Current Research in Food Science 5 (2022) 629–633

Contents lists available at ScienceDirect

Current Research in Food Science

journal homepage: www.sciencedirect.com/journal/current-research-in-food-science

Fungal inactivation on Mexican corn tortillas by means of thyme essential oil in vapor-phase

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ARTICLE INFO

Keywords:
Antifungal activity
Essential oil
Tortilla

ABSTRACT

Antifungal activity of thyme (*Thymus vulgaris*) essential oil (EO) in vapor-phase was tested against representative fungi in corn tortillas. The chemical composition of studied EO was analyzed by gas chromatography-mass spectroscopy, and its major components were linalool, thymol, and *p*-cymene. The antifungal activity was evaluated by determining the growth of *Aspergillus niger* or *Penicillium expansum* after exposure to EO vapors. The *in vitro* minimum inhibitory concentration (MIC) of the EO was determined by the inverted lid technique, while *in situ* MIC was determined on the corn tortillas inside an airtight container. The MICs obtained ranged from 160 to 200 μL of thyme EO/$L_{of\ air}$ for *in vitro* conditions and 550–850 μL of the EO/$L_{of\ air}$ in corn tortillas. The modified Gompertz model adequately described *in vitro* mold growth curves. Thyme EO was effective in preventing or significantly delaying growth of the contaminating molds on corn tortillas.

1. Introduction

Corn or maize (*Zea mays* L.) is one of the most widely cultivated cereals worldwide, with an extensive use for human and animal consumption, as well as in several industries. It can be used for various purposes, directly as an ingredient or transformed into different products, such as flours, flakes, and tortillas, among many others [Chavarri et al., 2014]. In some Latin-American countries, tortillas alone can provide up to 70% of total daily caloric intake and up to 50% of total daily protein, calcium, iron, and zinc intake [Rosentrater, 2006]. Corn tortillas are traditionally produced as follows: Corn is cooked and steeped in alkali solution, washed to remove excess alkali, and ground on a stone mill into *masa* (traditional *nixtamal*), which is then pressed into flat, circular tortillas. Commercial tortilla production demands optimum cooking of corn to produce a *masa* (dough) of proper consistency and repeatable quality [Khan et al., 1982; Vittadini et al., 2004]. Fresh corn tortillas become stale after only a few hours and are subjected to mold, yeast, and bacterial growth due to their high-water activity and moisture content [Clubbs et al., 2005]. Traditional foods made with tortillas include *enchiladas*, *quesadillas*, and *tacos*, among many other conventional dishes; so preserving them in good microbial quality is a challenge for the industry [Rosentrater, 2006]. Many industrially commercialized corn tortillas use several additives, including antimicrobials, to extend product shelf life.

To replace synthetic preservatives with natural additives, essential Oils (EOs) from several plants and species are considered as promising sources of food antimicrobials [Burt, 2004; Bendahou et al., 2008]. Currently, different EOs such as cinnamon, oregano, orange, clove, garlic, and basil have demonstrated to have antimicrobial effects against yeasts, molds, and bacteria [Adam et al., 2009]. However, their natural origin does not necessarily imply that their use are safe; to ensure this it is necessary to check their efficacy, safety, toxicity, and mechanism of action. Outcomes of these strict quality controls have resulted in distinguishing them as Generally Recognized as Safe (GRAS) by the US FDA [Yun et al., 2013]; thus, allowing their use in food products as additives. However, sometimes it is necessary to use them in higher concentrations to achieve the same effect as other commercial additives, which could cause undesirable effects on the product or even toxicity problems [Pateiro et al., 2021]. This makes it necessary to consider their use in combination with other preservatives or using different application techniques such as the vapor-phase tested.

