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Antifungal effect of clove essential oil and its main components on fungi isolated from corn tortillas

Ana Patricia Ibarra-Valenzuela, Rosalba Troncoso-Rojas, Alma Rosa Islas-Rubio, Elizabeth Peralta, Herlinda Soto-Valdez*, Coordinación de Tecnología de Alimentos de Origen Vegetal, Centro de Investigación en Alimentación y Desarrollo, A.C. Carretera Gustavo Enrique Astiazarán Rosas, No. 46. Col. La Victoria, CP. 83304, Hermosillo, Sonora, México; **Hayati Samsudin,** Food Technology Division, School of Industrial Technology, Universiti Sains Malaysia 11800 USM, Pulau Pinang, Malaysia.

Corresponding Author:
Herlinda Soto-Valdez
hsoto@ciad.mx

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ABSTRACT

Objective/Background. Corn tortillas are a staple food in México that have a shelf life of 1-2 days at 25 °C due to fungal growth. A natural alternative for controlling fungal growth is clove essential oil (AEC) and its major components: eugenol (E), isoeugenol (I), and eugenyl acetate (AE). Objective: to evaluate the antifungal effect of AEC on the identified fungi present in corn tortillas.

Materials and Methods. One kg samples of corn tortillas were obtained from the capitals of five states of Mexico (Sonora, Nuevo León, Michoacán, Oaxaca and Yucatán). Fungi were identified by their morphology and molecular biology. Moreover, the minimum inhibitory concentration (MIC) against AEC was determined. The effect of E, I, and AE on *Aspergillus niger* (previously identified) was evaluated with the Gompertz model.

Results. Two fungi were isolated from corn tortillas purchased in Nuevo León, Sonora, Yucatán, and Michoacán, and one fungus from those purchased in Oaxaca. The following fungi were identified by molecular biology in corn tortillas: *Aspergillus longivesica* and *Curvularia spicifera* from Nuevo León; *Aspergillus niger* and *Penicillium brevicompactum* from Sonora; *Aspergillus* sp. from Oaxaca; *Mucor* sp. and *Aspergillus flavus* from Yucatán; *Penicillium herquei* and *Curvularia racemosus* from Michoacán. The MICs were 200, 400, 800, 400, 800, 400, 800, 800, and 400 µg mL^{-1}, respectively. AEC, E, and I at a concentration of 800 µg mL^{-1} delayed the growth exponential phase of *Aspergillus niger*, while AE did not show any effect.

Conclusion. AEC could be a natural alternative for prolonging the corn tortillas' shelf life.

Key words. Eugenol, isoeugenol, eugenyl acetate, *Aspergillus niger*.

Introduction

Mexico belongs to the American continent and is located between the latitudes 14° 32' 27" North and 32° 43' 06" South, hence the arid climates in the north of the country and humid ones in the south (Hernández Cerda *et al*., 2018). Along the Mexican territory are 32 federative entities, each one with its diverse characteristics and traditions, but which share an essential common food: the corn tortilla. This food is also widely consumed in the United States of America and Central American countries (Rooney and Serna-Saldivar, 2016). The corn tortilla is considered an ethnic food; its origin dates back to the 5th century and to this day it is highly consumed in Mexico. Its nutritional value and versatility for the preparation of various dishes makes it a fundamental component of the Mexican diet (Serna-Saldivar, 2015). The annual consumption per capita of this food fluctuates between 75 and 120 kg (CONEVAL, 2012).

Corn tortillas are packaged in 1 to 2 kg portions in newsprint paper, kraft paper or high-density polyethylene bags (HDPE). Once the tortillas reach the households, they are stored in their original package and are often placed in tortilla holders (made of plastic, polystyrene or palm leaves) (Secretaría de Salud de Mexico, 2002). Some factors that contribute to the consumption of this food are custom, the cultural tradition, the income and also for the qualities of the tortilla itself, that is, its flavor, its texture and its aroma. It has been estimated that every Mexican eats an average of 67.5 kg of corn tortillas. Supposing that the total of inhabitants over 15 years of age in Mexico consume this amount every year, there would be a production of nearly seven million t of tortillas every year (CONEVAL, 2012; INEGI, 2020).

Corn tortillas have a percentage of humidity of 45-50% and an a_w of 0.98, therefore its composition and packaging type make the latter the adequate medium for the proliferation of microorganisms that limit their shelf life (Heredia-Sandoval *et al*., 2021). There are scarce reports that show specific fungal genera and species responsible for limiting the shelf life of corn tortillas. However, the main fungal genera that are known to proliferate in corn grains belong to *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp. (Martínez Padrón *et al*., 2013; Wall-Martínez *et al*., 2019). The development of different fungal genera in corn is directly related to the environmental characteristics (temperature and relative humidity of the

surroundings), where the grain is planted, harvested and stored (Wall-Martínez *et al*., 2019). Additionally, fungal development also relates to hygiene, i.e., the presence of fungal spores found around or inside tortilla stores and the type of package used for the corn tortillas. To reduce fungal development, several artificial conservatives have been added such as sodium propionate and potassium sorbate (Flores-Farías *et al*., 2002). The incorporation of artificial preservatives not only extends the shelf life of tortillas, but also modifies their characteristics, since the addition of sorbates or propionates (depending on the concentration) makes the color of the tortillas become more whitish, or it could also modify their taste and color (Báez-Aguilar *et al*., 2022). In Mexico, the NOM-187-SSA1/SCFI-2002 indicates the maximum potassium sorbate permitted in corn or wheat flour tortillas in Mexico, which is 2,000 mg kg^{-1} (Secretaría de Salud de México, 2002). Despite this, in 2002 it was reported that the concentration of additives in commercial corn flour was as high as 4,000 mg kg^{-1} of potassium sorbate, and if this is multiplied by the amount of corn tortillas eaten in one year, the consumption of this food may have adverse health effects (Flores-Farías *et al.*, 2002).

Nowadays, consumers request minimally processed foods, with natural ingredients. For this reason, the possibility of adding natural additives directly on foods is being studied. López Ortiz (2016) evaluated the addition of orange blossom essential oil (AEA), orange essential oil (AEN) and turmeric at 1% on the shelf life of corn tortillas stored at 5 °C. The addition of AEA and AEN extended the shelf life of corn tortillas by 11 and 2 days, respectively, in comparison with the corn tortillas without any additives (15 days).

Another way of adding these natural compounds is directly in the package. In this way, the effect of two antimicrobial packages with added AEA on the shelf life of corn tortillas was evaluated. The result of the study was that both packages extended the shelf life of the product by 8 and 10 days, respectively, in comparison with the control (15 days) (Ibarra-Valenzuela, 2019). Another example is clove essential oil (AEC), which includes eugenol (E), isoeugenol (I) and eugenyl acetate (AE), in a greater proportion, in its composition. These compounds have been proven to have antifungal properties against fungal genera that develop in corn (Abbaszadeh *et al.*, 2014).

