIC assay.*

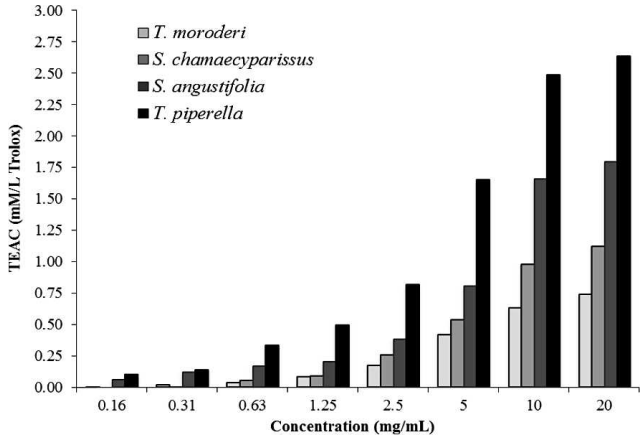

FIGURE 3. *Antioxidant activity of* T. piperella, T. moroderi, S. chamaecyparissus, *and* S. angustifolia *EOs at different concentrations (0.16 to 20 mg/ml) by the FRAP assay.*

piperella EO, at all concentrations assayed, showed the lowest ($P < 0.05$) values of Fe^{2+} chelating capacity. As mentioned above, the main component of *T. piperella* EO is carvacrol, a monohydroxylated compound unable to form a complex with Fe^{+2} *(7)*. The lowest concentration needed to obtain a 50% chelating effect (EC_{50}) was presented in the order *S. chamaecyparissus* EO < *S. angustifolia* EO < *T. moroderi* < *T. piperella* EO, with values of 3.9, 6.0, and 19.4, respectively, while the extrapolated EC_{50} value calculated for *T. piperella* was 425.7 mg/ml. The EC_{50} obtained for the positive control, EDTA, was 0.05 mg/ml.

The FRAP test is a simple, reproducible, rapid, and inexpensive procedure that measures the ability of antioxidative compounds to reduce the ferric ion Fe^{3+} to ferrous Fe^{2+}, as a measure of total antioxidant capacity. Figure 3 shows the ferric ion–reducing capacity obtained using the FRAP assay. A concentration-dependent ferric ion–reducing capacity was found for all the EOs studied. *T. piperella* EO, at all concentrations analyzed, showed the highest ($P < 0.05$) ferric ion–reducing capacity in terms of Trolox concentrations, with values ranging between 0.10 and 2.64 mM Trolox per liter. It was followed by *S. angustifolia* EO, with values comprised between 0.06 and 1.79 mM Trolox per liter. *T. moroderi* EO had the lowest ($P < 0.05$) ferric ion–reducing capacity among all of the EOs.

Figure 4 shows the inhibition of the formation of TBARs, which are a secondary metabolite of lipid oxidation. All EOs presented a high percentage of inhibition of TBARS, and this occurred in a concentration-dependent manner. At all concentrations assayed, *T. piperella* EO had the highest ($P < 0.05$) inhibition capacity. At high concentrations (5, 10, and 20 mg/ml), *S. chamaecyparissus* and *T. moroderi* EOs showed the lowest ($P < 0.05$) percent inhibition values. The values of IC_{50} were in the following order, *T. piperella* EO < *S. angustifolia* EO < *S. chamaecyparissus* EO < *T. moroderi,* with the values 6.30 and 12.3 for *T. piperella* and *S. angustifolia* EOs, while the extrapolated IC_{50} values calculated for *S. chamaecyparissus* and *T. moroderi* were 24.5 and 26.5 mg/ml, respectively, and the IC_{50} for the positive control, BHT, was 0.001 mg/ml.

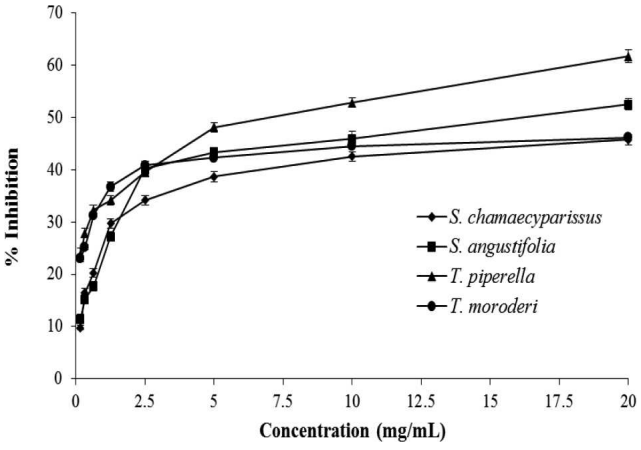

FIGURE 4. *Antioxidant activity of* T. piperella, T. moroderi, S. chamaecyparissus, *and* S. angustifolia *EOs at different concentrations (0.16 to 20 mg/ml) by the TBARs assay.*

This is the first study to analyze the antioxidant activity of the EOs obtained from *T. moroderi* and *T. piperella*. However, the antioxidant activities of several other thyme varieties have been widely studied. Tepe et al. *(40)* compared the antioxidant potential of two *Thymus* species, i.e., *Thymus sipyleus* subsp. *sipyleus* var. *sipyleus* and *Thymus sipyleus* subsp. *sipyleus* var. *rosulans,* by the DPPH assay. They reported that the free radical–scavenging activity of *T. sipyleus* subsp. *sipyleus* var. *rosulans* EO was superior to that of var. *sipyleus* EO, with IC_{50} values of 0.22 and 2.67 mg/ml, respectively. Viuda-Martos et al. *(43)* analyzed the antioxidant activity of *Thymus vulgaris* EO cultivated in Egypt. These authors reported that this EO showed in a DPPH assay an IC_{50} of 4.50 mg/ml, and in a TBARs assay an IC_{50} of 4.09 mg/ml while in the FIC assay the EC_{50} was 0.27 mg/ml. In a similar study, Viuda-Martos et al. *(47)* analyzed the antioxidant activity of the EO of *T. vulgaris* cultivated in Spain. They reported that the Spanish thyme showed, in a DPPH assay, an IC_{50} of 1.10 mg/ml, while in the FIC assay the EC_{50} was 17.32 mg/ml. Zouari et al. *(50)* investigated the antioxidant activity of *Thymus algeriensis* Boiss. et Reut EO, which grows wild in Tunisia. They reported that *T. algeriensis* EO was able to reduce the stable free radical DPPH with an IC_{50} of 0.8 mg/ml. Safaei-Ghomi et al. *(32)* analyzed the antioxidant activity of EO obtained from aerial parts of *Thymus caramanicus*. They reported that *T. caramanicus* EO had an IC_{50} of 0.26 mg/ml in the DPPH assay.

As with the EOs obtained from the *Thymus* species analyzed in this study, there are no scientific reports about the antioxidant activity of *S. angustifolia* EO. However, the antioxidant activities of other *Sideritis* species have been determined. Köse et al. *(23)* investigated the antioxidant activity of *Sideritis erythrantha* var. *erythrantha* and *S. erythrantha* var. *cedretorum* EOs and reported that the free radical–scavenging effects of the samples exhibited a dose-dependent increase but the EOs exhibited low radical-scavenging activity, 5.12% for *S. erythrantha* var. *cedretorum* and 4.62% for *S. erythrantha* var. *erythrantha* at concentrations of 2.0 mg/ml. Basile et al. *(6)* studied the antioxidant activity of *Sideritis italica* EOs obtained from leaves and flower heads. They reported that the antioxidant activities determined by ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid] and expressed as ascorbic acid equivalents were 4.29 and 1.98 mg/ml for leaf and flower head, respectively. As regards the antioxidant activity of EOs obtained from plants of the genus *Santolina,* there are no any studies in the scientific literature.

The antioxidant activity may be due to different mechanisms, such as prevention of chain initiation, decomposition of peroxides, and prevention of continued hydrogen abstraction, free radical scavenging, reducing capacity, and binding of transition metal ion catalysts *(24),* and could be attributed to the high content of monoterpenes, sesquiterpenes, and phenolic acids present in the EO, substances that are known for their increased levels of antioxidant activity *(12)*. These compounds exhibit in vitro and in vivo antioxidant activity, inhibiting lipid peroxidation by acting as chain-breaking peroxyl radical scavengers. In addition, phenols directly scavenge reactive oxygen species (hydroxyl radicals, peroxynitrite, and hypochlorous acid) *(44)*. However, it should be borne in mind that the activities of EOs such as antioxidants depend not only on their structural features but also on many other factors, such as concentration, temperature, light, type of substrate, and physical state of the system, as well as on microcomponents acting as pro-oxidants or synergists *(48)*.

It is interesting that for all EOs analyzed and all the antioxidant assays used, except for the FIC assay, there was a correlation between TPC and antioxidant activity. Djeridane et al. *(11)* and Katalinic et al. *(21)* demonstrated a linear correlation between the TPC and antioxidant capacity.

Antifungal activity. The antifungal activities of *T. moroderi, T. piperella, S. chamaecyparissus,* and *S. angustifolia* were evaluated against six yeasts and six molds.

The broth microdilution method was used to determine the antifungal activity of EOs against yeasts (Table 2). The results showed that all EOs had a substantial inhibitory effect on all assayed yeast strains. *S. angustifolia* EO had the lowest MICs for *R. mucilaginosa, S. cerevisiae, D. hansenii,* and *P. carsoni*. *T. moroderi* EO was the most active against *Y. lipolytica* with a MIC of 0.25 ml/100 ml. However, this EO had the highest MIC for *C. zeylanoides* and *S. cerevisiae*.

Aligianis et al. *(4)* proposed a classification for the antimicrobial activity of plant products based on MIC results, as follows: MIC $\leq$ 0.05 ml/100 ml, strong inhibitors; MIC between 0.06 and 0.15 ml/100 ml, moderate inhibitors; MIC $>$ 0.16 ml/100 ml, weak inhibitors. Thus, based on the MIC results from microdilution assays, the EOs analyzed presented a poor potential as antiyeast compounds, since most yeast strains showed a MIC of $>$0.16 ml/100 ml. As with antioxidant activity, this is the first work that analyzed the antiyeast activity of *T. moroderi, T. piperella, S. chamaecyparissus,* and *S. angustifolia* EOs. Nevertheless, some studies have found a

TABLE 2. *MICs of* T. moroderi, T. piperella, S. chamaecyparissus, *and* S. angustifolia *EOs against several yeast species*

Yeast	Concn (ml/100 ml) of EO from:			
	T. moroderi	*T. piperella*	*S. angustifolia*	*S. chamaecyparissus*
Y. lipolytica	0.25	0.50	0.50	0.50
C. zeylanoides	0.50	0.25	0.25	0.25
P. carsonii	0.50	0.50	0.25	0.50
S. cerevisiae	1.00	0.50	0.25	0.50
D. hansenii	0.25	0.25	0.12	0.25
R. mucilaginosa	0.50	0.50	0.25	0.50

strong antiyeast effect of EOs obtained from aromatic herbs or spices *(5, 29, 37, 41)*. Thus, Pinto et al. *(29)* reported that *Salvia officinalis* EO had MICs of 0.25 ml/100 ml against *Candida albicans, Candida krusei, Candida tropicalis,* and *Candida parapsilosis*. Souza et al. *(37)* showed that *Origanum vulgare* EO was effective in inhibiting the growth of *C. albicans, C. krusei, C. tropicalis, Pichia minuscula, Pichia ohmeri, Rhodotorula rubra,* and *S. cerevisiae,* with MICs between 2 and 0.06 ml/100 ml. Tserennadmid et al. *(41)* analyzed the antiyeast activities of clary sage, juniper, lemon, and marjoram EOs against *Geotrichum candidum, Pichia anomala, S. cerevisiae,* and *Schizosaccharomyces pombe*. These authors reported that the most sensitive yeast was *S. pombe* (0.006 to 0.0125 ml/100 ml) while *G. candidum* proved to be the most insensitive (MICs, 0.05 to 0.2 ml/100 ml). For antifungal activity against molds, the agar dilution method was used, and the results are summarized in Table 3. In the case of *A. niger,* all EOs at all concentrations assayed showed reduction in mycelial growth. *T. moroderi* EO produced the greatest ($P < 0.05$) MIG values, 29.01 and 52.54%, at concentrations of 0.1 and 0.2 ml/100 ml, respectively, followed by *T. piperella* EO with MIG values of 26.79 and 44.94%, respectively, at the same concentrations. At all concentrations, *S. angustifolia* EO caused the lowest MIG with values ranging between 6.06 and 33.74%. In the case of *A. flavus, S. chamaecyparissus* EO showed the highest ($P < 0.05$) MIG values (41.23%) at maximum concentration (0.2 ml/100 ml), while at concentrations of 0.05 and 0.1 ml/100 ml it was *T. moroderi* EO that showed the highest ($P <$

TABLE 3. *Percent mycelial inhibition growth (MIG) of* T. moroderi, T. piperella, S. angustifolia, *and* S. chamaecyparissus *EOs against several molds by the agar dilution method*[a]