According to Reyes-Jurado et al., [2020], the vapor phase of EOs has a particular impact against molds due to their superficial growth, which makes them more susceptible to EO volatile compounds. EO vapors

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https://doi.org/10.1016/j.crfs.2022.03.010
Received 30 December 2021; Received in revised form 11 March 2022; Accepted 19 March 2022
Available online 23 March 2022

affect molds at different stages of their life cycle, such as germination, hyphae growth, and sporulation. Inactivation of airborne conidia by volatile compounds of EOs in the vapor phase is a key part of the process in fungal inhibition since airborne conidia are generally difficult to inactivate, being stable when subjected to heat, light, and selected chemical compounds.

Thyme (*Thymus vulgaris*) is an important aromatic plant, its extracts and essential oil are utilized for flavoring sauces, meats, and beverages, while the volatile essential oil is applied for scenting perfumes and cosmetics [Baranauskiené et al., 2003; De Carvalho et al., 2015]. According to Guarda et al. (2011), thymol and carvacrol, the major components in thyme EO, are phenolic compounds responsible of its antimicrobial effects; these components are hydrophobic and thus likely to dissolve in the hydrophobic section of the cytoplasmic membrane of bacterial cells, between the lipidic acyl chains.

Furthermore, the modified Gompertz model is utilized to describe fungal growth parameters, including maximum colony diameter, maximum growth rate, and lag periods under the various plant-derived essential oil treatments, and has been demonstrated that the growth of tested fungi could be adequately estimated by this model [Hossain et al., 2016].

The purpose of this study was to evaluate the response of *Aspergillus niger* and *Penicillium expansum* when exposed to the vapor phase of thyme essential oil in corn tortillas.

2. Materials and methods

2.1. Essential oil

Thyme (CAS 8007-46-3) essential oil was purchased from Hersol Lab (Mexico state). The essential oil was kept in the dark stoppered flasks and under refrigeration conditions until utilized.

2.2. Chemical analysis

The EO was analyzed by gas chromatography coupled to mass spectrometry (GC–MS). Using a Network GC System (6850, Agilent Technologies, Santa Clara, CA) gas chromatograph coupled to a mass selective detector (5975C VL, Agilent Technologies) with triple-axis, and a split-splitless injector (1:10 split ratio). A fused silica capillary column (30 m by 0.250 mm; film thickness, 0.25 μm) HP-5MS (5% phenyl–95% polydimethylsiloxane) was utilized. The carrier gas was helium, at a flow rate of 1.1 mL/min. Samples were prepared by diluting the essential oil at 5:100 (v/v) in ethanol, and the injection volume was 1 μL. The column oven temperature was programmed from 60 °C (4 min) to 240 °C (10 min) at 4 °C/min. Injector and detector temperatures were set at 250 and 280 °C, respectively [Gómez-Sánchez et al., 2011]. The obtained spectra were compared with the respective mass spectra of pure compounds and the mass profile of the same compounds available from the US National Institute of Standard Technology (NIST) library [Ávila-Sosa et al., 2012].

2.3. Determination of water activity and moisture content of corn tortillas

Different corn tortilla samples were analyzed according to their composition using the following methods: moisture content was determined according to the method 44-15 (AACC, 2000), where 2 g of sample were placed in an oven at 105 °C for 12 h; water activity was determined using a dewpoint hygrometer (AquaLab TDL2, Decagon Devices, Pullman WA), previously calibrated with distilled water; every determination was performed by triplicate.

2.4. Isolation and identification of spoilage molds

Corn tortillas acquired from a local tortilla- shop (Puebla, Mexico) were kept in plastic bags at 25 °C for one week; once molds appeared on the surface, samples were plated on potato-dextrose agar (PDA, Merck, Merck-Mexico) and this was repeated until isolated colonies were obtained. Molds were plated on Czapek, Malt-Extract, and PDA agars (Merck, Merck-Mexico) and incubated at 25 °C for 5–7 days. Molds were identified based on their macro and microscopic characteristics after sub-culturing on each agar [Samson et al. 1981]. One strain of *P. expansum* and one strain of *A. niger* were identified. Molds species were cultured on PDA slants until sporulation at 25 °C. The spores were removed by washing the surface of the slants with 10 mL of sterile distilled water. Spore suspensions were adjusted to contain 1×10^6 spores/ml and utilized immediately for the antifungal assays; the total number of spores was counted using a hematocytometer under a light microscope. [Gómez-Sánchez et al., 2011; Chen et al., 2020]. The strains were continuously transferred to new PDA slants to maintain their viability and then kept refrigerated at 4 °C [López-Malo et al., 2006].