In order to evaluate the effect of antimicrobial agents added directly to the corn tortillas or the packaging material, shelf-life studies are carried out in which the growth of microorganisms that develop in this food are followed up. There are diverse microbial growth models whose assumptions are growth up to a maximum point, where the growth speed decreases and the subsequent growth is constant. The biphasic model is used to evaluate growth under thermal treatments; the Whiting model analyzes microbial survival curves during microorganism inactivation, while the Gompertz model is widely used to study asymmetric sigmoidal growth and

inactivation curves in microbial development (Bevilacqua *et al*., 2015). There are several programs containing applications to analyze data with prediction models. The dynamic fit model (DMFit) in an Excel tool which can be used to analyze data with the Gompertz model. DMFit provides three variables (μ_m-maximum growth speed, λ-latency phase and A-maximum fungal growth in the stationary phase) that can be used to evaluate the effect of adding antifungal agents on the growth of fungi. The first goal of this study was to identify the main fungi found in the corn tortillas from five Mexican state capitals. The second goal was to determine the Minimum Inhibiting Concentration (MIC) of AEC against those fungi. The third goal was to evaluate the antifungal effect of AEC, E, I and AE on the mycelial growth of *Aspergillus niger* identified in this investigation.

Materials and Methods

Five states, distributed throughout the country (Sonora, Nuevo León, Michoacán, Oaxaca and Yucatán) were chosen. One kg of tortillas was purchased from a *tortillería* (tortilla store) from each capital located in the five states in 2021. The inclusion criteria to choose the adequate *tortillerías* were: a local *tortillería* that included the greatest amount of nixtamal for the production of corn tortillas. This ensured the use of the least possible number of artificial preservatives, including commercial flour. The tortillas remained in their original package until their analysis (36 h); every individual tortilla was repackaged in a polyethylene bag, cooled to 25 °C and placed in a polystyrene cooler with two freezing packs. The cold tortillas in the coolers were sent by courier service and received on the following day in the Centro de Investigación en Alimentación y Desarrollo, A.C. (CIAD) located in Hermosillo, Sonora (Son). After analysis, the tortillas were stored at -20 °C.

Isolation of fungi in corn tortillas

The packages with the corn tortillas from the five states in Mexico were opened and left to temper to 25 °C. The samples were prepared and dilutions were performed, following the NOM-110-SSA1-1994, poured on potato dextrose agar (PDA), and the dishes were incubated at 25 °C for 72 h (Secretaría de Salud de México, 1994). Three samples were processed for every package of corn tortillas in triplicate. After the incubation period (72 h) every fungal culture was sub-cultured until each one was isolated. Once the cultures were isolated, the "Scotch tape" technique was followed, then staining with cotton blue to observe the fungal structure under an optical Amscope B020c® a 40 X microscope (Guerra *et al*., 2019). To identify each fungal genus, the macro and micro characteristics of each fungus were compared

using the reported bibliography (Barupal *et al.*, 2019; Nguyen *et al*., 2020; Rangel-Muñoz *et al*., 2020; Yin *et al*., 2021).

Molecular identification of fungi

For the extraction of deoxyribonucleic acid (DNA), the methodology reported by González-Mendoza *et al*. (2010) followed. The mycelium of each fungus was collected and frozen dried after seven days of growth. Twenty mg of mycelium were weighed and added with 200 µL of lysis Buffer (SDS 3%, Tris-HCl 0.1 mM, pH 8.0, EDTA 0.5 mM, pH 8.0, NaCl 1 M) and the mixture was shaken for 30 min. A total of 200 µL of chloroform/phenol was added (1/1) and it was incubated at 65 °C for 5 min in a dry bath, followed by centrifuging at 4 °C for 5 min at 10,000 *g.* The supernatant was poured into another tube where the same volume of pure ethanol was added. The content was slowly mixed, cooled to -20 °C for 20 min and centrifuged at 4 °C for 5 min at 10,000 *g*. The supernatant was discarded and the rest was resuspended in ethanol at 75% and centrifuged at 4 °C for 5 min at 10,000 *g*. The supernatant was discarded and the remaining volume was added 30 µL of Millipore® water. Finally, an electrophoresis was carried out in agarose gel at 1.5% to verify the DNA extraction of each isolation.

The endpoint polymerase chain reaction (PCR) was carried out to identify each isolation. For the amplification, primers were used with the Internal Transcribed Spacer (ITS)1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´). The final reaction mixture was of 25 µL: 12.5 µL of PCR Master mix (Promega®), 1 µL of platinum Taq polymerase (Invitrogen®), 1.5 µL of ITS1, 1.5 µL of ITS4, 2 µL of DNA and 6.5 µL of Millipore® water. The amplification reaction began at 30 s at 95 °C, followed by 35 cycles as follows: 1 min at 95 °C, 30 s at 50 °C and 1 min at 72 °C; the final phase lasted 5 min at 95 °C (Kim *et al*., 2020). Each amplification was quantified, purified and analyzed in the National Agricultural, Medical and Environmental Biotechnology Laboratory, located in San Luis Potosí. The sequences were compared with those published in the gene database of the National Center for Biotechnology Information (NCBI, 2023).

Minimum inhibitory concentration (MIC) of AEC against the isolated fungi

To evaluate the MIC of the AEC on filamentous fungi, the proposal of the "Clinical Laboratory Standard Institute" was followed, with some modifications (Standards Clinical and Laboratory Institute, 2018). The commercial AEC used was acquired from Young Living ®. The following AEC concentrations were used: 3.12, 6.25, 12.5, 25, 50, 100, 200, 400, 800 and 1,600 µg mL^{-1} and the culture medium used was PDA. Every fungal isolation was re-cultured and on the 5th day of growth, it

was taken to quantify in a Neubauer chamber and adjusted at 2,500 conidia mL^{-1}. Once the inoculant was standardized, 100 µL of AEC and 100 µL of inoculant were deposited in 2 mL Eppendorf tubes, in triplicate. They were incubated at 25 °C for 24 h for the species of *Mucor* spp. and for 48 h for the remaining fungal genera. After the incubation time, the colony-forming units (CFUs) were quantified by plate pouring and the concentration at which no fungal growth was observed was taken as the MIC and reported as µg mL^{-1} for 250 conidia.