Mold	EO concn (ml/100 ml)	%MIG			
		T. moroderi	*T. piperella*	*S. angustifolia*	*S. chamaecyparissus*
A. niger	0.025	6.42 ± 0.12 a A	8.44 ± 0.02 b A	4.06 ± 0.15 c A	6.68 ± 0.12 a A
	0.05	13.73 ± 0.24 a B	14.33 ± 0.01 b B	12.91 ± 0.09 c B	14.50 ± 0.06 d B
	0.1	29.01 ± 0.15 a C	26.79 ± 0.12 b C	22.10 ± 0.11 c C	22.24 ± 0.07 c C
	0.2	52.54 ± 0.23 a D	44.94 ± 0.05 b D	33.74 ± 0.13 c D	42.82 ± 0.12 d D
A. flavus	0.025	10.10 ± 0.11 a A	3.94 ± 0.07 b A	8.43 ± 0.08 c A	7.06 ± 0.11 d A
	0.05	19.89 ± 0.23 a B	10.76 ± 0.14 b B	13.61 ± 0.10 c B	13.58 ± 0.08 c B
	0.1	26.79 ± 0.10 a C	16.21 ± 0.09 b C	21.88 ± 0.07 c C	23.26 ± 0.14 d C
	0.2	34.84 ± 0.11 a D	28.22 ± 0.10 b D	29.54 ± 0.09 c D	41.23 ± 0.09 d D
A. alternata	0.025	12.83 ± 0.06 a A	0.00 ± 0.00 b A	14.47 ± 0.01 c A	14.48 ± 0.06 c A
	0.05	24.57 ± 0.12 a B	15.80 ± 0.06 b B	33.24 ± 0.12 c B	23.96 ± 0.07 d B
	0.1	39.27 ± 0.14 a C	20.64 ± 0.08 b C	50.29 ± 0.12 c C	32.83 ± 0.11 d C
	0.2	54.69 ± 0.06 a D	29.43 ± 0.09 b D	55.70 ± 0.09 c D	43.96 ± 0.14 d D
P. chrysogenum	0.025	13.84 ± 0.05 a A	0.00 ± 0.00 b A	10.51 ± 0.12 c A	1.17 ± 0.11 d A
	0.05	30.47 ± 0.09 a B	5.56 ± 0.11 b B	21.69 ± 0.14 c B	7.24 ± 0.15 d B
	0.1	51.57 ± 0.11 a C	8.33 ± 0.05 b C	60.56 ± 0.05 c C	38.13 ± 0.06 d C
	0.2	63.94 ± 0.15 a D	28.33 ± 0.13 b D	79.81 ± 0.04 c D	53.94 ± 0.01 d D
M. circinelloides	0.025	4.92 ± 0.06 a A	7.01 ± 0.03 b A	0.00 ± 0.00 c A	1.60 ± 0.20 d A
	0.05	13.98 ± 0.07 a B	9.23 ± 0.04 b B	1.12 ± 0.01 c B	6.79 ± 0.12 d B
	0.1	19.01 ± 0.09 a C	29.14 ± 0.14 b C	26.88 ± 0.14 c C	33.59 ± 0.09 d C
	0.2	22.57 ± 0.13 a D	44.57 ± 0.07 b D	42.81 ± 0.12 c D	45.60 ± 0.14 d D
M. racemosus	0.025	1.46 ± 0.04 a A	3.58 ± 0.04 b A	0.00 ± 0.00 c A	0.00 ± 0.00 c A
	0.05	17.54 ± 0.12 a B	5.58 ± 0.14 b B	12.61 ± 0.08 c B	0.00 ± 0.00 d A
	0.1	30.29 ± 0.08 a C	18.28 ± 0.14 b C	49.60 ± 0.07 c C	14.93 ± 0.18 d B
	0.2	40.02 ± 0.09 a D	38.63 ± 0.17 b D	62.24 ± 0.11 c D	29.06 ± 0.15 d C

[a] For the same mold and at the same concentration, values followed by the same lowercase letter within the same row are not significantly different ($P > 0.05$) according to Tukey's multiple range test. For the same mold and the same EO, values followed by the same uppercase letter within the same column are not significantly different ($P > 0.05$) according to Tukey's multiple range test.

0.05) MIG values (19.89 and 26.79%, respectively). *T. piperella* at all concentrations showed the lowest ($P < 0.05$) MIG values. *S. angustifolia* EO, at high concentrations (0.1 and 0.2 ml/100 ml) was the most effective EO ($P < 0.05$) in reducing the growth of *A. alternata, P. chrysogenum,* and *M. racemosus* with MIG values of 50.29 and 55.70%, 60.56 and 79.81%, and 49.60 and 62.24%, respectively. For these molds, the next most ($P < 0.05$) effective EO at high concentrations was that of *T. moroderi* with MIG values of 39.27 and 54.69%, 51.57 and 63.94%, and 30.29 and 40.02% for *A. alternata*, *P. chrysogenum,* and *M. racemosus,* respectively, at high concentrations. For *A. alternata* and *P. chrysogenum,* the *T. piperella* EO at all concentrations showed the lowest ($P < 0.05$) MIG values. Insofar as *M. circinelloides* is concerned, *S. chamaecyparissus* EO showed the highest ($P < 0.05$) MIG values (33.59 and 45.60%) at high concentrations (0.1 and 0.2 ml/100 ml, respectively). This was followed by *T. piperella* EO with slightly lower reductions (29.14 and 44.57% at the same concentrations). *S. angustifolia* showed the lowest ($P < 0.05$) MIG values at low concentrations. The antifungal activity of aromatic herbs and spices EOs against molds has been widely demonstrated *(19, 25, 36, 45).* Indeed, Viuda-Martos et al. *(45)* demonstrated that thyme, oregano, or clove EOs at concentrations of 0.04 ml/100 ml provoked a total inhibition of growth of *A. niger* and *A. flavus.* Sokmen et al. *(36)* demonstrated the capacity of thyme EO at 10 ml to inhibit the growth of molds such as *Alternaria* spp., *A. flavus*, *Fusarium* spp., and *Penicillium* spp. Gonçalves et al. *(19)* reported that *Thymus zygis* subsp. *sylvestris* EO had MICs between 0.016 and 0.12 ml/100 ml against *A. niger, A. flavus,* and *Aspergillus fumigatus.*

The mechanisms behind the antifungal activity of EOs against molds and yeasts are not fully understood because these substances are a mixture of several compounds that do not act on specific targets in the fungal cells. However, there are many studies that try to explain them.

As regards yeasts, Uribe et al. *(42)* reported that terpenes present in EOs inhibited the respiratory activity in intact yeast cells and also in isolated mitochondria, while Adegoke et al. *(2)* reported that EOs caused cell membrane degradation and loss of cytoplasmic components in the yeast *C. tropicalis*. Ferguson and von Borstel *(14)* reported that the cytotoxicity of the EOs is accompanied by the induction of cytoplasmic ''petite'' mutations, indicating mitochondrial damage and impairment of oxidative metabolism. In the case of the molds, Soylu et al. *(38)* found that monoterpenes present in the EOs cause alterations in the hyphal morphology and hyphal aggregates, resulting in reduced hyphal diameters and lyses of hyphal wall interacting with the cell membrane of the pathogen. For Sharma and Tripathi *(34),* the EOs would act on the hyphae of the mycelium, provoking the exit of components from the cytoplasm and the loss of rigidity and integrity of the hyphal cell wall, resulting in its collapse and death of the mycelium. Omidbeygi et al. *(26)* have suggested that antimicrobial components of the EOs cross the molds' cell membrane, interacting with the enzymes and proteins of the membrane, thus producing a flux of protons toward the cell exterior that induces changes in the cells and, ultimately, their death.

The results obtained in this study suggest the possibility of using the essential oils of *T. moroderi, T. piperella, S. chamaecyparissus,* and *S. angustifolia* as natural antioxidant food preservatives, emerging from a growing tendency to replace synthetic preservatives by natural ones. In addition, these EOs may have a great potential to be used as safe and ecofriendly antifungal ingredients compounds in the food industry. However, in general, the levels of EOs and their compounds necessary to inhibit microbial growth are higher in foods than in culture media. This is due to interactions between phenolic compounds and the food matrix and should be considered for commercial applications. In this respect, studying the results obtained with native species may be of great interest.

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ABSTRACT

The aim of this work was to evaluate chitosan edible films incorporated with the EOs of two aromatic herbs, *Thymus moroderi* and *Thymus piperella* for (i) the growth inhibition of some bacterial strains (ii) their total phenolic content (TPC), and (iii) their antioxidant activity by means of three different antioxidant tests to define if the chitosan edible films incorporated with these EOs could be used as natural active films for food use. The agar disc diffusion method was used to determine the antibacterial activities of chitosan edible films. For the antioxidant activity, three different analytical assays were used (DPPH, FRAP and FIC). The chitosan films containing *T. piperella* EO (CH + TPEO) were more effective ($p < 0.05$) against *Serratia marcenscens* and *Listeria innocua* than chitosan films containing *T. moroderi* EO (CH + TMEO), while no statistically differences were found ($p > 0.05$) between CH + TPEO and CH + TMEO against *Aeromonas hydrophila* and *Achromobacter denitrificans*. The CH + TMEO films showed lower ($p < 0.05$) antioxidant activity, at all concentrations and with all methods assayed, than CH + TPEO. The antioxidant activity occurred in a concentration dependent manner.

The results showed that chitosan edible films incorporated with *T. piperella* and *T. moroderi* EOs could be used as active films due to its excellent antibacterial and antioxidant activities.

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1. Introduction

At present there is an interest in biodegradable edible packaging due to the excellent biodegradability, biocompatibility, edibility and their potential applications. These films may operate as carriers of many functional ingredients. Such ingredients may include antioxidants, antimicrobial agents, flavours, spices and colourants which improve the functionality of the packaging materials by adding novel or extra functions (Salmieri & Lacroix, 2006).

In particular, chitosan, poly-β-(1/4) N-acetyl-D-glucosamine, is obtained by deacetylation of chitin, a biopolymer that is abundant in a variety of crustacean shells, such as crab shells, crawfish shells and shrimp shells (Kim et al., 2011). Chitosan has a great potential for a wide range of food applications due to its biodegradability, biocompatibility, antimicrobial and antioxidant activities, non-toxicity and film-forming capacity (Tharanathan & Kittur, 2003). Chitosan films have selective permeability to gases (CO_2 and O_2) and good mechanical properties. However, due to their hydrophilic nature, they are poor barriers to moisture, which limits their uses (Ojagh, Rezaei, Razavi, & Hosseini, 2010). This is an important drawback since an effective control of moisture transfer is a desirable property for most foods. Usually, hydrophobic compounds, such as lipids, are incorporated into this type of hydrophilic hydrocolloid films to improve their water barrier properties. One possibility is the use of essential oils (EOs). Several studies have shown that incorporation of EOs into chitosan films or coatings may not only enhance the film's antimicrobial and antioxidant properties but also reduce water vapour permeability and down lipid oxidation of the product on which the film is applied (Kanatt, Chander, & Sharma, 2008) mainly meat and meat products. On the other hand, the purpose of incorporating antimicrobial compounds into an edible film instead of applying them directly onto the meat surface by spraying or dipping was to extend delivery of the antimicrobials during meat storage rather than delivering them in a single massive dose (Ouattara et al., 2000). Thus, the use of edible chitosan films in meat and meat products have been comprehensively explained (Ouattara et al., 2000; Park, Marsh, & Dawson, 2010; Petrou, Tsiraki, Giatrakou, & Savvaidis, 2012).

EOs extracted from plants or spices are rich sources of biologically active compounds such as terpenoids and phenolic acids. It has been long recognized that some of the EOs have antimicrobial

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and antioxidant properties (Viuda-Martos, El Gendy, et al., 2010; Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez, 2008; Viuda-Martos, Ruiz-Navajas, Sánchez-Zapata, Fernández-López, & Pérez-Alvarez, 2010). Although most of the EOs are classified as Generally Recognized as Safe (GRAS) their use as food preservatives is often limited due to flavouring considerations since effective antimicrobial doses may exceed organoleptically acceptable levels (Viuda-Martos et al., 2008). To avoid this problem an alternative is the incorporation of EOs within edible films. Edible films can reduce the diffusion of antimicrobial compounds into the product since the EO forms part of the chemical structure of the film and interacts with the polymer and the plasticizer. Antimicrobial compounds release from the edible films depends on many factors, including electrostatic interactions between the antimicrobial agent and the polymer chains, osmosis, structural changes induced by the presence of antimicrobial, and environmental conditions (Avila-Sosa et al., 2012). Compared with direct application, smaller amounts of antimicrobial agents would be needed when edible films are used as carriers in order to achieve a specific food shelf life due to a gradual release on food surfaces (Ponce, Roura, del Valle, & Moreira, 2008).

Within a great variety of spices, Thymus species are considered to be one of the most active. Thymus L. (Labiatae) consists of about 215 species of herbaceous perennials and sub-shrubs (Nickavar, Mojab, & Dolat-Abadi, 2005). Growing wild in the Mediterranean environment, there are several ecotypes of thyme, which differ in their morphological characteristics (Tedone, D'andrea, & Marzi, 2001). Two species found in the Thymus genus are *Thymus piperella* and *Thymus moroderi*. The first of these is an endemic eastern Iberian taxon, extending over approximately 800 km^2 of the province of Valencia. *T. moroderi*, which is closely related to common thyme (*Thymus vulgaris* L.), is also endemic to south-eastern Spain.

The aim of this work was to evaluate chitosan edible films incorporated with the EOs of two aromatic herbs, *T. moroderi* and *T. piperella* for (i) the inhibition growth of some bacterial strains (ii) their total phenolic content (TPC), and (iii) their antioxidant activity by means of three different antioxidant tests to define if the chitosan edible films incorporated with these EOs could be used as natural active films for food use mainly in meat and meat products.