2.5. Antifungal activity testing by inverted lid technique

For each studied mold, Petri dishes containing solidified PDA were inoculated in the center with 5 μL of spore suspension (10^6 spores/ml). A selected concentration of thyme EO (0, 20, 40, 60, 80, 100, 120, 140, 160, 180, or 200 μL of EO/$L_{of\ air}$) was poured on a Whatman filter paper and placed on the lid of the plates; then, the plates were immediately inverted and sealed with Parafilm® and incubated at 25 °C for 20 days [Suhr and Nielsen, 2003; Kloucek et al., 2012]. For each colony, the mean diameter was calculated by measuring both directions at right angles to each other with a digital caliper every day during 16 days of incubation at 25 °C; growth controls were prepared in parallel to assure that viable organisms were present. The colony diameter (in mm) was plotted versus the time (days) needed to reach the end of the Petri dish, which varied depending on the strain as well as on the conditions tested. The minimum inhibitory concentration (MIC) was defined as the lowest tested concentration of the EO that inhibited fungal growth (i.e. prevented any increase in colony diameter) for 20 days. Each experiment was performed in triplicate.

2.6. Antifungal activity by vapor contact in tortillas

The activity of the vapor phase of thyme EO on corn tortillas was tested by a modified micro-atmosphere method in airtight chambers; 5 μL of spore suspension (10^6 spores/ml) from *A. niger* or *P. expansum* were inoculated in a 12 mm diameter corn tortilla and placed in a 1.7 L airtight chambers. The selected concentration of the thyme essential oil (0, 50, 100, 150, 200, 250, 300, 350, 400, 500, 600, 700, 800, or 850 EO/$L_{of\ air}$) was poured in a glass plate (5 cm of diameter) that was placed in the airtight chamber separated from the corn tortilla; also 10 mL of a sodium chloride solution (30% w/v) was placed in the airtight box to maintain the water activity (0.97) of tortillas [Segvić Klarić et al., 2007]. The airtight boxes were incubated at 25 °C for 20 days, and the MIC of the essential oil that did not allow fungal growth for 20 days was recorded. The MIC values were expressed as μL of EO/·L of air. Each experiment was performed in triplicate.

2.7. Modeling growth kinetics

Mold growth curves were generated from experimental data and fitted using the modified Gompertz equation since nonlinear behavior was observed for most tested cases; growth parameters were obtained using nonlinear regression. Tested modified Gompertz equation is the following:

$$D_t = A\ exp\left[- exp\left[\left(\frac{Vm^*e}{A} \right)(\lambda - t) + 1 \right] \right] \quad \text{(Eq. 1)}$$

where D_t (mm) is the average colony diameter at time t (h), A is the maximum mold growth achieved during the stationary phase, V_m is the

maximum specific growth rate (1/h), λ is the lag phase (h), and *e* is Euler's number.

3. Results and discussion

3.1. Moisture content and water activity of corn tortillas

Water content in food and grains is one of the most important criteria for the preservation of their quality and its commercialization [Vittadini et al., 2004]. The moisture content of studied corn tortillas was 42.43 ± 0.18% while their water activity was 0.973 ± 0.004; thus, corroborating that a relatively high moisture content is correlated with tortillas of good-quality textural characteristics [Vázquez-Carrillo et al., 2011; Gaytán-Martinez et al., 2011].