Effect of the AEC, E, I and AE on the mycelial growth of *Aspergillus niger*

One of the fungi isolated due to its prevalence (*Aspergillus niger*) was chosen to evaluate the effect of AEC, E, I and AE on its mycelial growth. For this assay, the procedure by Díaz-García *et al*. (2024) was chosen, with some modifications. Initially, the inoculant was standardized to a concentration of 1×10^6 spores mL^{-1}. From it, 100 µL were taken, equivalent to 100,000 conidia, which were deposited in a container, 0.9 cm in diameter, placed in the center of each Petri dish with PDA. Subsequently, in a second container, 100 µL of each antifungal agents AEC, E, I and AE were placed, at four concentrations (2,000, 4,000, 8,000 and 16,000 µg mL^{-1}), in such a way that one compound and one concentration were evaluated per Petri dish by triplicate. In addition, a control with water and another with ethanol were included to discard the possible antifungal activity of the diluent. All dishes were incubated at 25 °C for 96 h and the diameter of the mycelium was measured every 12 h for the first 48 h, followed by every 24 h. The final measurements were considered to be one day after the diameters of the water and ethanol controls started becoming stationary. The measurement of each mycelial diameter was carried out in the vertical direction of the containers, starting on the right edge of the central container and towards the left edge, until the point to which the fungal growth extended. The results of the mycelial growth and the percentage of inhibition of each compound were calculated using equations 1 and 2:

$$Mycelial\ growth(\%) = \left(\frac{diameter\ of\ the\ fungal\ growth\ in\ the\ treatment}{diameter\ of\ the\ negative\ control}\right) \times 100$$

Equation 1

$$Inhibition\ (\%) = 100 - growth\ (\%)$$

Equation 2

The kinetic parameters (λ: latency phase, μ_m: maximum growth speed, A: maximum growth of the fungus in the stationary phase) of the effect of the AEC, E and I were estimated using the Excel DMFit tool. The tool wa fed the data of the mycelial diameter (cm) obtained in each treatment and each sampling time (12, 24,

48, 72 and 96 h). The Gompertz model helps us obtain every kinetic variable and compare the effect of each compound on λ, μ_m and *A*. The Gompertz model is based on equation 3 (Bevilacqua *et al.,* 2015; Soro *et al*., 2021; Zwietering *et al*., 1990).

$$y = A\ exp\left\{-exp\left[\frac{\mu_m * e}{A}(\lambda - t) \quad + 1\right]\right\} \qquad \text{Equation 3}$$

where *y* is the mycelial diameter in a particular incubation time, t is time and μ_m is the maximum speed growth, λ is the latency phase and *A* is the maximum fungal growth in the stationary phase. The latency phase (λ) and the maximum fungal growth in the stationary phase (*A*) were analyzed in the NCSS 2023 statistical package and a means comparison was carried out with Tukey-Kramer ($p \leq 0.05$) (NCSS, 2023).

Results

Isolation and identification of fungi in corn tortillas

One of the fungal isolations of the corn tortillas from Monterrey, Nuevo León (N.L.) (Figure 1B), Hermosillo, Son (Son) (Figure 1H), Mérida, Yucatán (Yuc) (Figure 1E) and the only isolation from Oaxaca, Oaxaca (Oax) (Figure 1C) showed morphological similarities with the *Aspergillus* genus. This genus is characterized for having hyaline hyphae with an even circular conidiophore, which displays conidia on all its surface, which detach to form another fungal colony. The coloring of the *Aspergillus* spp. cultures on gelified culture medium can be white, black or green (Figure 2 B, C y E) (Ibarra-Valenzuela, 2019; Rangel-Muñoz *et al*., 2020).

On the other hand, the presence of the genus *Penicillium* was found in one fungal isolation (out of two) of the tortillas from Morelia, Michoacán (Mich) and Hermosillo, Son (Figures 1A and 1D, respectively). The cultures are usually velvety in texture with a white circumference in green tones that turn dark brown during maturation (Ibarra-Valenzuela, 2019; Yin *et al*., 2021).

Curvularia was also found in one of the two isolations of the tortillas from Monterrey, N.L. (Figure 1G). This genus presents septated hyphae with oval-shaped microconidia and macroconidia with 3 to 5 curved divisions. The cultures of these genera go from olive green to dark maroon and are considered dematiaceous, since they have a brown color, due to its own production of melanin (Barupal *et al*., 2019).

Mucor spp. was found in one of the two isolates of the tortillas from Morelia, Mich (Figure 2F) and Mérida, Yuc (Figure 2I). This genus presents hylaine hyphae that are joined in the uniform circular conidiophore and, unlike the rest of the

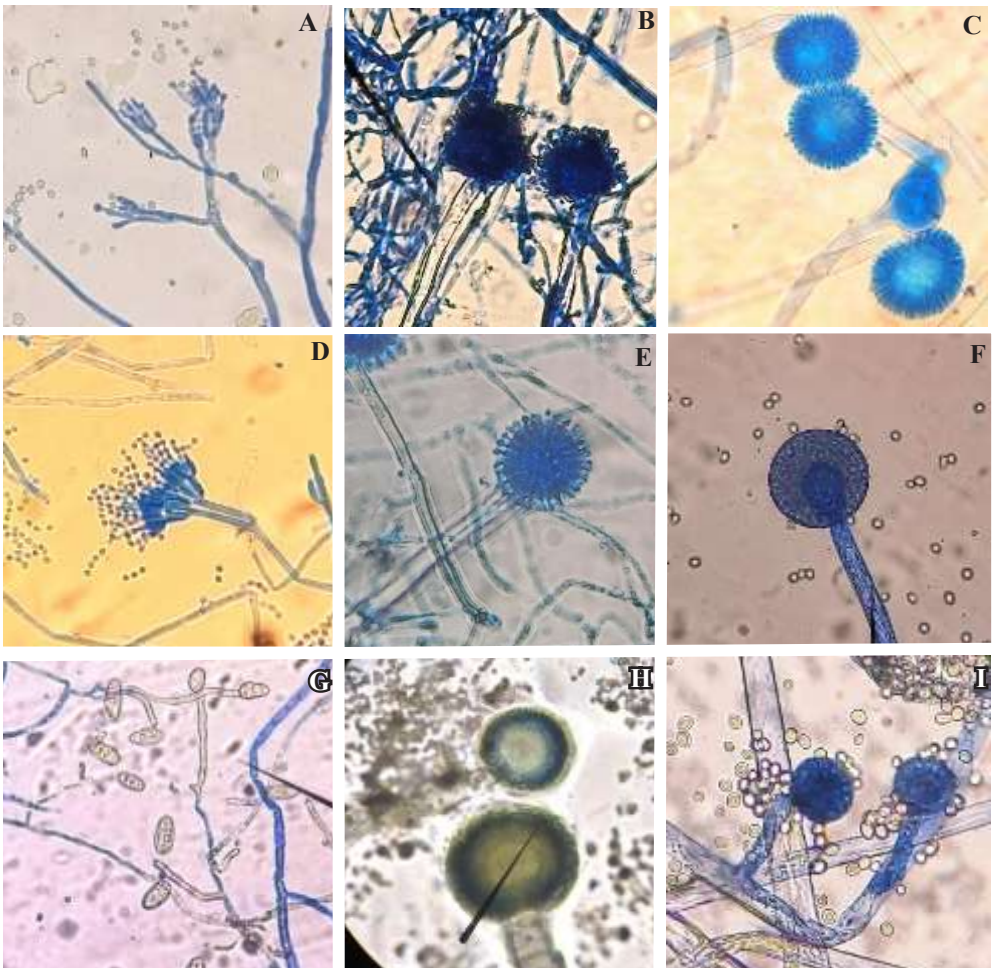

Figure 1. Fungal structure by optic microscopy (40X), isolated from corn tortillas from Morelia, Mich (A: *Penicillium* sp. and F: *Mucor* sp.), Monterrey, N.L. (B: *Aspergillus* sp. and G: *Curvularia* sp.), Oaxaca, Oax (C: *Aspergillus* sp.), Hermosillo, Son (D: *Penicillium* sp. and H: *Aspergillus* sp.) and Mérida, Yuc (E: *Aspergillus* sp. and I: *Mucor* sp.).