2. Materials and methods

2.1. Extraction of the essential oils

T. moroderi (TM) and *T. piperella* (TP) were collected during their flowering period. The EOs of TM and TP were extracted from whole plant (stems, leaves and flowers) by hydro-distillation using a Clevenger-type apparatus for 3 h. The oily layer obtained on top of the aqueous distillate was separated and dried with 0.5 g of anhydrous sodium sulphate (Panreac Química, Barcelona, Spain). The extracted EOs was kept in sealed air-tight glass vials and covered with aluminium foil at 4 °C until further analysis.

2.2. Preparation of edible films

Chitosan-based film was prepared by dissolving chitosan (high molecular weight 75–85% deacetylated, Sigma–Aldrich Chemical Co., Steinheim, Germany) in a lactic acid aqueous solution (1% v/v) (Sigma–Aldrich Chemical Co., Steinheim, Germany) at a concentration of 2% (w/v) while stirring on a magnetic stirrer/hot plate, following the indications of Ojagh et al. (2010) with some modifications. The chitosan solution was stirred at room temperature until it was completely dissolved (24 h). The resultant chitosan solution was filtered through a Whatman No. 3 filter paper to remove any undissolved particles. After filtration the solution was returned to the magnetic stirrer/hot plate and glycerol (Panreac Quimica, Barcelona, Spain) was added to a level of 0.75 mL/g chitosan as a plasticizer. The plasticizer was mixed into the solution for 15 min. Then, Tween 80 (Panreac, Quimica, Barcelona, Spain) at level of 0.2% (v/v) of EO, was added as an emulsifier to assist EO dispersion in film forming solutions. After 15 min of stirring, *T. moroderi* (TMEO) or *T. piperella* (TPEO) EOs were added to chitosan solution to reach a final concentration of 0.5%, 1%, and 2% (v/v).

Both chitosan TMEO (CH + TMEO) and chitosan TPEO (CH + TPEO) mixtures were emulsified at room temperature using a rotor-stator homogenizer (Ultraturrax DI 25, Janke & Kunkel, Staufen, Germany) at 20,000 rpm for 3 min. These emulsions were degasified at room temperature using an ultrasonic water bath (Selecta S.A. Barcelona, Spain), without temperature control, during 2 h. The film forming solutions (15 g) were casted into 90 mm inner diameter sterile Petri dishes (0.23 g/cm^2) covers and then dried for 48 h at 37 °C. Dried films were peeled and stored in a desiccator at 25 °C and 51% relative humidity until evaluation. Saturated magnesium nitrate (Panreac Quimica, Barcelona, Spain) solution was used to meet required relative humidity.

2.3. Microbial strains

The chitosan films incorporate with TPEO and TMEO were individually tested against *Listeria innocua* CECT 910, *Serratia marcenscens* CECT 854, *Aeromonas hydrophila* CECT 5734, *Achromobacter denitrificans* CECT 449, and *Alcaligenes faecalis* CECT 145. These species were supplied by the Spanish Type Culture Collection (CECT) of the University of Valencia. These bacteria were chosen based on they are commonly isolated from refrigerated foods (mainly meat and meat products), or as indicators or models of food pathogenic bacteria.

2.4. Agar disc diffusion method

The agar disc diffusion method described by Tepe, Daferera, Sokmen, Sokmen, and Polissiou (2005) with some modifications was used to determine the antibacterial capacity of chitosan edible films incorporated with TMEO or TPEO. Briefly, a suspension (0.1 mL of 10^6 cfu/mL) of each microorganism was spread on the solid medium plates. Nutrient Agar II (Oxoid, Basingstoke, Hampshire, England) in the case of *S. marcenscens* and *A. hydrophila*; Nutrient Agar I (Oxoid, Basingstoke, Hampshire, England) in the case of *A. faecalis* and *A. denitrificans*; and Brain Heart Infusion agar (Sharlab, Barcelona, Spain) for *L. innocua*. CH + TMEO or CH + TPEO edible films discs, 10 mm in diameter, were aseptically obtained and placed on the inoculated plates; these plates were incubated at 37 °C for 24 h in the case of *A. faecalis* and *L. innocua*; at 26 °C for 24 h for *S. marcenscens*, *A. hydrophila* and *A. denitrificans*. The diameters of the inhibition zones were measured in millimetres. All tests were performed in triplicate.

2.5. Total phenol content

The total phenol content (TPC) was determined using the Folin–Ciocalteu reagent (Singleton & Rossi, 1965). Every sample (25 mg) of each film was dissolved in 3 mL of methanol and then a volume of 0.3 mL was introduced into the test tubes followed by 2.5 mL of Folin Ciocalteu's reagent (Sigma–Aldrich Chemical Co., Steinheim, Germany) (diluted 10 times with water) and 2 mL of sodium carbonate (Panreac Quimica, Barcelona, Spain) (7.5% w/v). The tubes were vortex-mixed, covered with parafilm and incubated at 50 °C for 5 min. Absorption at 760 nm was measured with an HP

8451 spectrophotometer (Hewlett–Packard, Cambridge, UK) and compared to a gallic acid calibration curve. The results were expressed in mg gallic acid equivalents (GAE)/g of films, as mean of three replicates.

2.6. Antioxidant activity

2.6.1. DPPH (2,2′-diphenyl-1-picrylhydrazyl) radical scavenging assay

The efficacy of the films to scavenge 2,2′-diphenyl-1-picrylhydrazyl (DPPH) (Sigma–Aldrich Chemical Co., Steinheim, Germany) radicals was determined using spectrophotometry method on basis of bleaching of the bluish-red or purple colour of DPPH solution as a reagent, according to Siripatrawan and Harte (2010) with minor changes. Every sample (25 mg) of each film was dissolved in 3 mL of methanol, and then 2.8 mL of film extract solution were mixed with 0.2 mL of 1 mM methanolic solutions of DPPH. The mixtures were well shaken in a Vortex (2500 rpm) for 1 min and then placed in a dark room. The decrease in absorbance at 517 nm was determined with an HP 8451 spectrophotometer (Hewlett Packard, Cambridge, UK) after 1 h for all samples. Results are expressed in mg Trolox equivalent/g film as mean of three replicates.

2.6.2. Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) of the different films samples was determined by using the potassium ferricyanide-ferric chloride method (Oyaizu, 1986). Every sample (25 mg) of each film was dissolved in 3 mL of methanol and then 1 mL of different dilutions was added to 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide (Panreac Quimica, Barcelona, Spain) (1%). The mixtures were incubated at 50 °C for 20 min, after which 2.5 mL trichloroacetic acid (Panreac Quimica, Barcelona, Spain) (10%) were added. An aliquot of the mixture (2.5 mL) was taken and mixed with 2.5 mL water and 0.5 mL 0.1% $FeCl_3 \cdot 6H_2O$ (Panreac Quimica, Barcelona, Spain). The absorbance at 700 nm was measured after allowing the solution to stand for 30 min. Results were expressed in mg Trolox equivalent/g film as mean of three replicates.

2.6.3. Ferrous ion-chelating ability assay

Ferrous ions (Fe^{2+}) chelating activity (FIC) was measured by inhibiting the formation of Fe^{2+}–ferrozine complex after treatment of test material with Fe^{2+}, following the method of Carter (1971) with some modifications. Every sample (25 mg) of each film was dissolved in 3 mL of methanol and then 1 mL of different dilutions was mixed with 0.1 mL $FeCl_2 \cdot 4H_2O$ (Panreac Quimica, Barcelona, Spain) (2 mM) and 3.7 mL of methanol. After 5 min incubation, the reaction was initiated by the addition of 0.2 mL of ferrozine (Panreac Quimica, Barcelona, Spain) (5 mM). The mixture was shaken vigorously and after a further 10 min incubation period, the absorbance of the solution was measured spectrophotometrically at 562 nm. The inhibition percentage of ferrozine–Fe^{2+} complex formation was calculated by using the following equation:

$$\text{Chelating effect}(\%) = [(A_C - A_S)/A_C] \times 100,$$

where A_C = absorbance of control sample; (The control contains $FeCl_2$ and ferrozine, complex formation molecules) and A_S = absorbance of a tested sample.

2.7. Statistical analysis

Conventional statistical methods were used to calculate means and standard deviations of three simultaneous assays carried out with the different methods. Data collected for antioxidant and antibacterial properties were analysed by two-way analysis of variance to test the effects of two fixed factors: concentration (levels: 0.5, 1 and 2%) and EO (levels: *T. piperella*, *T. moroderi*). The Tukey post hoc test was applied for comparisons of means, differences were considered significant at $p > 0.05$. Statistical analysis and comparisons among means were carried out using the statistical package Statgraphics 5.1 for Windows.

3. Results and discussions

The chemical composition of the essential oils used in this work was previously determined by Ruiz-Navajas, Viuda-Martos, Sendra, Pérez-Álvarez, and Fernández-López (2012). In the TMEO the main components were camphor (26.74%), 1.8-cineol (24.99%), myrcene (5.63%) and α-pinene (4.35%) while in TPEO the predominant compounds were carvacrol (31.92%), *para*-cymene (16.18%), γ-terpinene (10.11%) and α-terpineol (7.29%).

3.1. Antibacterial activity

The antibacterial effects of chitosan edible films incorporated with the EOs of two aromatic herbs, *T. moroderi* and *T. piperella* at different concentrations, against five Gram-positive or Gram-negative bacteria are shown in Table 1.

Films containing only chitosan were not effective against any of the five tested bacteria. These results are in concordance with Ojagh et al. (2010), Zivanovic, Chi, and Draughon (2005) and Wang et al. (2011) who reported that no significant inhibition zone was observed for the pure chitosan film against *Escherichia coli* and *Staphylococcus aureus*. This effect of chitosan may be related to the fact that chitosan does not diffuse through the adjacent agar media in the agar diffusion test method, so that only organisms in direct

Table 1
Antibacterial effect of chitosan edible films, incorporated with different concentrations of *T. moroderi* and *T. piperella* EOs, against five Gram-positive or Gram-negative bacteria, by disc diffusion method.

Films	Diameter (mean and SD) of inhibition zone (mm) including film (10 mm)				
	Serratia marcenscens	*Aeromonas hydrophila*	*Alcaligenes faecalis*	*Achromobacter denitrificans*	*Listeria innocua*
CH	N.A.	N.A.	N.A.	N.A.	N.A.
CH + 0.5% TPEO	N.A.	12.00 ± 0.00^{aA}	N.A.	20.00 ± 0.00^{aA}	12.00 ± 0.00^{aA}
CH + 1% TPEO	13.50 ± 0.71^{aA}	17.00 ± 1.41^{bBC}	13.50 ± 0.71^{aA}	25.00 ± 0.00^{bB}	15.00 ± 0.00^{bB}
CH + 2% TPEO	$19.50 \pm B0.71^{b}$	18.50 ± 0.71^{bBD}	20.50 ± 0.71^{bB}	30.00 ± 0.00^{cC}	19.00 ± 1.41^{cC}
CH + 0.5% TMEO	N.A.	N.A.	23.00 ± 0.00^{aC}	20.00 ± 0.00^{aA}	N.A.
CH + 1% TMEO	N.A.	16.50 ± 0.71^{aC}	24.50 ± 0.71^{bD}	24.00 ± 1.41^{bB}	13.00 ± 0.00^{aD}
CH + 2% TMEO	N.A.	19.50 ± 0.71^{bD}	25.00 ± 0.00^{bD}	29.50 ± 0.71^{cC}	16.50 ± 0.71^{bE}

N. A.: Non active.
For a same essential oil, values followed by the same lower case letter are not significantly different ($p > 0.05$) according to Tukey's Multiple Range Test.
For a same bacteria, values followed by the same upper case letter are not significantly different ($p > 0.05$) according to Tukey's Multiple Range Test.

contact with the active sites of chitosan are inhibited. In addition the antimicrobial performance of the chitosan needs the positively charged amino groups of chitosan monomer units, which could react with the anionic groups of the microbial cell surface (Wang et al., 2011). However, Li, Wang, Chen, Huangfu, and Xie (2008) reported that chitosan solution at 0.10 mg/mL markedly inhibited the growth of *Xanthomonas* pathogenic bacteria from different geographical origins while Du, Zhao, Dai, and Yang (2009) showed that chitosan had good inhibition activities against *Bacillus subtilis*, *E. coli* and *S. aureus*.

As regards CH + TPEO, this edible film showed an inhibitory effect, at all concentrations, on 3 of the 5 bacteria assayed and this inhibitory effect was concentration-dependent. For *A. hydrophila,* the inhibition halos ranged from 12.00 to 18.50 mm with no statistically differences ($p > 0.05$) between CH + 1% TPEO and CH + 2% TPEO. *L. innocua* showed inhibition halos ($p < 0.05$) that varied from 12.00 to 19.00 mm. *A. denitrificans* showed the highest inhibition halos with values comprise between 20.00 and 30.00 mm with statistically differences ($p < 0.05$) between samples. For *S. marcenscens* and *A. faecalis* no antibacterial activity was achieved at the lowest concentration of EOs (0.5%). For CH + 1% TPEO and CH + 2% TPEO the inhibition halos ranged between 13.50 and 19.50 mm, respectively, in the case of *S. marcenscens*, while for *A. faecalis* the inhibition halos varied between 13.50 and 20.50 mm. In the case of CH + TMEO no antibacterial activity was obtained, at all concentrations, against *S. marcenscens*. In the same way, for *L. innocua* and *A. hydrophila,* no inhibitory effect was obtained at the lowest concentration (0.5%). For *A. faecalis,* the inhibition halos ranged from 23.00 to 25.00 mm with no statistically differences ($p > 0.05$) between CH + 1% TPEO and CH + 2% TPEO. *A. denitrificans* showed the highest inhibition halos, at the higher concentration, with values comprised between 20.00 and 29.50 mm with statistically differences ($p < 0.05$) between samples.