3.2. Composition of essential oil

The chemical composition of tested thyme EO is depicted in Table 1; a total of 15 compounds were identified by CG-MS in this EO, being *p*-cymene (19.80%), linalool (14.61%), and thymol (12.13%) its major components. The presence of other compounds with antimicrobial activities (e.g. carvacrol) were also detected but in lower proportions. According to Baranauskiené et al. [2003], phenolic compounds are major constituents and the aroma principles of thyme EO; however, in this study monoterpenic alcohols such as linalool and *p*-cymene were found in great abundance in the tested essential oil. Although the percentage obtained of chemical compounds detected differs from other reports [Consentino et al., 1999; Baydar et al., 2004; Imelouane et al., 2009], several components detected in tested thyme EO such as linalool, thymol, carvacrol, α-pinene, and ρ-cymene agree with previous reports.

Because of their great number of constituents, EOs seem to have no specific cellular targets; as lipophiles, they pass through the cell wall to the cytoplasmic membrane, disrupting the structure of their different layers of polysaccharides, fatty acids, and phospholipids and permeabilize them; EOs cytotoxicity appears to include such membrane damage [Bakkali et al., 2008]. Therefore, the chemical composition determines the antimicrobial activity of EOs, and major components are primarily responsible for its activity, so if there is an increased presence of certain components in a particular EO, it will possess better antimicrobial activity [Hyldgaard et al., 2012]. This was confirmed by Segvić Klarić et al. (2007), whom evaluated the antifungal effect in the vapor phase of thyme EO and pure thymol against different fungi; in that study, they reported that pure thymol produced approximately three-times stronger inhibition than the EO of thyme.

Table 1
Detected compounds and relative percentage composition of studied thyme (*Thymus vulgaris*) essential oil.

Detected compound	%	Retention time (min)
p-cymene	19.80	17.37
Linalool	14.61	20.23
Thymol	12.13	27.87
ϒ- terpinene	9.86	17.81
α- pinene	7.55	10.23
Carvacrol	7.11	29.32
Isoborneol	6.72	24.14
Caryophyllene	6.23	30.12
Borneol	3.81	24.3
Camphene	3.49	11.2
Terpineol	2.40	26.19
β- myrcene	2.15	14.04
α-terpineol	2.81	26.19
β-pinene	0.96	12.97
Caryophyllene oxide	0.37	34.67

3.3. Screening of thyme essential oil in vapor phase for antifungal activity

According to Table 2, thyme EO exhibited an antifungal effect against the studied molds with MICs from 160 to 850 μL of EO ·/L of air. Lower MICs were obtained *in vitro* (inverted lid technique) in comparison to *in situ* tests (in corn tortillas); *P. expansum* was more sensitive in both conditions than *A. niger* to EO. The growth of *A. niger* was also inhibited with 200 μL of EO ·/L of air and 850 μL of EO ·/$L_{of\ air}$ for *in vitro* and *in situ*, respectively.

In the growth curves for both studied molds can be observed that there is an increased lag in radial growth initiation as the concentration of thyme EO increases (Figs. 1 and 2). For *A. niger*, thyme EO (160–200 μL/$L_{of\ air}$) caused inhibition only at higher concentrations (Fig. 1). Concentrations <160 μL/L exhibited only a small delay in the lag phase. *A. niger* reached a maximum radial growth of 48 mm, *P. expansum* (Fig. 2) displayed a lower maximum growth and reduced specific growth rates, as well as an increase in its lag time when exposed to increasing amounts of thyme EO.

Our results agree with other studies that emphasize the toxicity of thyme EO against selected fungal species; Segvić Klarić et al. (2007) evaluated the antifungal effect in the vapor phase of thyme EO against different species of *Aspergillus*; reporting MICs below 20 μg of EO/$L_{of\ air}$. Omidbeygi et al. [2007] determined the antifungal activity of thyme EO against *Aspergillus flavus* in liquid medium and tomato paste; their results indicated a strong antifungal effect, observing complete inhibition of *A. flavus* at 350 ppm of thyme EO *in vitro* and above 500 ppm of the EO in the tomato paste. Viuda-Martos et al. [2007] confirmed the antifungal potential of thyme EO against *A. niger* and *A. flavus* using the agar dilution method, obtaining MICs of 8 μL. Edris and Farrag [2003] stated that EOs affect the three stages of the life cycle of filamentous fungi. Likewise, Mani-Lopez et al., (2021) reported that EOs affect fungi via inhibition of sporulation or producing cell damage, which leads to irreversible coagulation or denaturation of the cellular components. The EOs are capable of penetrating and disrupting the fungal cell wall and cytoplasmic membranes via a permeabilization process that can reach the mitochondrial membranes. Changes in the fluidity of the plasma membrane may cause the electrolytes or cellular contents to leak, altering protein metabolism and calcium ion concentration.