fungi mentioned, it produces and keeps all conidia inside the conidiophore. Once the conidiophore reaches its highest point of maturation, its cell wall breaks and releases all the conidia to form a new colony. The colonies of this genus were cottonlike, brown to gray and with the characteristic of completely filling up the culture dish in a maximum of 48 h. Additionally, due to its quick proliferation, most tests must be done after 24 h (Nguyen *et al*., 2020).

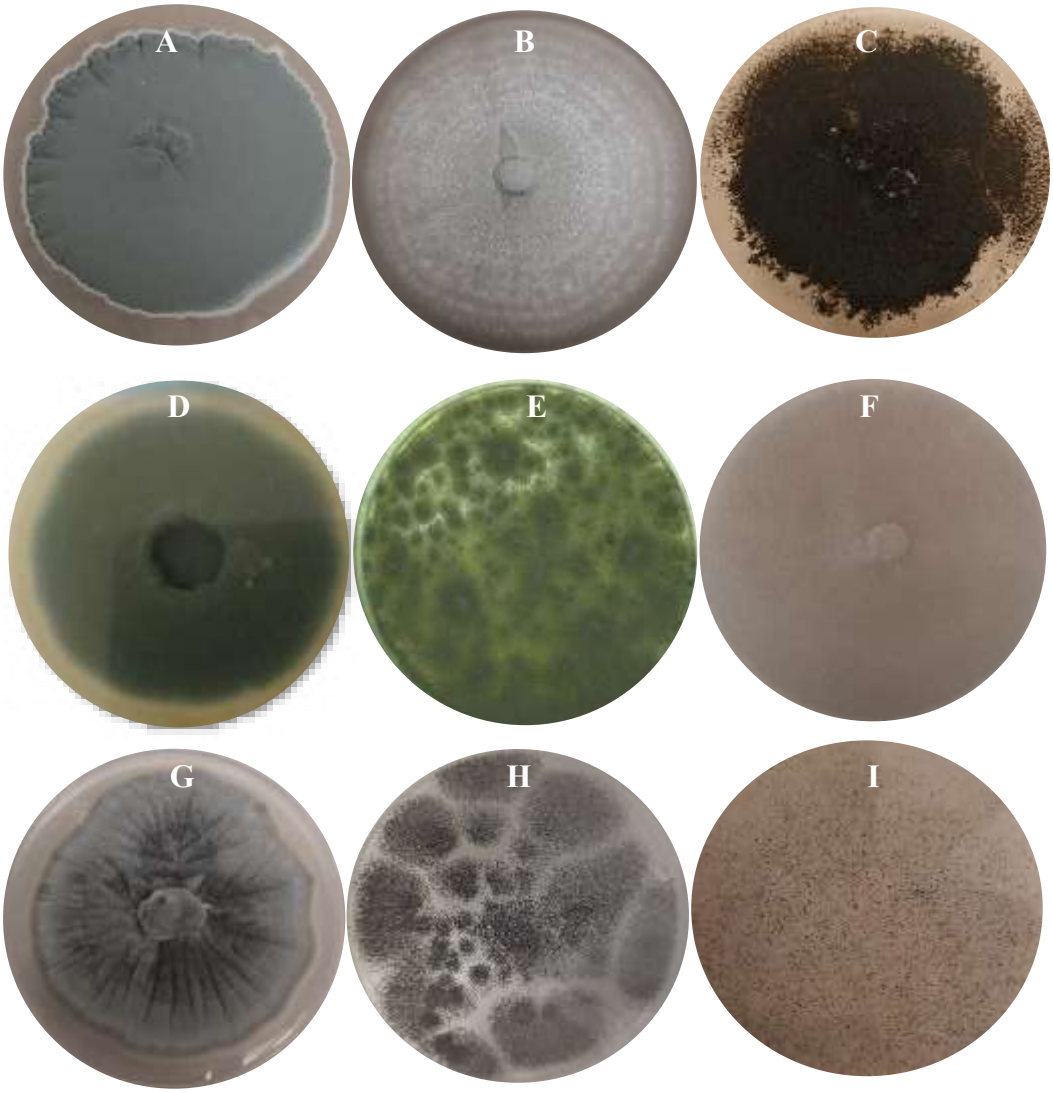

Figure 2. Growth of fungi in PDA isolated from corn tortillas from Morelia, Mich (A, *Penicillium* sp. and F, *Mucor* sp.), Monterrey, N.L. (B, *Aspergillus* sp. and G, *Curvularia* sp.), Oaxaca, Oax (C, *Aspergillus* sp.), Hermosillo, Son (D, *Penicillium* sp. and H, *Aspergillus* sp.) and Mérida, Yuc (E, *Aspergillus* sp. and I, *Mucor* sp.).

Molecular identification of fungi isolated from corn tortillas

During the amplification of the ITS, DNA fragments were obtained with a size between 500 and 750 pb. The sequences obtained verified the genus of each fungus and helped identify the species of each one. *M. racemosus* and *P. herquei* were identified on the Morelia Mich isolations, with an identity of 100%. Later, *A. longivesica* and *C. spicifera* were identified in the isolations from Monterrey, N.L.

with a 100% identity. Then, *A. niger* and *P. brevicompactum* in the isolations from Hermosillo, Son with a 100% identity. *A. flavus* was identified in one of the two isolations from Mérida, Yuc with a 94.23% identity, whereas the sequence from the second isolation that corresponds to *Mucor* sp. was not found in the NCBI database. Finally, the isolate from Oaxaca, Oax, which was identified as *Aspergillus* sp., was not amplified with the ITSs used.

MIC of the AEC against fungi in corn tortillas

The MICs of the fungi isolated from Michoacán, Nuevo León, Oaxaca, Sonora and Yucatán went from 200 to 800 µg mL^{-1}, which corresponds to between 20 and 80 µg added AEC to inhibit the growth of 250 conidia (Table 1). The least resistant fungus to AEC was *A. longivesica*, since it only required 200 µg mL^{-1} to inhibit its growth, followed by *M. racemosus*, *P. herquei*, *C. spicifera* and *P. brevicompactum* with a MIC of 400 µg mL^{-1}. Finally, the most resistant fungi to AEC were *Aspergillus* sp*., A. niger, A. flavus* and *Mucor sp* with a MIC of 800 µg mL^{-1}. *Aspergillus* sp. was the most prevalent genus in corn tortillas, as well as the most resistant to AEC with the highest MIC. Therefore, controlling this genus is crucial to guarantee the security and quality of corn tortillas.