CH + TPEO was more effective ($p < 0.05$) against *S. marcenscens* and *L. innocua* than CH + TMEO, while no statistically differences were found ($p > 0.05$) between CH + TPEO and CH + TMEO against *A. hydrophila* and *A. denitrificans*. For *A. faecalis*, CH + TMEO showed higher ($p > 0.05$) inhibition halos, at all concentrations, than CH + TPEO.

This is the first study to analyse the antibacterial activity of edible films incorporated with *T. moroderi* and *T. piperella* EOs. However, the antibacterial activity of edible films of chitosan incorporated with EOs from several spices has been studied. Thus, Hosseini, Razavi, and Mousavi (2009) reported that addition of thyme, clove, and cinnamon essential oils to chitosan films, in general, inhibited the growth of *Listeria monocytogenes*, *S. aureus*, *Salmonella enteritidis*, and *Pseudomonas aeruginosa*. In other study Sánchez-González, Cháfer, Hernández, Chiralt, and González-Martínez (2011) analysed the antimicrobial chitosan films prepared with different concentrations of bergamot, lemon and tea tree EOs. These authors concluded that CH-EO composite films, containing bergamot, lemon and tea tree EOs showed a significant antimicrobial activity (bacteriostatic effect) against *E. coli*, *L. monocytogenes*, and *S. aureus*. Wang et al. (2011) investigated the synergistic antimicrobial activities of three natural EOs (clove bud oil, cinnamon oil, and star anise oil) added in chitosan films. These authors reported that chitosan-cinnamon EO and chitosan-clove EO films showed a good antimicrobial activity against *E. coli* and *S. aureus* while chitosan-star anise EO film did not show antibacterial activity.

The antibacterial activity of CH + TMEO or CH + TPEO films can be attributed to the EOs. The antibacterial activity of the different *Thymus* spp. is widely studied. Thus, Ruiz-Navajas et al. (2012) reported a strong inhibitory effect by the direct application of TPEO or TMEO against the same bacteria tested in this study. Viuda-Martos, Mohammadey, et al. (2011) analysed the antibacterial activity of *T. vulgaris* EO cultivated in Egypt. These authors reported that *T. vulgaris* EO, at concentration of 40 μL, showed inhibition zones of 41.00, 20.25 and 23.50 mm for *L. innocua*, *S. marcescens* and *Pseudomonas fluorescens* respectively. The antibacterial activity of EO isolated from the aerial parts of Tunisian *Thymus capitatus* was analysed by Bounatirou et al. (2007). They reported that *T. capitatus* EO, at concentrations of 4 μL, showed inhibitions zones of 9.7, 10.1, 13.9 and 19.4 mm for *Salmonella* spp. *B. cereus*, *L. innocua* and *S. aureus*, respectively.

The antibacterial activity could be due to one sole component, such as carvacrol or camphor. Thus antimicrobial activity of this compound has been confirmed on bacteria such as verocytotoxigenic *E. coli* (Rivas et al., 2010), *Shigella sonnei* and *S. flexneri* (Bagamboula, Uyttendaele, & Debevere, 2004) *E. coli* and *P. aeruginosa* (Demetzos, Angelopoulou, & Perdetzoglou, 2002). However, it is a more widely held point of view that the action is due to a synergistic effect between various components, whether major or minor ones (Daferera, Ziogas, & Polissiou, 2003).

The EOs affect microbial cells by various antimicrobial mechanisms, including attacking the phospholipid bilayer of the cell membrane, disrupting enzyme systems, compromising the genetic material of bacteria, and forming fatty acid hydroperoxidase caused by oxygenation of unsaturated fatty acids (Arques, Rodriguez, Nunez, & Medina, 2008; Burt et al., 2007). The EOs can coagulate the cytoplasm and damage lipids and proteins. Their mechanism of action would be similar to other phenolics, i.e. the disturbance of the proton motive force, electron flow, active transport and coagulation of cell contents. Instead, enzymes such as ATPases are known to be located in the cytoplasmic membrane and to be bordered by lipid molecules (Burt, 2004).

3.2. Total phenol content

Total phenolic content of chitosan edible films incorporated with TPEO and TMEO was shown in Fig. 1. CH films showed a TPC of 1.95 mg GAE/g film. This result was in agreement with the results reported by Siripatrawan and Harte (2010) or Moradi et al. (2012) where a low TPC for chitosan film was obtained. This finding might probably be attributed to the formation of chromogens, due to the reaction of Folin and Ciocalteu reagent with non-phenolic reducing substances which can be detected spectrophotometrically (Moradi et al., 2012). The TPC of CH + TPEO, expressed as gallic acid equivalent, ranged ($p < 0.05$) from 7.07 to 28.55 mg/g of film. While, as expected, TPC of CH + TMEO increased significantly by incorporating TMEO and the highest value (2.58 mg GAE/g film)

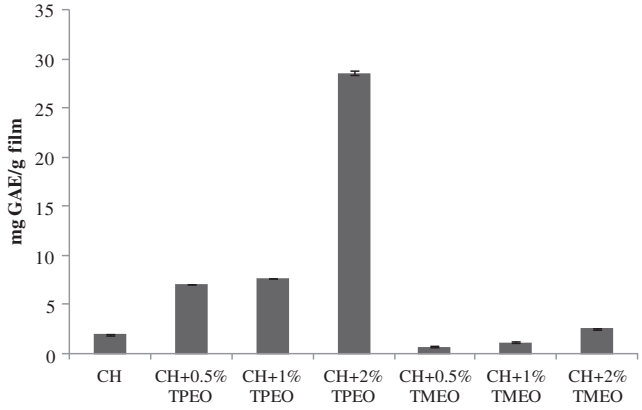

Fig. 1. Total phenol content of chitosan edible films incorporated with *T. moroderi* and *T. piperella* EOs.

was for the film formulated with 2% TMEO. The CH films made with TPEO showed higher TFC values ($p < 0.05$), at all concentrations, than the CH films elaborated with TMEO.

The phenolic content can be used as an important indicator of antioxidant capacity which can be used as a preliminary screen for any product when intended as a natural source of antioxidants in functional foods (Viuda-Martos, Ruiz-Navajas, et al., 2011). Thus, the polyphenolic compounds are very important plant constituents, by virtue of their antioxidant activity by chelating redox-active metal ions, inactivating lipid free radical chains and preventing hydroperoxide conversion into reactive oxyradicals (Cabral de Oliveira et al., 2009).

3.3. Antioxidant activity

Since the antioxidant capacity of food is determined by a mixture of different antioxidants with different action mechanisms, among which synergistic interactions, it is necessary to combine more than one method in order to determine *in vitro*, the antioxidant capacity of foodstuffs (Pérez-Jiménez et al., 2008).

Therefore the antioxidant activities of the edible films of CH + TPEO and CH + TMEO were investigated using DPPH radical-scavenging assay, FIC assay as well as FRAP assay.

DPPH is a compound that consists of a nitrogen free radical which is easily quenched by proton radical scavenger of hydrogen donating antioxidant and subsequently transformed into a non-radical form (DPPH-H) (Prior, Wu, & Schaich, 2005). CH films (Table 2) showed a slight scavenging activity ct activity (0.003 mg Trolox Equivalent (TE)/g film). The scavenging mechanism of chitosan is related to the fact that free radical can react with the residual free amino (NH_2) groups to form stable macromolecule radicals, and the NH_2 groups can form ammonium (NH_3^+) groups by absorbing a hydrogen ion from the solution (Yen, Yang, & Mau, 2008). The scavenging capacities of the CH + TPEO films (Table 2) significantly increased ($p < 0.05$) with increasing TPEO concentration. The results showed that DPPH scavenging activity of the films varied ($p < 0.05$) from 0.69 to 1.09 mg TE/g film. In the case of CH + TMEO films, the antioxidant activity values ranged between 0.42 and 0.63 mg TE/g film, and occur in a concentration-dependent manner. The CH + TMEO films showed lower ($p < 0.05$) antioxidant activity, at all concentrations assayed, than CH + TPEO.

The FRAP assay is commonly used to study the antioxidant capacity of plant materials. The antioxidant capacity of films is determined by the ability of the antioxidants in these extracts to reduce ferric to ferrous iron in FRAP reagent. CH film showed a slight ability to reduce ferric to ferrous iron (0.21 mg TE/g film). The analysed CH + TPEO showed a wide range of antioxidant capacities, with values ($p < 0.05$) between 2.79 and 10.09 mg TE/g of film. As in the case of DPPH the antioxidant capacity occur in a concentration dependent manner. As regards CH + TMEO film, at low concentrations (0.5 and 1%) no antioxidant activity was found. CH + 2% TMEO showed a low ability to reduce ferric to ferrous iron (0.67 mg TE/g film). The CH + TPEO films showed higher ($p < 0.05$) ability to reduce ferric to ferrous ion activity, at all concentrations assayed, than CH + TMEO.

FIC assay measures how effective the compounds in it can compete with ferrozine for ferrous ion. By forming a stable iron (II) chelate, an extract with high chelating power reduces the free ferrous ion concentration thus decreasing the extent of Fenton reaction (Halliwell & Gutteridge, 1990). Analysis of metal ion-chelating properties (Fig. 2) showed that CH + TPEO films studied were capable of chelating iron (II) and did so in a concentration-dependent manner ($p < 0.05$). In the same way, CH + TMEO showed a high ability to chelating iron (II) and as in the case of CH + TPEO its occur in a concentration-dependent manner ($p < 0.05$). At all concentrations (except 0.5%) CH + TMEO films were better chelators of iron (II) ($p < 0.05$) than CH + TPEO films. In general terms it can be said that edible chitosan films showed high chelating activity, which is of great significance because the chelation of transition metals is of great potential interest in the food industry (Viuda-Martos, Ruiz-Navajas, et al., 2010).

Few studies have examined the antioxidant activity of chitosan edible films incorporated with spices EOs (Altiok, Altiok, & Tihminlioglu, 2010; Moradi et al., 2012). This antioxidant activity could be related with the presence, in the edible films, of bioactive compounds such as phenolics acids of terpenoids coming from the essential oils. Thus, the phenolic and terpenoids compounds present in the chemical composition of EOs are closely associated with their antioxidant function mainly due to their redox properties exerted by various possible mechanisms: free-radical scavenging activity, hydrogen donors, transition-metal-chelating activity, and/or singlet-oxygen-quenching capacity (Liyana-Pathirana & Shahidi, 2006).

The antioxidant activity of EOs obtained from plants belonged to genus Thymus is widely demonstrated (Sarikurkcu et al., 2010; Viuda-Martos, El Gendy, et al., 2010; Zouari et al., 2011). The activities of EOs such as antioxidants depend on several structural features of the molecules and are primarily attributed to the high reactivity of hydroxyl groups substituents (Číž et al., 2010), but also on many other factors, such as concentration, temperature, light, type of substrate, physical state of the system, as well as on micro-

Table 2
Antioxidant effect of chitosan edible films incorporated with different concentrations of *T. moroderi* and *T. piperella* EOs by means of two different antioxidant test (DPPH and FRAP assays).

	DPPH (mg TE/g)	FRAC (mg TE/g)
CH	0.003 ± 0.00^{aA}	0.21 ± 0.00^{aA}
CH + 0.5% TPEO	0.69 ± 0.00^{aB}	2.79 ± 0.13^{aB}
CH + 1% TPEO	1.03 ± 0.01^{bC}	5.42 ± 0.07^{bC}
CH + 2% TPEO	1.07 ± 0.01^{cD}	10.09 ± 0.13^{cD}
CH + 0.5% TMEO	0.42 ± 0.00^{aE}	N.A.
CH + 1% TMEO	0.54 ± 0.00^{bF}	N.A.
CH + 2% TMEO	0.63 ± 0.00^{cG}	0.67 ± 0.09^{aE}

For a same essential oil, values followed by the same lower case letter are not significantly different ($p > 0.05$) according to Tukey's Multiple Range Test.
For a same antioxidant assay, values followed by the same upper case letter are not significantly different ($p > 0.05$) according to Tukey's Multiple Range Test.

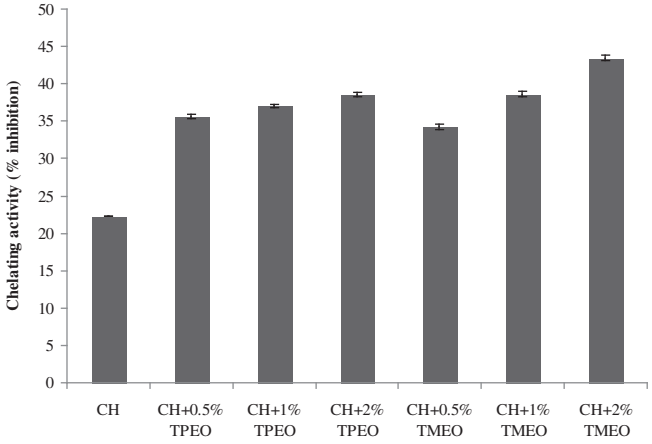

Fig. 2. Chelating activity of chitosan edible films incorporated with the EOs, evaluated by means of FIC assay, of *T. moroderi* and *T. piperella* at different concentrations.

components acting as pro-oxidants or synergists may influence the antioxidant activity (Yanishlieva-Maslarova, 2001).