It is challenging to compare published results since there are many different methods utilized to evaluate the antimicrobial activity of EOs, making the reported results not always directly comparable [Hammer et al., 1998; Hammer et al., 1999]; our results demonstrated that tested thyme EO, which is rich in monoterpenic alcohols such as linalool and *p*-cymene and phenolic compounds such as thymol and carvacrol was very effective against tested molds as expected following Sivropoulou et al. [1996] and Baydar et al. [2004] for an EO with that composition.

3.4. Growth modeling

The Gompertz model is one of the most applied to describe microbial growth under controlled conditions [Peleg, 1997]. Every studied mold growth curve was suitably fitted by tested modified Gompertz model.

Table 2
Antifungal activity of vapor phase *Thymus vulgaris* essential oil assessed *in vitro* and *in situ* against studied molds.

Mold	MIC of Thyme Essential Oil (μL of EO/$L_{of\ air}$)[a]			
	In vitro conditions	Re-growth[b]	Corn tortilla	Re-growth[b]
Aspergillus niger	200	ND	850	ND
Penicillium expansum	160	ND	400	ND

[a] MIC: minimum inhibitory concentration.
[b] ND: no re-growth was detected after 20 days of incubation in an essential oil-free atmosphere.

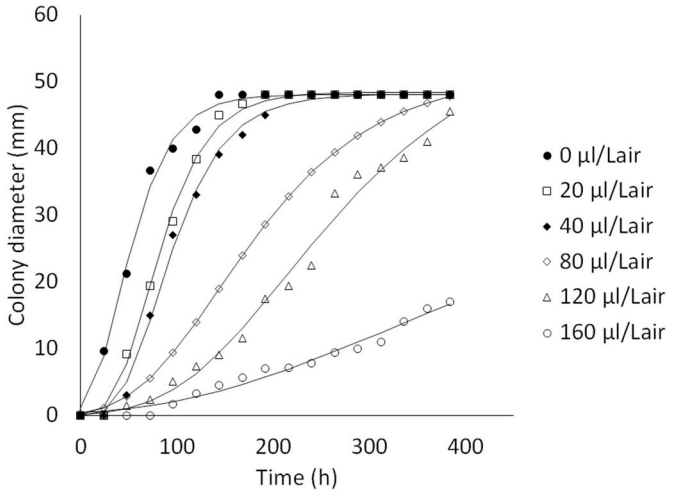

Fig. 1. Effect of *Thymus vulgaris* essential oil concentration (µL of EO/$L_{of\ air}$) in vapor phase on the growth of *Aspergillus* niger. Symbols are experimental data, lines are tested model fits assessed by the inverted lid technique.

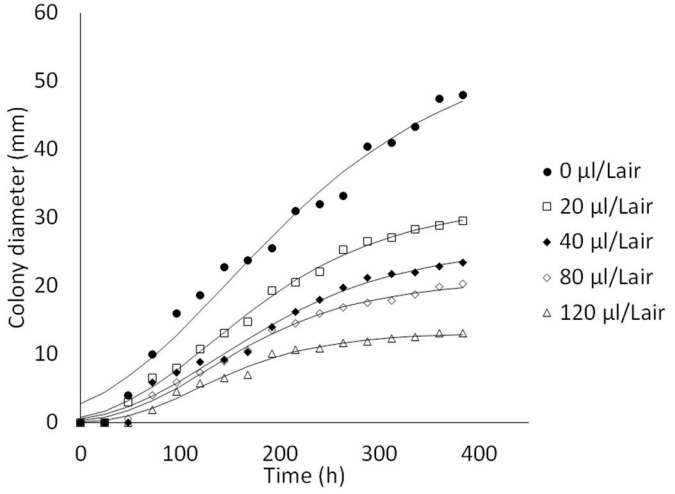

Fig. 2. Effect of *Thymus vulgaris* essential oil concentration (µL of EO/$L_{of\ air}$) in vapor phase on the growth of *Penicillium.* Symbols are experimental data; lines are tested model fits assessed by the inverted lid technique.