Table 1. Minimum inhibiting concentration (MIC) of clove essential oil on fungi (250 conidia) isolated from corn tortillas.

States	Fungi	MIC (µg mL^{-1})
Michoacán	*M. racemosus*	400 ± 0
	P. herquei	400 ± 0
Nuevo León	*A. longivesica*	200 ± 0
	C. spicifera	400 ± 0
Oaxaca	*Aspergillus* sp.	800 ± 0
Sonora	*A. niger*	800 ± 0
	P. brevicompactum	400 ± 0
Yucatán	*A. flavus*	800 ± 0
	Mucor sp.	800 ± 0

Effect of the AEC, E, I and AE on the mycelial growth of *Aspergillus niger*

After the incubation period, it was observed that the AEC (4,000 and 2,000 µg mL^{-1}) presented the same fungistatic effect as E (8,000, 4,000 and 2,000 µg mL^{-1}) and I (16,000, 8,000, 4,000 and 2,000 µg mL^{-1}) after 24 h; these are found in a more significant proportion in this oil (Figure 3). The highest percentage of

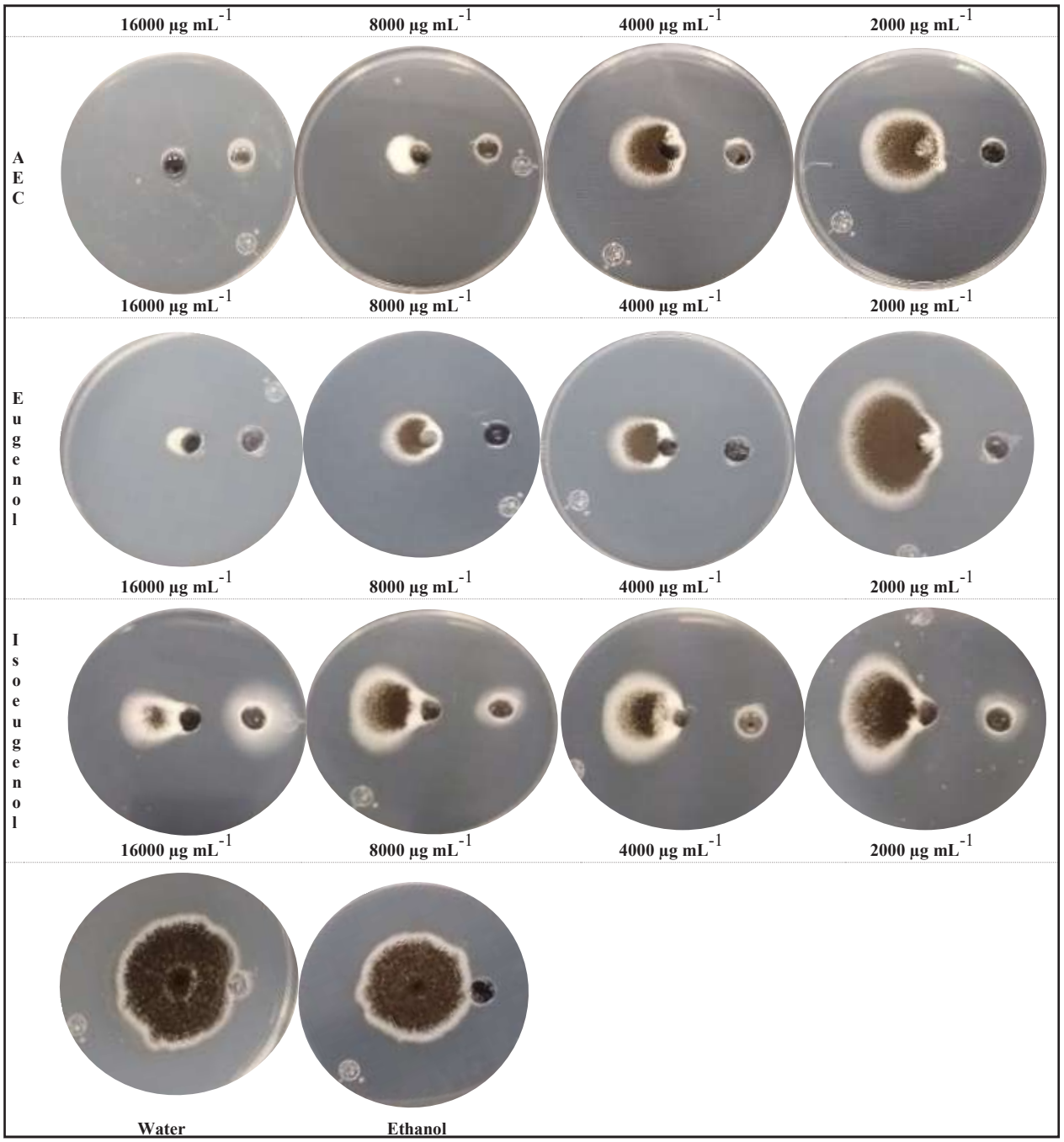

Figure 3. Antifungal effect of AEC, E and I on the growth of *Aspergillus niger* on PDA 96 h after de incubation at 25 ± 1 °C.

inhibition was produced by AEC at 16,000 µg mL^{-1}. The fungicidal effect of this concentration was maintained for at least 96 h (Figure 4). The AEC (16,000 and 8,000 µg mL^{-1}) and E (16,000 µg mL^{-1}) produced the highest percentages of inhibition (100, 57.1 and 67.3%), respectively. The inhibition of AEC (4,000 µg mL^{-1}, 27.6%), E (8,000 µg mL^{-1}, 34.7% and 4,000 µg mL^{-1}, 21.4%) and I (16,000 µg mL^{-1}, 22.4% and 8,000 µg mL^{-1}, 22.4%) presented no significant differences with each other. Meanwhile, the addition of AEC and E (16,000 µg mL^{-1}) reduced the growth speed of *Aspergillus niger* by five and six times in comparison with the water and ethanol controls, respectively. On the other hand, none of the AE concentrations presented an antifungal effect on the mycelial growth of *A. niger*.

Discussion

Isolation and identification of fungi in corn tortillas

Corn tortillas are eaten every day throughout México. However, there is scarce evidence of fungal species that limit their shelf life. The fungal development of *Aspergillus* spp. and *Penicillium* spp. in foods is important, since these produce several mycotoxins. The incidence of these fungi on corn cannot be eliminated with the tortilla processing conditions, hence they develop once they adapt to the storage conditions of the product at a domestic level.