In order to infer a major contributing effect of the total phenolic content (TPC) to the total antioxidant activities of methanolic extracts of edible films of CH + TPEO and CH + TMEO, linear correlation studies were performed with the three antioxidant assays (DPPH, FRAC and FIC). Thus, a significant correlation was observed between TPC of CH edible films incorporated with TPEO and TMEO and the DPPH radical-scavenging activity ($R^2 = 0.928$). In the same way, for FRAP assay, there was again a high correlation between this assay and the TPC of the CH edible films ($R^2 = 0.914$). However, a low correlation was obtained between FIC assay and TPC of the CH edible films added with EOs ($R^2 = 0.051$). A positive and significant correlation has also been obtained between the TPC and antioxidant activity by other authors (Baltrušaityte, Venskutonis, & Ceksteryte, 2007; Contreras-Calderón, Calderón-Jaimes, Guerra-Hernández, & García-Villanova, 2011; Ruiz-Navajas et al., 2011).

4. Conclusions

The incorporation of *T. piperella* and *T. moroderi* essential oils to chitosan edible films may have supplementary applications in food packaging. Thus, the results showed that chitosan edible films incorporated with these essential oils could be used as active films due to its excellent *in vitro* antibacterial and antioxidant activities. However, further studies are necessary to consider, the chitosan edible film, an additional stress factor for preserving food products, assuring its quality as well as a prolonged shelf-life.

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Abstract: The aim of this work was to develop chitosan edible films added with essential oils obtained from two Thymus species, Thymus moroderi (TMEO) and Thymus piperella (TPEO) to determine their application for enhancing safety (antioxidant and antibacterial properties) and shelf-life of cooked cured ham (CCH) storage at 4 ºC during 21 days.
Addition of TMEO and TPEO into chitosan films decreased the aerobic mesophilic bacteria (AMB) and lactic acid bacteria (LAB) counts in coated cooked cured ham samples as compared with uncoated samples. Both AMB and LAB showed the lowest counts in CCH samples coated with chitosan films added with TPEO at 2%. As regards lipid oxidation, the CCH samples coated with chitosan films added with TMEO or TPEO had lower degrees of lipid oxidation than uncoated control samples. Again, chitosan films added with TPEO at 2% showed the lowest values.
The addition of TPEO or TMEO in chitosan films used as coated in CCH improved their shelf life

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Effect of chitosan edible films added with *Thymus moroderi* and *Thymus piperella* essential oil on shelf-life of cooked cured ham

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Abstract

The aim of this work was to develop chitosan edible films added with essential oils obtained from two Thymus species, *Thymus moroderi* (TMEO) and *Thymus piperella* (TPEO) to determine their application for enhancing safety (antioxidant and antibacterial properties) and shelf-life of cooked cured ham (CCH) storage at 4 ºC during 21 days.

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Keywords: Essential oils, chitosan, films, Shelf-life, Cooked cured ham

1. Introduction

At the present there is great variety of ready-to-eat (RTE) foods including pre-packed and precooked meats, in individual or family-size packages, with very different presentations. However, the transformation into RTE food of meat product such as cooked ham, involves additional manipulation such as cutting, slicing, dicing and packaging aimed at facilitating its consumption at home (Gil-Díaz, Santos-Delgado, Rubio-Barroso & Polo-Díez, 2009). This may contribute considerably to the contamination of RTE meat products with pathogenic bacteria. For this reason there is a need for continuous supervision of RTE food products to ensure safety to consumers in the industrial countries. The increased demands by consumers for better quality and improved freshness of RTE food products have given rise to the development and implementation of edible films (Beverlya, Janes, Prinyawiwatkula & No, 2008).

Edible films and coatings are thin layers of edible material formed or placed on or between foods or food components (Bravin, Peressini & Sensidoni, 2006) which can play an important role on their preservation, distribution and marketing of food products (Falguera, Quintero, Jiménez, Muñoz & Ibarz, 2011). They are prepared from biopolymers; the major constituents of these are polysaccharides, proteins and lipids.

In particular, chitosan has a great potential for a wide range of food applications due to its biodegradability, biocompatibility, antimicrobial and antioxidant activities, nontoxicity and film-forming capacity (Tharanathan & Kittur, 2003). The use of edible films helps to maintain product quality, enhance sensory properties, improve product safety, and increase the shelf life of various RTE food products (Beverlya et al., 2008). Moreover, they can act as carriers of active substances, such as antioxidant, antimicrobial or flavoring compounds, resulting in shelf-life extension and safety improvement of the food product. One of these active substances is the essential oils

(EO). The ability of EOs to protect foods against pathogenic and spoilage microorganisms as well as the oxidation have been reported by several researchers (Ruiz-Navajas, Viuda-Martos, Sendra, Perez-Alvarez & Fernández-López, 2013; Alves-Silva et al. 2013). In order to achieve effective antimicrobial activity in direct food applications, high concentrations of essential oils are generally needed, which might impact inappropriate flavors and odors in the product (Seydim & Sarikus, 2006). To avoid this problem, the EOs could be incorporated into bioactive film coatings which would allow us to fix and retain the compound on the product surface, thus increasing its effectiveness (Sánchez-González, González-Martínez, Chiralt & Cháfer, 2010). In these coatings, the major compounds are biodegradable polymers and a relatively reduced amount of EOs can be used. Consequently, the application costs of essential oils and/or other problems, such as the intense aroma and potential toxicity, could be minimized (Sánchez-González et al. 2010).

Several studies have shown that incorporation of EOs into chitosan films or coatings may not only enhance the film's antimicrobial and antioxidant properties but also reduce water vapor permeability and down lipid oxidation of the product on which the film is applied (Kanatt, Chander & Sharma, 2008) mainly meat and meat products. There are several scientific works where the edible films elaborated with isolate whey protein, isolate soy protein, alginate and so on and incorporated with essential oils have been used to prevent the lipid oxidation and microbial growth in meat and meat product (Zinoviadou, Koutsoumanis & Biliaderis, 2009; Emiroğlu, Yemiş, Coşkun & Candoğan, 2010; Juck, Neetoo & Chen, 2010). However, the applications of chitosan edible films added with EOs are lesser analyze.

The aim of this work was to develop chitosan edible film added with EOs obtained from two *Thymus* species, *Thymus moroderi* and *Thymus piperella* to determine their

application for enhancing safety (antioxidant and antibacterial properties) and shelf-life of cooked cured ham storage at 4 ºC during 21 days.

2. Materials and Methods

2.1. Extraction of the essential oils

Thymus moroderi (TM) and *Thymus piperella* (TP) were collected during their flowering period and the identification of the plant material was made by Prof Dra. Concepcion Obon de Castro, Biology Department of Miguel Hernandez University (Spain). The EOs of TM and TP were extracted from whole plant (stems, leaves and flowers) by hydro-distillation using a Clevenger-type apparatus for 3 h. The oily layer obtained on top of the aqueous distillate was separated and dried with 0.5 g of anhydrous sodium sulphate (Panreac Química, Barcelona, Spain). The extracted EOs was kept in sealed air-tight glass vials and covered with aluminium foil at 4 ºC until further analysis.

2.2. Preparation of edible films

Chitosan-based film was prepared by dissolving chitosan (high molecular weight 75-85% deacetylated, Sigma-Aldrich Chemical Co., Steinheim, Germany) in a lactic acid aqueous solution (1% v/v) (Sigma-Aldrich Chemical Co., Steinheim, Germany) at a concentration of 2% (w/v) while stirring on a magnetic stirrer/hot plate, following the indications of Ojagh, Rezaei, Razavi and Hosseini (2010) with some modifications.

The chitosan solution was stirred at room temperature until it was completely dissolved (24 h). The resultant chitosan solution was filtered through a Whatman No. 3 filter paper to remove any undissolved particles. After filtration the solution was returned to the magnetic stirrer/hot plate and glycerol (Panreac Quimica, Barcelona, Spain) was added to a level of 0.75 mL/g chitosan as a plasticizer. The plasticizer was

126 mixed into the solution for 15 min. Then, Tween 80 (Panreac, Quimica, Barcelona,
127 Spain) at level of 0.2% (v/v) of EO, was added as an emulsifier to assist EO dispersion
128 in film forming solutions. After 15 min of stirring, the following five solutions were
129 prepared by adding *T. moroderi* (TMEO) or *T. piperella* (TPEO) EOs into chitosan
130 solution: (i) chitosan without TMEO and TPEO; (ii) chitosan with 1% TMEO; (iii)
131 chitosan with 2% TMEO; (iv) chitosan with 1% TPEO; (v) chitosan with 2% TPEO.
132 Both chitosan TMEO (CH+TMEO) and chitosan TPEO (CH+TPEO) mixtures were
133 emulsified at room temperature using a rotor-stator homogenizer (Ultraturrax DI 25,
134 Janke & Kunkel, Staufen, Germany) at 20,000 rpm for 3 min. These emulsions were
135 degasified at room temperature using an ultrasonic water bath (Selecta S.A. Barcelona,
136 Spain), without temperature control, during 2 h. The film forming solutions (7 g) were
137 casted into 60 mm inner diameter sterile Petri dishes (0.25 g/cm^2) covers and then dried
138 for 48 h at 37 ºC. Dried films were peeled and stored in a desiccator at 25 ºC and 51%
139 relative humidity until evaluation. Saturated magnesium nitrate (Panreac Quimica,
140 Barcelona, Spain) solution was used to meet required relative humidity.

141 **2.3 Film application on cooked cured ham**

142 Cooked cured hams (CCH) were purchased directly from a local producer and
143 transported immediately to the laboratory under refrigerated conditions and then they
144 were sliced (3 mm thick and 12.5 g weight). Each film with different formulations was
145 placed between two slices and was placed in a sterile bags made of polyethylene and
146 polyamide laminate of 1.1 g/m^2/24 h water vapor permeability at 23 ºC 10 cm^3/m^2/24 h,
147 nitrogen permeability at 23 ºC, 140 cm^3/m^2/24 h carbon dioxide permeability at 23 ºC,
148 and 30 cm^3/m^2/24 h oxygen permeability at 23 ºC (Fibran, Girona, Spain). Slices
149 without any chitosan film were also prepared as control CMH samples. The bags were

heat-sealed and stored at 4±1 ºC. The packs were stored for 21 d. Samples from each treatment were taken at 0, 7, 14, and 21 d (storage time) and analyzed on the same day.

2.4. Physico-chemical analysis

The CIEL*a*b* color space was studied following the procedure of Cassens et al. (1995). The following color coordinates were determined: lightness (L*), redness (a*, ± red-green), and yellowness (b*, ± yellow-blue). Color determinations were made, at 12±2 ºC by means of a Minolta CM-2600D (Minolta Camera Co., Osaka, Japan) spectrophotometer with illuminant D_{65}, 10º observer angle, 11 mm aperture for illumination and 8 mm for measurement. American Meat Science Association guidelines for color measurements were followed and spectrally pure glass (CRA51, Minolta Co., Osaka, Japan) was put between the samples and the equipment (AMSA, 2012).

The pH was measured by blending a 5 g sample with 50 mL deionized water for 2 min The pH of the resultant suspension was measured with a Crison pH meter (Model 507, Crison, Barcelona, Spain) equipped with a Crison combination electrode (Cat. nº. 52, Crison, Barcelona, Spain).

2.5. Lipid oxidation

Lipid oxidation was assessed in triplicate by the 2-thiobarbituric acid (TBA) test following the recommendations of Buege and Aust (1978). TBARS values were calculated from a standard curve of malonaldehyde (MAD) and expressed as mg MAD/kg sample.

2.6. Microbiological analysis

At each time (0, 7, 14, and 21 d), films were removed from the middle of two slices with a forceps and then the slices (two slices from the same treatment, weighing 25 g) were homogenized with sterile 1.5% peptone water (225 mL) in a Stomacher 400

175 (Colworth, London, UK) for 2 min. Total viable counts were determined on Plate Count
176 Agar, *Enterobacteriaceae* using Violet Red Bile Glucose Agar (VRBGA) and lactic
177 acid bacteria (LAB) were counted on double layer MRS Agar at pH 5.6. In all cases,
178 plates were incubated at 37 ºC for 48 h. molds and yeasts were determined on Rose
179 Bengal plates with chloramphenicol incubated at 28 ºC for 5 days. All results are
180 reported as log10 colony forming per gram (CFU).

181 **2.7. Release of phenolic compounds from film into cooked cured hams**

182 On each sampling day (0, 7, 14, and 21 d), films were removed from the middle of
183 two slices and total phenolic content (TPC) of films was determined with Folin-
184 Ciocalteu reagent (Singleton & Rossi, 1965). Every sample of each film was extracted
185 with 5 mL of methanol using an ultrasonic water bath (Selecta S.A. Barcelona, Spain)
186 without temperature control during 2 h. Then, the mixtures were centrifuged at 3600 g
187 for 15 min at 4 °C. Then a volume of 0.3 mL of supernatant was introduced into the test
188 tubes followed by 2.5 mL of Folin Ciocalteu's reagent (SigmaeAldrich Chemical Co.,
189 Steinheim, Germany) (diluted 10 times with water) and 2 mL of sodium carbonate
190 (Panreac Quimica, Barcelona, Spain) (7.5% w/v).

191 The tubes were vortex-mixed, covered with parafilm and incubated at 50 ºC for 5
192 min. Absorbance at 760 nm was measured with an HP 8451 spectrophotometer (Hewlett
193 Packard, Cambridge, UK) and compared to a gallic acid calibration curve. The results
194 were expressed in mg gallic acid equivalents (GAE)/g of films, as mean of three
195 replicates.