The effects of thyme EO in vapor phase on Gompertz parameters are presented in Table 3 where the growth parameter (*A*) depends on the maximum growth diameter, which in this study is the Petri dish diameter (48 mm); while the lag time (λ) was the most affected by the tested thyme EO concentrations.

Figs. 1 and 2 exhibit the modified Gompertz model fits the experimental data for *A. niger* and *P. expansum*, respectively. The tested model is useful to compare antifungal effects among the studied concentrations; the effect of an increasing concentration of thyme EO in the vapor phase for each mold shows a decrease in growth rates while increasing lag times. The biological parameters *A*, *Vm*, and λ obtained by the tested modified Gompertz model (Table 3) corroborate these tendencies for both studied molds; i.e., their lag times are higher when exposed to higher concentrations of thyme EO. Gómez-Sánchez et al. [2011] mentioned that the effect of EO concentration on mold lag time can be explained by the action of the essential oil at the germination phase of mold conidia. Modeling the behavior of microorganisms in food systems enables us to choose selected application techniques, in order to ensure quality and microbiological safety aspects of food products. The Gompertz model helps to describe and predict growth while estimating biological parameters of food-borne microorganisms [Jafari et al., 2021].

Table 3
Parameters of tested modified Gompertz model that describe studied mold growth curves subjected to selected concentrations of *Thymus vulgaris* essential oil in vapor phase.

Mold	Thyme Essential Oil Concentration (µL of EO/$L_{of\ air}$)	Parameters		
		λ (h)	V_m (h^{-1})	*A*
Aspergillus niger	0	9.9	0.5931	48.1
	20	34.6	0.5201	48.4
	40	41.2	0.4595	48.1
	80	53.9	0.2100	51.2
	120	95.7	0.1768	57.2
	160	104.2	0.0596	34.5
	200	*	*	*
Penicillium expansum	0	14.5	0.1538	55.9
	20	32.1	0.1181	32.1
	40	35.7	0.0925	25.9
	80	39.8	0.0882	20.7
	120	45.3	0.0690	13.2
	160	*	*	*

A: maximum mold growth in the stationary phase.
V_m: maximum specific growth rate.
λ: lag phase.
*: no growth was observed.

4. Conclusions

Thyme essential oil can be utilized in the vapor phase to control the growth of *A. niger* and *P. expansum* in a closed system, having a great impact on their lag phases. Tested modified Gompertz model adequately described experimental data. Results demonstrate the possibility of protecting corn tortillas against mold spoilage by applying the vapors of thyme essential oil. Thus, it is strongly recommended that the application of EO in the vapor phase be considered by the corn tortilla industry as it can effectively reduce mold contamination which is very common and prevalent. Future work in this regard should focus on sensory evaluation (odor, flavor, and texture) when applying thyme EO on tortillas.

CRediT authorship contribution statement

Fatima Reyes-Jurado: Methodology, Experimentation, Writing – original draft. **Zyanya Bárcena-Massberg:** Methodology, Experimentation. **Nelly Ramírez-Corona:** Statistical Formal analysis, Writing – review & editing. **Aurelio López-Malo:** Conceptualization, Formal analysis, Writing – review & editing. **Enrique Palou:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Financial support for the project [grant CB-2016-01-283636] from the National Council for Science and Technology (CONACyT) of Mexico and Universidad de las Américas Puebla (UDLAP) is gratefully recognized. Author Reyes-Jurado acknowledges financial support for her Ph. D. studies in Food Science from CONACyT and UDLAP.

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