Aspergillus spp. and *Penicillium* spp. are found in corn from harvest (Ullah *et al*., 2010), whereas *Curvularia* spp. and *Mucor* spp. are related to contamination during the production process of the food, hence they can contaminate the seeds at the moment of being packaged. *Curvularia* spp. had not been related to the corn tortillas or the reduction of their shelf life, although they had been related to infecting the corn grain, while *Mucor* spp. was identified in corn tortillas produced with five different corn races (López-Morales *et al*., 2023). *Curvularia* spp. is associated with the contamination of storage rooms and silos for corn grains and, in turn, *Mucor* spp. has been isolated from silos with corn grains during storage, since the latter is considered a fungal genus that contaminates the environment and not corn itself (Akwuobu *et al.*, 2019; García-Leaños *et al*., 2007).

MIC of the AEC against fungi in corn tortillas

The antifungal effect of the AEC is due to the terpenic compounds contained in this oil, and specifically E and I are compounds with lypophilic characteristics, that modify the fungal membrane and interrupt the exchange of ions, which produces

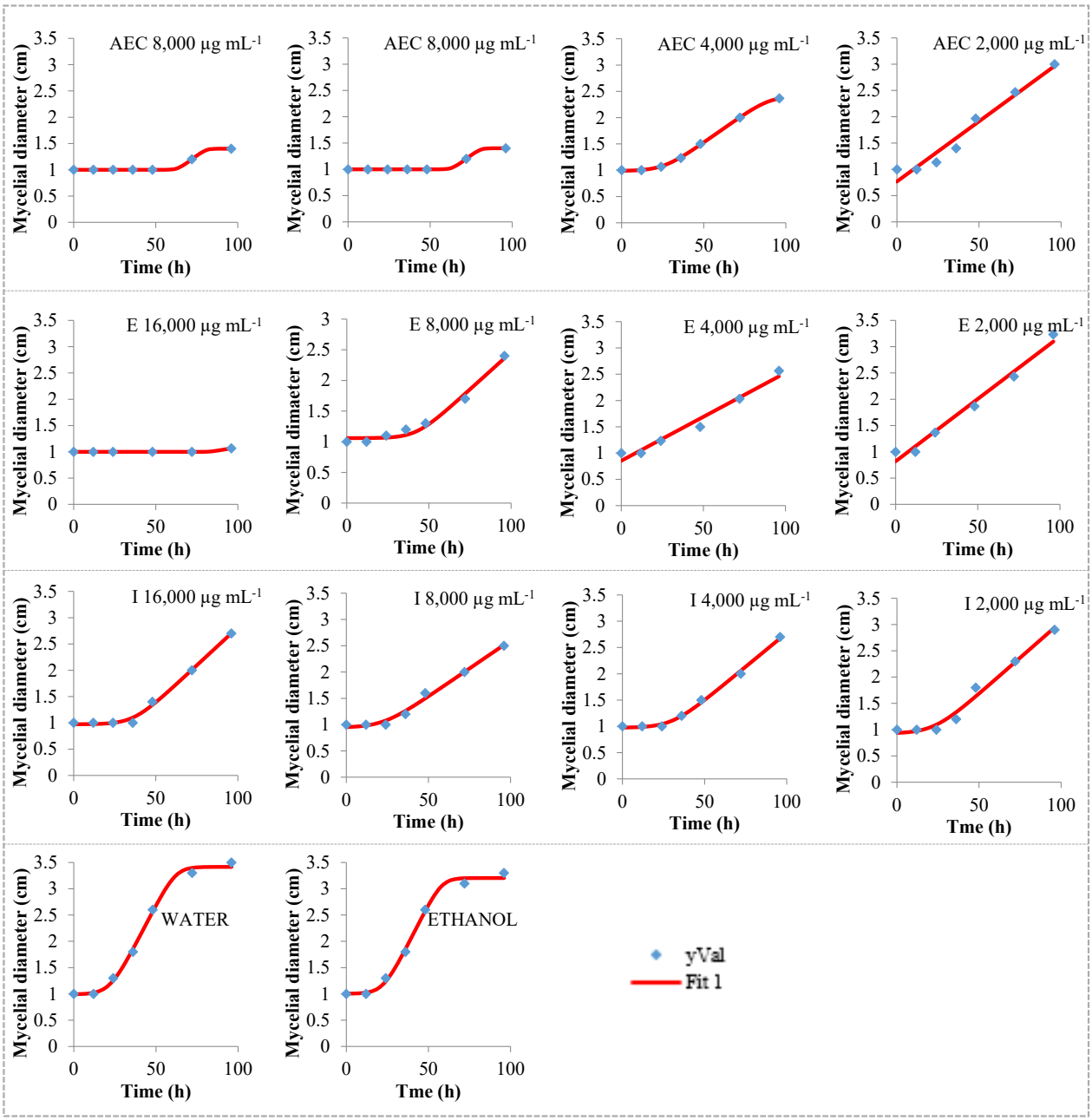

Figure 4. Kinetics of mycelial growth of *Aspergillus niger* on PDA for 96 h of incubation at 25 ± 1 °C. yVal= y values; Fit 1=curve with adjusted data.

cell lysis (de Oliveira Pereira *et al*., 2013). The results of the MIC (200-800 µg mL^{-1}) obtained for *Penicillium* spp. and *Aspergillus* spp. against AEC are found in the same magnitude as the MIC (350-400 µg mL^{-1}) reported for *Aspergillus* sp. and for *Penicillium* sp. against E (Abbaszadeh *et al.*, 2014). For *Curvularia spicifera*, no reports were found comparable to the AEC, but rather with other essential oils, such as the oil from *Psidium guajava* (0.2%), which has limonene (29%) and caryophyllene (15%). In a greater proportion, this oil inhibited 53% of *Curvularia Lunata* (Chaturvedi *et al.*, 2019). Heer *et al*. (2017) evaluated the effect of *Cinnamomum tamala*, which contains E (52%), against *Curvularia lunata*; the MIC of the extract against *Curvularia lunata* was of 2,117 µg mL^{-1}. To date there are no reports on the effect of AEC, E, I or AE against *Mucor* spp. that have used the technique described in the Materials and methods section, although there are two reports of the MIC of E against *Rhizopus oryzae*, which was of 512 µg mL^{-1} (Prajapati *et al*., 2023) and 350 µg mL^{-1} (Abbaszadeh *et al.*, 2014). Due to the importance of keeping foods free of any fungi and their secondary metabolites, it is important to have a natural alternative such as AEC to reduce their development in foods. The results of the MIC of the AEC against each fungal species suggest that 1,600 µg mL^{-1} are enough to exert a fungicidal activity on each case.