196 **2.8. Statistical assay**

197 Statistical analysis and comparisons among means were carried out using the
198 statistical package SPSS 19.0 (SPSS Inc., Chicago, IL.). All the data collected for pH,
199 color, lipid oxidation, microbiological analysis and release of phenolic compounds were

analyzed by two-way analysis of variance (ANOVA) to test the effects of two fixed factors: film samples (levels: control, CH, CH+TPEO 1%, CH+TPEO 2%, CH+TMEO 1% and CH+TMEO 2%) and time (levels: 0, 7, 14 and 21 days). Tukey's post hoc test was applied for comparisons of means; differences were considered significant at $p<0.05$. Generalized Additive Models were used in order to estimate the relationship between variables with different links and transformations of the variables following the recommendations of Wood (2006).

3. Results and discussion

The chemical composition of the essential oils used in this work was previously determined by Ruiz-Navajas, Viuda-Martos, Sendra, Perez-Alvarez and Fernández-López (2012). In the *Thymus moroderi* (TMEO) the main components were camphor (26.74%), 1.8-cineol (24.99%), myrcene (5.63%) and α-pinene (4.35%) while in *Thymus piperella* (TPEO) the predominant compounds were carvacrol (31.92%), *para*-cymene (16.18%), γ-terpinene (10.11%) and α-terpineol (7.29%).

3.1. Physico-chemical analysis

Figure 1 showed the effect of chitosan films formulated with TMEO and TPEO essential oils on pH values in cooked cured ham storage at 4 ºC during 21 days. In all uncoated and coated samples, the pH values decreased with the storage time. In uncoated CCH samples there was a slight fell ($p<0.05$) of pH values (6.24 at day 0 to 6.12 at day 21). Nevertheless, in coated CHH samples the decrease in pH values was most accentuated. At the end of storage time all coated CHH samples showed pH values comprised between 5.63 and 5.68 with no statistical differences ($p>0.05$) between them, except for the sample coated with CH+TPEO2% that showed higher ($p<0.05$) values (5.81). This decreased in pH values of coated CHH samples could be attributed to the

225 release of lactic acid present in chitosan films into meat product. Additionally, the
226 gradual growth of lactic bacteria in the CHH samples, which could have generate lactic
227 acid, which would lead to the gradual decrease of pH.

228 As regards to color parameters, Table 1 shows the effect of chitosan films formulated
229 with TMEO and TPEO essential oils on lightness (L*), redness (a*) and yellowness
230 (b*) coordinates in cooked cured ham storage at 4 ºC during 21 days. For lightness (L*),
231 at the end of storage time, the type of film used had no significant ($p>0.05$) effect on
232 this coordinate except for the sample coated with CH+TPEO 1% that showed higher
233 ($p<0.05$) values. In the same way, storage time had no significant ($p>0.05$) effect in the
234 samples coated with CH, CH+TMEO and CH+TPEO, except for the sample coated with
235 CH+TPEO 1%. The absence of any modification in this parameter may have been due
236 to the protective effect of the packing film against oxygen. However, the storage time
237 had a significant ($p<0.05$) effect in the uncoated CCH sample. In these samples, L*
238 values decreased from 60.43 at the outset to 58.95 at the end of the experiment. Some
239 authors reported that L* values in meat and meat products are related to (i) surface
240 water, (ii) water vapor exchanges between the products and the environment and (iii)
241 modifications of the different states of hemopigments (Fernández-López, Pérez-Alvarez
242 & Aranda-Catalá, 2000).

243 As regards redness (a*) coordinate, as occur with lightness, at the end of storage
244 time, there no were differences ($p>0.05$) between uncoated and coated CCH samples,
245 except for CH+TPEO 1% that showed lowest ($p<0.05$) values. The storage time,
246 however, had an effect ($p<0.05$), and the redness values fell in both uncoated and coated
247 CCH samples. This coordinate is affected by the structural integrity of the food, the
248 pigment content and disposition (water or lipid-soluble) and surface water availability
249 (Fernández-López, Sayas-Barberá, Navarro, Sendra & Pérez-Álvarez, 2005). Moreover,

250 decreases in redness have been related to oxidation of lipids and hemopigments
251 (Fernandez-Lopez, Sayas-Barberá, Sendra & Pérez-Alvarez, 2006).

252 For yellowness (b*) coordinate, at day 21, there no were differences ($p>0.05$)
253 between uncoated CCH samples and samples coated with CH or CH+1%TMEO.
254 Similarly, storage time had no effect on either of these samples. On the other hand, the
255 CCH samples coated with CH+2%TMEO, CH+1%TPEO and CH+2%TPEO were
256 affected ($p<0.05$) by type of films used and storage time. The behavior of b* depends to
257 a great extent on the food matrix, and it is recognized that changes (pH, oxidation
258 extent, water activity, etc.) in the matrix have the greatest influence on this coordinate in
259 many foods (Cofrades et al., 2004).

260 **3.2. Lipid oxidation**

261 There are several scientific works that shown the beneficial effects on lipid oxidation
262 of chitosan when it is added to meat and meat products as ingredient (Georgantelis,
263 Blekas, Katikou, Ambrosiadis & Fletouris, 2007; Sayas-Barberá et al. 2011). However,
264 to our knowledge, few studies have been conducted to analysis the antioxidant effect of
265 chitosan films or chitosan films added with EO on meat and meat products is limited.

266 Figure 2 shows the effect of chitosan films formulated with *Thymus* TMEO and
267 TPEO essential oils on TBA values in cooked cured ham storage at 4 ºC during 21 days.
268 At day 0, there was no significant difference ($p>0.05$) in TBA values between uncoated
269 and coated CCH samples. On day 7, the CCH samples coated with CH+TPEO 2% and
270 CH+TMEO 2% showed the lowest ($p<0.05$) oxidation degree with TBA values of 2.79
271 and 2.67 mg MA/kg sample, respectively. The CCH sample coated with chitosan film
272 without EOs added showed a slight reduction in oxidation degree ($p<0.05$) with regards
273 to uncoated sample. The antioxidant ability of CH films is thought to be due to
274 chelation of free ion which is released from hemoproteins of meat product during

storage (Shahidi, Arachchi & Jeon, 1999). At day 14, again the CCH samples in which CH+TPEO 2% and CH+TMEO 2% was used showed the lowest TBA values ($p<0.05$) with reductions in the oxidation degree when compared with control of 26.86 and 37.17% respectively with statistically differences ($p<0.05$) between them. At the end of experiment (day 21) the CCH sample coated with CH+TPEO 2 % showed the lowest ($p<0.05$) TBA values with a reduction in the oxidation degree regarding to uncoated CCH sample of 28.37% followed by the CCH sample coated with CH+TMEO 2% ($p<0.05$) with a reduction in the oxidation degree regarding to uncoated CCH sample of 25.25%. These results were in agreement with Moradi, Tajik, Rohani and Oromiehie (2012) who reported that a cooked meat product coated with chitosan films containing *Zataria multiflora* Boiss EO had lower degrees of lipid oxidation than uncoated control sample. In the same way, Suman et al. (2010) showed that coating ground beef patties with chitosan reduced TBARS values and improved the surface red color of patties as compared to uncoated samples. Chitosan film incorporating green tea extract was analyzed as active packaging for shelf life extension of pork sausages by Siripatrawan and Noipha (2012). These authors reported that during the storage time (20 days), significantly higher TBA values were evident in control samples than those coated with chitosan film or chitosan film incorporating green tea extract.

The decrease in the lipid oxidation of samples coated with CH, CH+TPEO or CH+TMEO may be, probably, due to a sharp release of active compounds from those films during first days of storage as mentioned Moradi et al. (2012). These results showed that the incorporation of EOs in the films improved the protection of the meat samples against lipid oxidation. The antioxidant activity of EOs obtained from plants belonged to genus *Thymus* is widely demonstrated (Viuda-Martos et al., 2010a; Zouari et al., 2011). The antioxidant activity of EOs obtained from plants belonging to genus

300 *Thymus* can be credited to the presence of its major phenolic compounds, particularly
301 thymol and carvacrol, and their recognized impact on lipid oxidation. The antioxidant
302 activity of phenolic compounds is related to the hydroxyl groups linked to the aromatic
303 ring, which are capable of donating hydrogen atoms with electrons and stabilizing free
304 radicals (Dorman, Peltoketo, Hiltunen & Tikkanen, 2003; Yanishlieva, Marinova &
305 Pokorny, 2006).

306 Moreover, the storage conditions (4 ºC and protected from light) as well as the low
307 oxygen permeability characteristics of chitosan films may contributed to the inhibition
308 of lipid oxidation.

309 **3.3. Microbial counts**

310 Effect of chitosan films formulated with TMEO and TPEO essential oils on Aerobic
311 Mesophilic Bacteria (AMB) and Lactic Acid Bacteria (LAB) in cooked cured ham
312 storage at 4 ºC during 21 days are given in Figures 3 and 4. Microbial counts (aerobic
313 mesophilic bacteria and lactic acid bacteria) were ($p<0.05$) affected by storage time and
314 the application of chitosan edible films added with TMEO or TPEO. In our experiment,
315 no moulds and yeast were found in any of the treatments regardless of time of storage,
316 probably due to the aseptic slicing process, together with the presence of the sodium
317 chloride in the products (Viuda-Martos, Ruiz-Navajas, Fernandez-Lopez & Perez-
318 Alvarez, 2010b). In the same way, no enterobacteria were found. Ouattara et al. (2000)
319 reported that indigenous *Enterobacteriaceae* in meat products (bologna, beef pastrami
320 and cooked ham) were inhibited by the use of antimicrobial films, containing chitosan
321 and acetic acid.

322 As regards to aerobic mesophilic bacteria (Figure 3), the log CFU/g of the uncoated
323 and those samples coated with chitosan added with TMEO and TPEO showed no
324 significant difference ($p>0.05$) at day 0. However, on day 7, the CCH samples coated

with CH+TMEO or CH+TPEO at 2% showed the lowest counts ($p<0.05$) with 2.57 and 2.61 log cycle reduction, respectively, with regard to uncoated sample. CCH sample coated with CH showed a 0.62 log reduction relating to uncoated sample demonstrating a certain antibacterial activity of chitosan. The antimicrobial effect of chitosan is thought to be related to electrostatic interaction between a positive charge on the NH^{+3} group of glucosamine monomer in chitosan molecules and negative charge of microbial cell membrane that leads to the leakage of intracellular constituents (Dutta, Tripathi, Mehrotra & Dutta, 2009). At day 14, CCH samples coated with CH+TMEO 2%, CH+TPEO 1% and CH+TPEO 2% showed the lowest counts ($p<0.05$) with no statistically differences between them ($p>0.05$). At the end of experiment (day 21) the CCH sample coated with CH+TPEO 2 % showed the lowest counts ($p<0.05$) in aerobic mesophilic bacteria with a reduction of 0.87 log cycles regarding to uncoated CCH sample. CCH samples coated with CH+TMEO 1%, CH+TMEO 2% and CH+TPEO 1% showed a 0.34, 0.53 and 0.37 log cycle reduction, respectively, in aerobic mesophilic bacteria counts when compared to the control, with no statistically differences between them ($p>0.05$).

In the case of Lactic acid bacteria (Figure 4), as occur with mesophilic bacteria, at day 0 no statistically differences were found ($p>0.05$) between uncoated and coated CCH samples. On day 7, the CCH sample coated with CH+TPEO 2% showed the lowest ($p<0.05$) counts with 2.16 log cycle reduction with regard to uncoated CCH sample. In CCH samples coated with CH+TMEO 1%, CH+TMEO 2% and CH+TPEO 1% no significant difference between them ($p>0.05$) were found. The reduction of the counts, when compared with the uncoated CCH sample, was 1.89, 1.91 and 1.93 log cycles, respectively. The same behavior was observed at day 14. At the end of the analysis, day 21, no statistically differences ($p>0.05$) were found between uncoated

CCH sample and the coated CCH sample with chitosan. Again, the CCH samples coated with CH+TPEO 2% showed the lowest ($p<0.05$) counts for lactic acid bacteria followed by the coated CCH sample in which CH+TMEO 2% was employed, with a reduction in counts, when compared with the uncoated CCH sample of 1.14 and 0.84 log cycles. In coated CCH samples in which CH+TMEO 1% and CH+TP 1% was used no significant differences ($p>0.05$) between them were found, However, significant differences ($p<0.05$) were observed between these samples and uncoated CCH sample.

The use of chitosan edible films or chitosan edible films added with essential oils in meat, meat product and RTE meat products helps to maintain product quality, improve product safety, and increase the shelf life. The purpose of incorporating antimicrobial compounds into an edible film instead of applying them directly onto the meat surface by spraying or dipping was to extend delivery of the antimicrobials during meat storage rather than delivering them in a single massive dose (Ouattara et al., 2000). The scientific literature describes how chitosan film added or not with essential oils improves the shelf-life of RTE meat products. Therefore, Giatrakou, Ntzimani, Zwietering and Savvaidis (2010) informed that a 5-day microbiological shelf-life extension was obtained for a poultry product (ready to cook chicken-pepper kebab) treated with either thyme oil (0.2% v/w) or chitosan (1.5% w/v). The chitosan films and chitosan-oregano EO films were applied on inoculated bologna samples and stored 5 d at 10 ºC were analyzed by Zivanovic, Chi and Draughon (2005). They reported that pure chitosan films reduced *Listeria monocytogenes* by 2 log cycles, whereas the films with 1% and 2% oregano EO decreased the numbers of *L. monocytogenes* by 3.6 to 4 log cycles, respectively, and *Escherichia coli* by 3 log cycles. Siripatrawan and Noipha (2012) analyzed chitosan films incorporating green tea for improve the shelf life extension of pork sausages. These authors reported that incorporation of chitosan film

with green tea enhanced the antimicrobial properties of the film, as total aerobic counts, yeasts, and molds in the sausages wrapped with chitosan-tea film were lower than those wrapped with chitosan film. Moradi et al. (2012) studied the effectiveness of chitosan films containing *Z. multiflora* Boiss essential oil (ZEO) and grape seed extract (GSE) on microbial (lactic acid bacteria, aerobic mesophiles and inoculated *L. monocytogenes*) characteristics of *mortadella* sausage. They informed that the growth of *L. monocytogenes* was significantly inhibited by ZEO-GSE containing films especially during storage of the sausages for 6 days. Aerobic mesophiles and lactic acid bacteria were the most sensitive and resistant groups to films by 0.1-1.1 and 0.1-0.7 log cycles reduction, respectively. Zinoviadou et al. (2009) used antimicrobial films that were prepared by incorporating different levels of oregano EO into whey protein isolate films. These authors observed that Total viable count and *Pseudomonas* were significantly reduced while the growth of lactic acid bacteria was completely inhibited.

The antibacterial activity of chitosan films incorporated with EOs can be attributed to the EOs. The antibacterial activity could be due to one sole component, such as carvacrol, camphor or thymol. However, it is a more widely held point of view that the action is due to a synergistic effect between various components, whether major or minor ones (Daferera, Ziogas & Polissiou, 2003). The EOs affect microbial cells by various antimicrobial mechanisms, including attacking the phospholipid bilayer of the cell membrane, disrupting enzyme systems, compromising the genetic material of bacteria, and forming fatty acid hydroperoxidase caused by oxygenation of unsaturated fatty acids (Burt et al. 2007; Arques, Rodriguez, Nuñez & Medina, 2008).

3.4 Release of phenolic compounds from the film into cooked cured hams

Figure 5 shows the release of phenolic compounds from chitosan films formulated with TMEO and TPEO essential oils into cooked cured ham storage at 4 ºC during 21

400 days. In the first seven days of storage, a pronounced release of phenolic compounds
401 from the film to the meat product shown in all samples analyzed. This release was
402 higher in the CH films added with 2% TMEO or TPEO (7.27 and 8.34 mg GAE/g film,
403 respectively) than CH films added with 1% TMEO or TPEO (0.87 and 1.46 mg GAE/g
404 film, respectively). In this way, Contini (2013) informed that antioxidant components
405 are released from the coating films into the meat as soon as the meat comes in contact
406 with the films. From day 7 until the end of storage time (21 days) the release of
407 phenolic compounds is more gradual, where all samples, except for CH+TPEO2%, had
408 values comprised between 0.13 and 0.49 mg GAE/g film. For Cagri, Ustunol and Ryser
409 (2004) the migration of bioactive compounds from edible film is dependent on many
410 factors, including food composition, electrostatic interactions between the bioactive
411 compounds and polymer chains, ionic osmosis and structural changes induced by the
412 presence of antimicrobial and environmental conditions (pH, *aw* and storage
413 temperature)

414 **3.5 Correlation for different variables.**

415 To identify variables with a great correlation, an 8x8 matrix was constructed in
416 which the TPC, lipid oxidation (MA), pH, aerobic mesophilic bacteria (AMB), lactic
417 acid bacteria (LAB), lightness (L*), redness (a*) and yellowness (b*) were included
418 (Figure 6), where the matrix is depicted with squares for greater clarity. Each square has
419 a different color, indicating the correlation value of the two associated variables. The
420 red color indicates maximum positive correlation and the blue maximum negative
421 correlation. This figure illustrates how AMB and LAB show a greater degree of positive
422 correlation with the lipid oxidation with values of 0.93 and 0.94 respectively in the
423 different samples assayed. Lipid oxidation also shows a considerable negative
424 correlation with the other variables, such as TPC, pH and color coordinate redness with

values of -0.45, -0.44 and -0.44. These results indicated that higher values of TPC, pH and redness lower values of lipid oxidation. As regards color coordinates (L*, a* and b*) there was a lower correlation between them with values close to zero.

4. Conclusions

The results of this study clearly demonstrated that addition of *Thymus moroderi* and *Thymus piperella* essential oil in active chitosan films used as coated in cooked cured ham improve their shelf life, due to a decreased in Aerobic Mesophilic Bacteria, Lactic Acid Bacteria and lipid oxidation compared to uncoated control samples. This increase in shelf life can be attributed to the protective effects of bioactive compounds release from chitosan films into cooked cured ham.

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591 **FIGURE CAPTIONS**

592 **Figure 1.** Effect of chitosan films formulated with *Thymus moroderi* (TMEO) and
593 *Thymus piperella* (TPEO) essential oils on pH values in cooked cured ham storage at 4
594 ºC during 21 days.

595 **Figure 2.** Effect of chitosan films formulated with *Thymus moroderi* (TMEO) and
596 *Thymus piperella* (TPEO) essential oils on TBA values in cooked cured ham storage at
597 4 ºC during 21 days.

598 **Figure 3.** Effect of chitosan films formulated with *Thymus moroderi* (TMEO) and
599 *Thymus piperella* (TPEO) essential oils on Aerobic Mesophilic Bacteria (AMB) in
600 cooked cured ham storage at 4 ºC during 21 days.

601 **Figure 4.** Effect of chitosan films formulated with *Thymus moroderi* (TMEO) and
602 *Thymus piperella* (TPEO) essential oils on Lactic Acid Bacteria (LAB) in cooked cured
603 ham storage at 4 ºC during 21 days.

604 **Figure 5.** Release of phenolic compounds from chitosan films formulated with *Thymus*
605 *moroderi* (TMEO) and *Thymus piperella* (TPEO) essential oils into cooked cured ham
606 storage at 4 ºC during 21 days.

607 **Figure 6.** Correlation matrix for different variables.

Highlights

- Chitosan films incorporated with *Thymus moroderi* or *Thymus piperella* EOs were analyzed.
- These films decrease aerobic and lactic acid bacteria counts in cooked cured ham (CCH).
- Lipid oxidation degree decrease in CCH samples where these films were used.
- Application of these films extend the shelf life of CCH

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Figure 1.

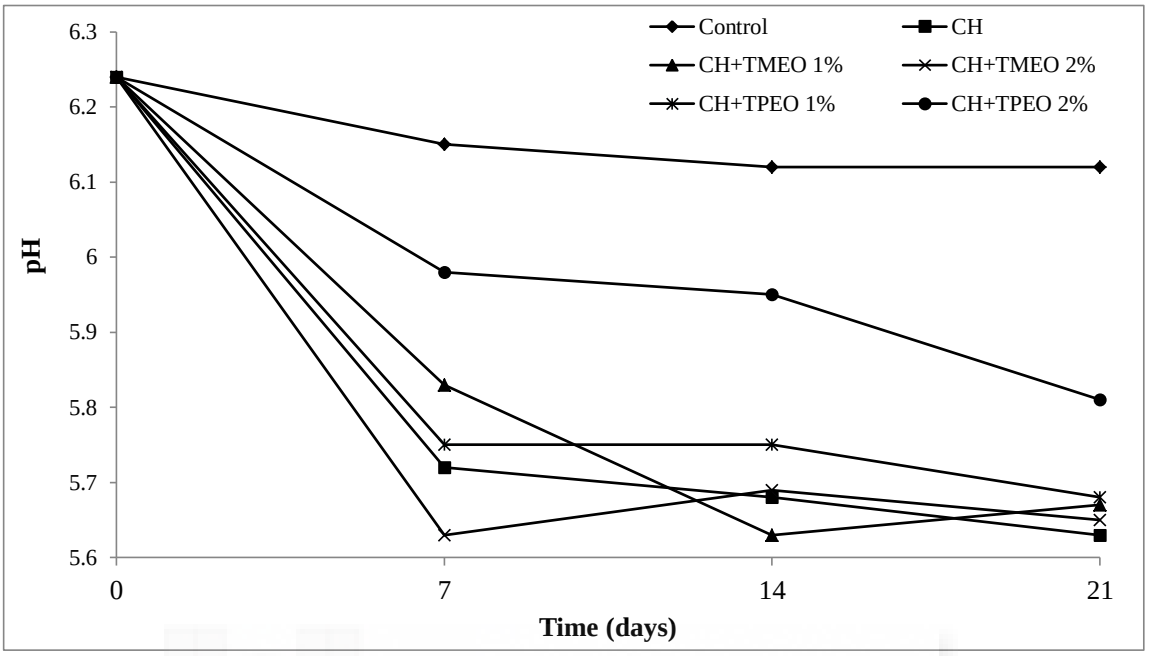

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Figure 2

Figure 2. Effect of chitosan films formulated with *Thymus moroderi* (TMEO) and *Thymus piperella* (TPEO) essential oils on TBA values in cooked cured ham storage at 4 ◦C during 21 days.

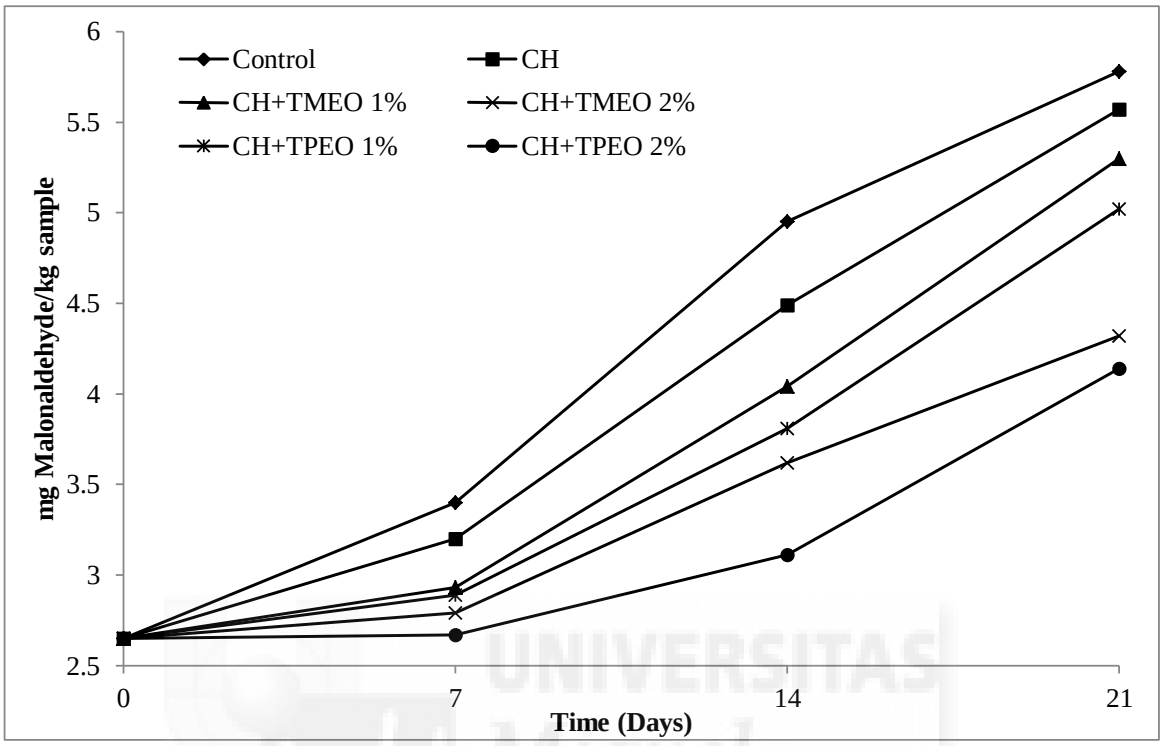

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Figure 3.

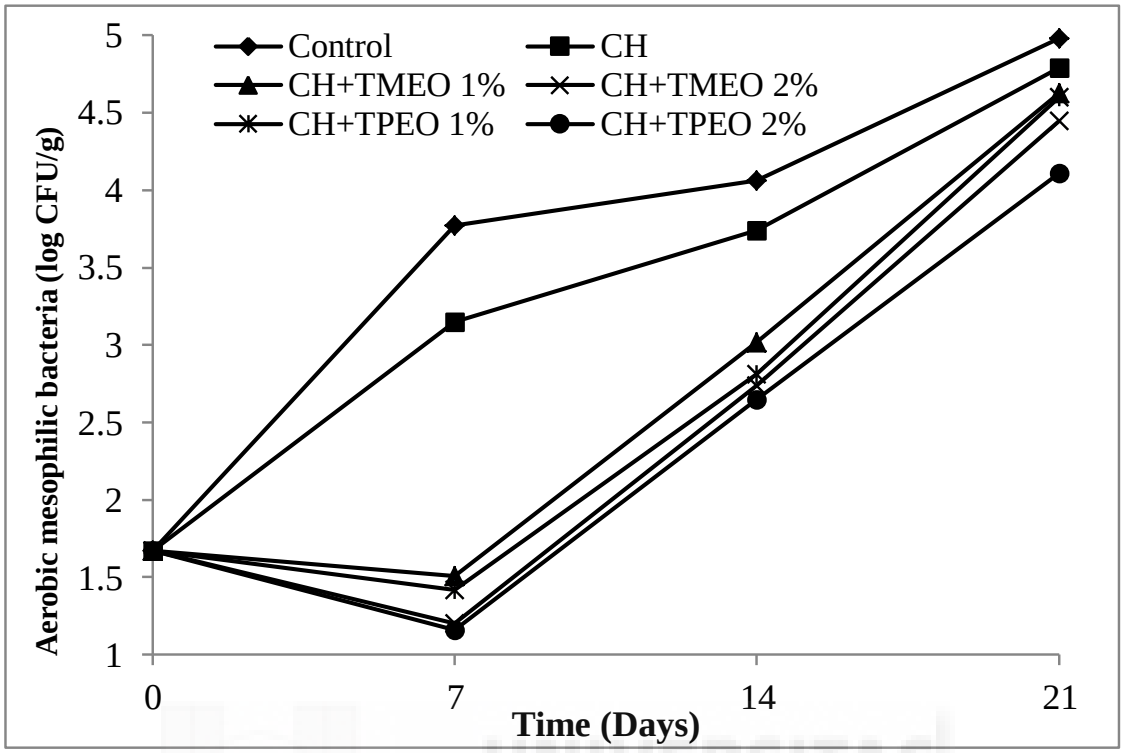

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Figure 4.