Effect of the AEC, E, I and AE on the mycelial growth of *Aspergillus niger*

The growth of the diameters of the controls (water and ethanol) reduced after 72 h, therefore the measurements were considered to be completed after 96 h. *Aspergillus niger* began developing in the water and ethanol controls after 17 and 22 h respectively, whereas in AEC (16,000 µg mL^{-1}) and in E (16,000 µg mL^{-1}) they took ≥96 and 87.43 h, respectively. The highest concentration of AEC hindered the fungal growth during the entire incubation period (96 h). Pereira *et al*. (2013) and Tarhan (2021) reported AEC is due to E, since it is found in a greater proportion of this oil. Nevertheless, the results of the effect of AEC, E and I on the growth of *Aspergillus niger* suggest that there is a greater antifungal effect by AEC than by E and I separately in the same concentrations. This occurs because in AEC, the mechanisms of action of E and I exert a synergistic effect. Specifically, E is reported to interact with the ergosterol molecule in the fungal membrane, whereas I inhibits the formation of components of the fungal extracellular membrane (Gupta *et al*., 2022; Pereira *et al*., 2013). The hydroxyl groups of E and I promote a synergic antifungal effect when combined in AEC. Consequently, applying a lower concentration of AEC (4,000 µg mL^{-1}) leads to the same effect on the latency phase (λ) as adding a higher concentration of E (8,000 µg mL^{-1}) or I (16,000 µg mL^{-1}) separately (Table 2).

Table 2. Kinetic parameters of the effect of the clove essential oil (AEC), eugenol (E) and isoeugenol (I) on the mycelial growth of *Aspergillus niger* incubated at 25 ± 1 °C for 96 h, obtained with Excel DMFit 3.5.

Treatment	Concentration (µg mL^{-1})	λ (h)	μ_m (cm h^{-1})	A (cm)	R^2 (%)
AEC	16,000	≥96±0.00^{b}	0.00±0.00×10^{-2a}	1.00±0.00^{a}	100.0±0.00
	8,000	63.33±0.00^{b}	2.25±0.05×10^{-2bd}	1.40±0.00^{a}	100.0±0.00
	4,000	25.17±6.82^{ab}	2.31±1.27×10^{-2bd}	2.36±0.88^{b}	98.83±0.83
	2,000	26.05±5.02^{ab}	2.94±0.77×10^{-2b}	3.01±0.29^{b}	97.39±3.20
E	16,000	87.43±7.42^{b}	0.43±0.40×10^{-2ad}	1.06±0.05^{a}	100.0±0.00
	8,000	34.55±9.07^{ab}	1.84±0.65×10^{-2abd}	2.11±0.29^{b}	98.10±0.62
	4,000	31.07±2.65*^{ab}	2.26±1.32×10^{-2bd}	2.47±0.37^{b}	93.64±5.56
	2,000	27.03±7.70^{ab}	3.22±0.04×10^{-2b}	3.21±0.04^{b}	98.64±0.01
I	16,000	30.15±8.70^{ab}	2.26±0.90×10^{-2bd}	2.53±0.24^{b}	95.61±0.07
	8,000	27.04±3.82^{ab}	2.26±0.19×10^{-2bd}	2.52±0.08^{b}	98.39±0.01
	4,000	32.91±3.59^{ab}	2.60±0.25×10^{-2b}	2.64±0.08^{b}	97.57±0.02
	2,000	23.36±0.57^{ab}	2.71±0.49×10^{-2b}	3.00±0.21^{b}	95.44±0.06
WATER		17.56±4.06^{a}	5.56±0.68×10^{-2c}	3.47±0.08^{b}	99.22±0.00
ETHANOL		22.34±0.01^{a}	6.17±0.01×10^{-2c}	3.17±0.06^{b}	99.01±0.00

λ = phase of latency of the fungus (h); μ_m = maximum growth speed (cm h^{-1}); A = maximum growth of the fungus in the stationary phase (cm). Values are mean ± standard deviation of triplicates. *= estimated result of the average of the triplicate with DMFit. Different letters per column indicate significant differences ($p \leq 0.05$).

The water and ethanol controls began developing at the same time as the low concentrations of AEC and E, although these concentrations reduced the speed of growth of *Aspergillus niger* from 1.6 to 2.3 times regarding the water control. This shows that the additions of AEC and E at low concentrations have a greater impact on the maximum diameter of the fungus in comparison with the impact they exert on the time of adaptation during the period of incubation. Therefore, low concentrations of these compounds have a greater effect on the development of hyphae and conidiophores in comparison with the lower effect exerted on the conidia of the fungus. When considering the possible addition of AEC as a food additive, it is fair to mention that this compound could have the ability to reduce the growth rate of fungi present in corn tortillas. The addition of AEC (8,000 µg mL^{-1}) to corn tortillas could extend their shelf life by at least 63 hours at room temperature, as this treatment delays fungal growth during this period. The trials of the antifungal effect were performed at 25 °C and the corn tortillas were stored at 5 °C, therefore the addition of AEC to this food, along with the low temperatures of its storage, could boost the antifungal effect which could extend the shelf life of the corn tortillas for longer than 63 h.

The effects of AEC, E and I on the maximum growth of *Aspergillus niger* presented a different behavior in the latency phase (λ). After 96 h of incubation, AEC (16,000 and 8,000 µg mL^{-1}) and E (16,000 µg mL^{-1}) presented a more significant antifungal effect ($p \leq 0.05$) on the maximum growth of the fungus in the stationary phase (*A*). The above, followed by AEC (4,000 µg mL^{-1}), E (8,000 and 4,000 µg mL^{-1}), and I (16,000, 8,000 and 4,000 µg mL^{-1}) similar to each other. Finally, AEC (2,000 µg mL^{-1}), E (2,000 µg mL^{-1}), I (2,000 µg mL^{-1}), water and ethanol presented no significant differences with each other ($p>0.05$). The results suggest that each molecule has a function in the antifungal effect against *Aspergillus niger*, although in the latency phase (λ), I (8,000 µg mL^{-1}) it presents no differences with E (8,000 µg mL^{-1}). After 22.04 h, the antifungal effect is reduced. Consequently, after 96 h, the maximum fungal growth in the stationary phase (*A*) in I (8,000 µg mL^{-1}) is similar to AEC (2,000 y 4,000 µg mL^{-1}) and greater than AEC (8,000 µg mL^{-1}) and E (8,000 µg mL^{-1}). The latency phase (*λ*) is important in the study of the antifungal effect of AEC on *Aspergillus niger*, but it is more important on mycelial growth, since reducing the hyphal growth can lead to a reduction in the production of mycotoxins (Hua *et al*., 2014; Mirza Alizadeh *et al*., 2022). Allam *et al*. (2012) reported a reduction of mycelial growth and a 100% eradication of ocratoxin A, a mycotoxin produced by *Aspergillus niger*, when using a concentration of 250 µL L^{-1} of AEC.