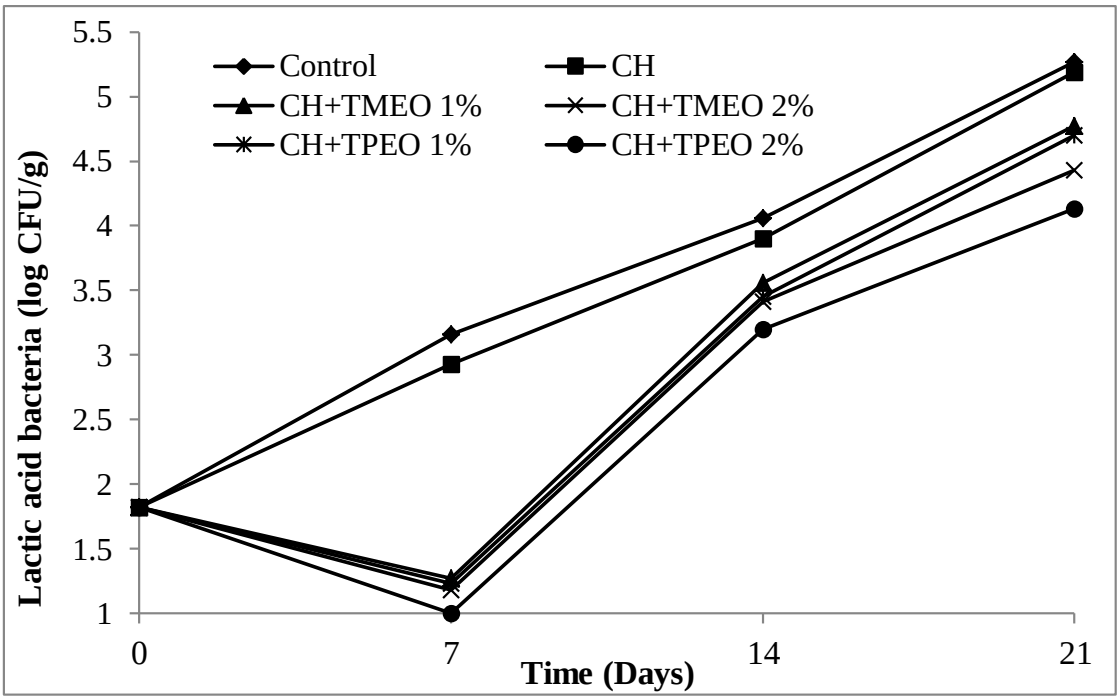

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Figure 5.

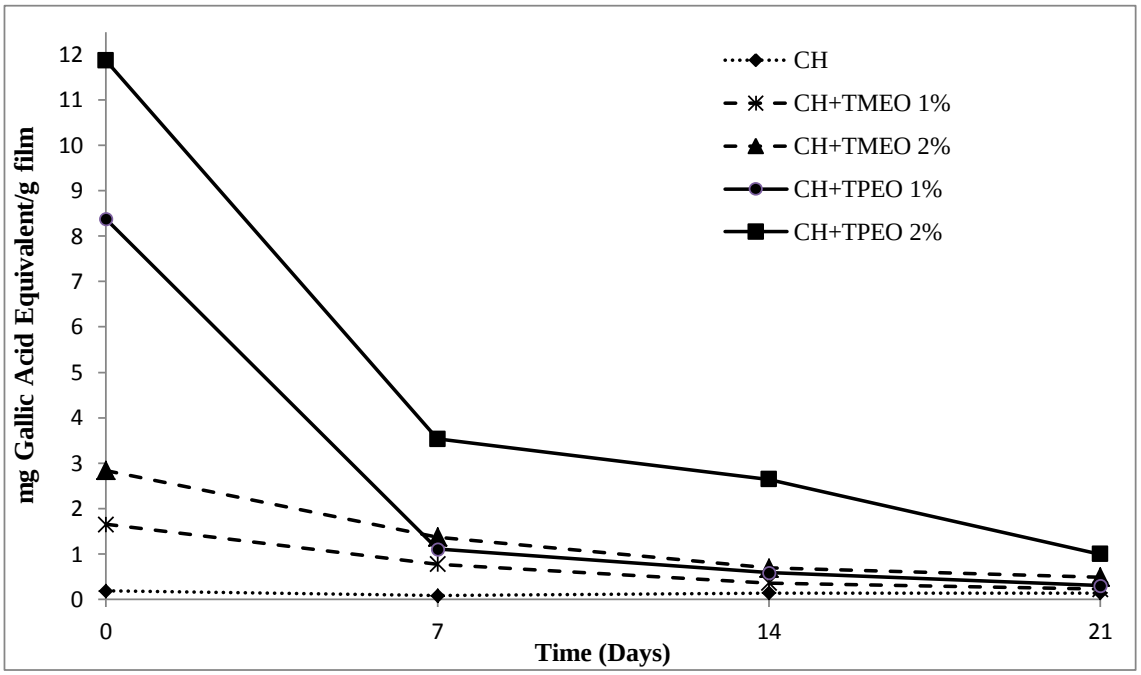

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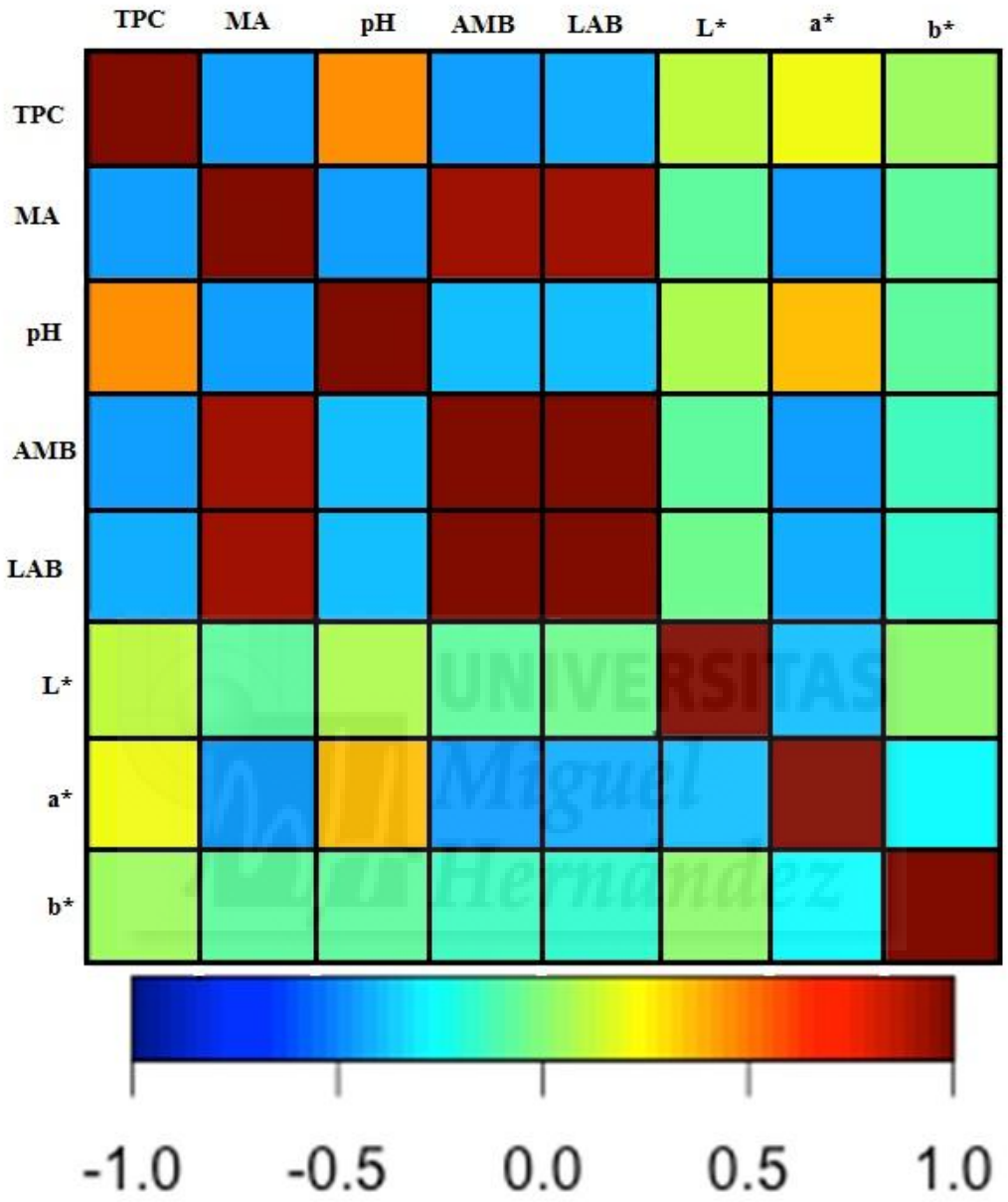
Figure 6.



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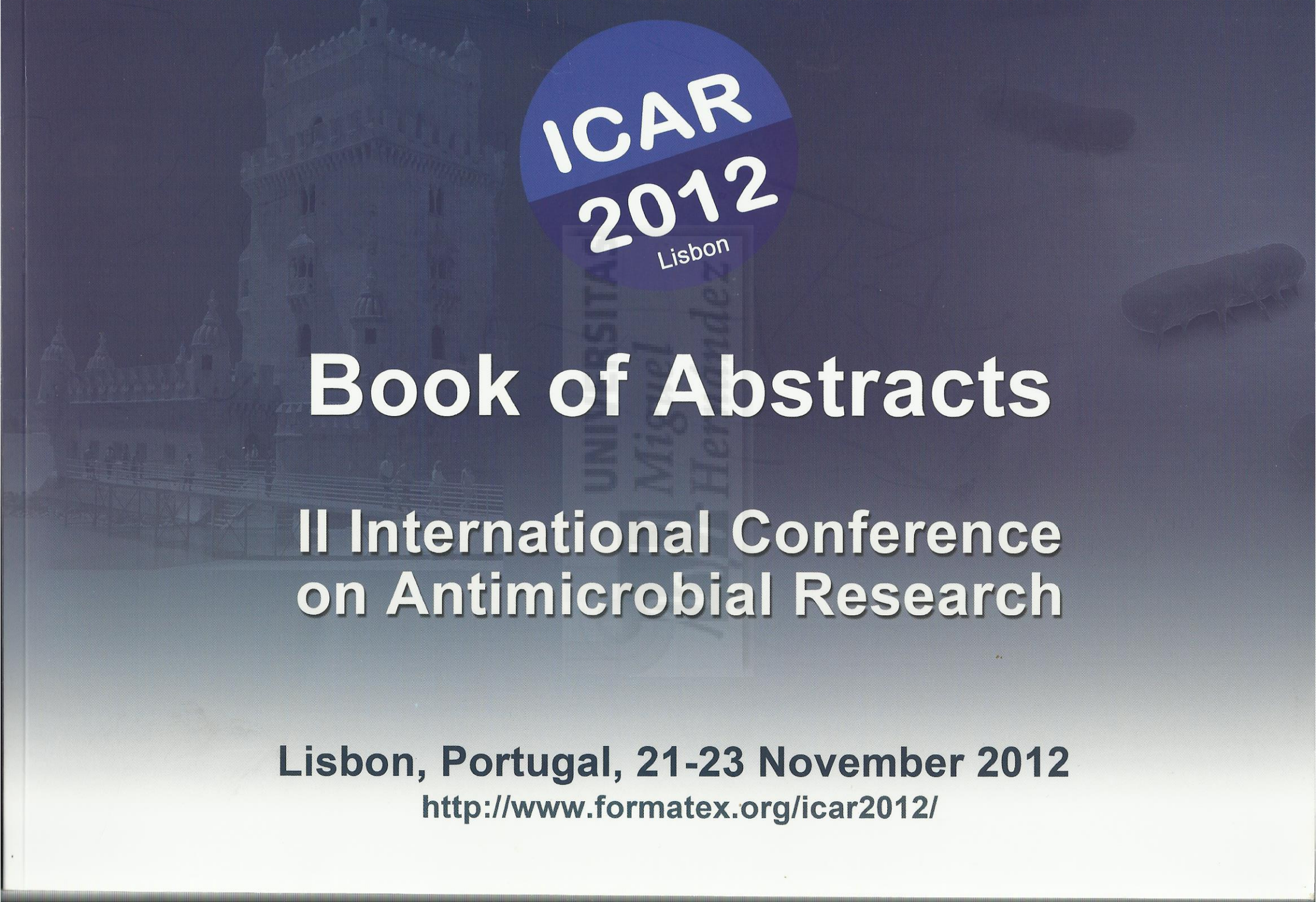

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