Before the search for natural compounds such as AEC for fungal control in foods, diverse conventional preservatives, such as sorbates and propionates, were used to control the development of fungi and the production of mycotoxins in corn tortillas. The NOM-247-SSA1-2008 establishes the maximum limit of some preservatives for corn tortillas such as sorbic acid (1,000 mg kg^{-1}) and benzoic acid (1,000 mg kg^{-1}) (Secretaría de Salud de México, 2009). Meanwhile, the "Agreement establishing the additives and processing aids in foods, beverages and dietary supplements, their use and health regulations" (Diario Oficial de la Federación de México, 2012) published a list of additives for various foods. It establishes the upper limit for the addition of other preservatives such as calcium benzoate (1,000 mg kg^{-1}) and potassium sorbate (2,000 mg kg^{-1}) in pre-packaged tortillas. The addition of every preservative and its prolonged use can have adverse effects on human health; therefore, a natural alternative can be the addition of natural compounds such as AEC. In an attempt to find an equivalence, the AEC treatment (8,000 µg mL^{-1}) in PDA dishes (15 g of agar) results in a lower concentration (53.3 mg kg^{-1}) than the maximum concentration of artificial preservatives established for corn tortillas (1,000-2,000 mg kg^{-1}). Therefore, AEC could be a natural alternative to reduce the growth of fungi on corn tortillas, thus extending their shelf life.

This study identified the presence of the *Aspergillus* genus on corn tortillas. In the tortilla industry, artificial additives are applied, mainly potassium sorbate

and sodium propionate; their permitted limits are found in the official Mexican norms (Secretaría de Salud de México, 2009). The incorporation of any additive modifies the food's characteristics and the tortilla is no exception since the addition of sorbates or propionates causes it to change. Depending on the concentration of food additives, it can modify its flavor and color (Báez-Aguilar *et al*., 2022). The Mexican Health Secretariat (2002) includes the maximum limit permitted for potassium sorbate in corn or wheat flour tortillas, which indicates a limit of 2,000 mg kg^{-1}. However, in 2002, the concentration of additives in commercial corn flour was evaluated and it was found that the MASECA® brand corn flour contained 4,000 mg kg^{-1} of potassium sorbate (Flores Farías *et al*., 2002).

Due to the modifications undergone by corn tortillas when added artificial preservatives such as potassium sorbate and the adverse effects on health due to their consumption, natural compounds are being investigated that may exert the same antimicrobial properties (Jarkvist *et al*., 2020; Pepper *et al*., 2020). This study found that AEC concentrations are required which are lower than the permitted limit of artificial preservatives (potassium sorbate, 2,000 mg kg^{-1}) to reduce the maximum growth speed (μ_m) and delay the maximum growth of the fungus in the stationary phase (A). In addition, the acute oral median lethal dose (DL 50) of AEC (5,000 mg kg^{-1}) is higher than the DL 50 of E (1,930 mg kg^{-1}) and I (1,560 mg kg^{-1}). This means that a larger amount of AEC must be ingested to cause any adverse effects, suggesting that AEC could be a less toxic alternative (Parvitra, 2024; Roth, 2024). Regarding the price of AEC in the market (MXN$3.7/g), it is 2.3 and 4.1 times lower than the prices of E and I (MXN$8.6/g and MXN$15.2/g), respectively (Sigma-Aldrich, 2023a, 2023b, 2023c). This indicates that AEC is more affordable and has a greater antifungal effect than E or I, making it more effective at reducing the presence of fungi and extending the shelf life of corn tortillas.

Conclusions

Nine fungi were identified in the corn tortillas from five states in Mexico using optical microscopy, seven of which were confirmed molecularly. *Mucor racemosus* and *Penicillium herquei* were found in Morelia, Mich, *Aspergillus longivesica* and *Curvularia spicifera* in Monterrey, N.L., *Aspergillus* sp. in Oaxaca, Oax, *Aspergillus niger* and *Penicillium brevicompactum* in Hermosillo, Son, *Aspergillus flavus* and *Mucor* sp. in Mérida, Yuc. Applying AEC against the growth of *Aspergillus niger* proved to be more efficient to increase the phase of latency (λ), reduce the maximum growth of the fungus in the stationary phase (A) and the maximum growth speed (μ_m), in regard to E, I and AE. In addition, DL 50 and the price of the AEC in the market is lower than each one of its components, which increases its potential to control the growth of fungi in corn tortillas and thus extend their shelf life.

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References

Abbaszadeh S, Sharifzadeh A, Shokri H, Khosravi AR and Abbaszadeh A. 2014. Antifungal efficacy of thymol, carvacrol, eugenol and menthol as alternative agents to control the growth of food-relevant fungi. Journal de Mycologie Médicale 24(2): 51–56. https://doi.org/10.1016/j.mycmed.2014.01.063

Akwuobu CA, Antiev WS and Ofukwu RA. 2019. Fungal contaminants of smoke-dried fish sold in open markets in Makurdi , Benue State. Scientific Research Publishing 10: 290–297. https://doi.org/10.4236/fns.2019.103022

Allam NG, El-shanshoury AER, Emara HA and Zaky AZ. 2012. Decontamination of ochratoxin-A producing *Aspergillus niger* and ochratoxin *A* in medicinal plants by gamma irradiation and essential oils. Journal of International Environmental Application and Science 7(1): 161–169. https://www.researchgate.net/profile/Nanis-Allam/publication/266944166_Decontamination_of_Ochratoxin-A_Producing_Aspergillus_niger_and_Ochratoxin_A_in_Medicinal_Plants_by_Gamma_Irradiation_and_Essential_Oils/links/543fa14d0cf23da6cb5b5839/Decontamination-

Báez-Aguilar ÁM, Arámbula-Villa G, Prinyawiwatkul W, López-Espíndola M, Ramírez-Rivera EJ, Contreras-Oliva A and Herrera-Corredor JA. 2022. Effect of calcium hydroxide mixed with preservatives on physicochemical characteristics and sensory shelf-life of corn tortilla. Journal of the Science of Food and Agriculture 102(2): 688–695. https://doi.org/10.1002/jsfa.11399

Barupal T, Meena M and Sharma K. 2019. Inhibitory effects of leaf extract of *Lawsonia inermis* on *Curvularia lunata* and characterization of novel inhibitory compounds by GC–MS analysis. Biotechnology Reports 23. https://doi.org/10.1016/j.btre.2019.e00335

Bevilacqua A, Speranza B, Sinigaglia M and Corbo M. 2015. A focus on the death kinetics in predictive microbiology: benefits and limits of the most important models and some tools dealing with their application in foods. Foods 4(4): 565–580. https://doi.org/10.3390/foods4040565

Chaturvedi T, Singh S, Nishad I, Kumar A, Tiwari N, Tandon S, Saikia D and Verma RS. 2019. Chemical composition and antimicrobial activity of the essential oil of senescent leaves of guava (*Psidium guajava L*). Natural Product Research 35(8): 1393-1397. https://doi.org/10.1080/14786419.2019.1648462

CONEVAL, Consejo Nacional de Evaluación de la Política de Desarrollo Social. 2012. Contrucción de las líneas de bienestar. https://www.coneval.org.mx/Informes/Coordinacion/INFORMES_Y_PUBLICACIONES_PDF/Construccion_lineas_bienestar.pdf (consulta, enero 2024).

de Oliveira Pereira F, Mendes JM and de Oliveira Lima E. 2013. Investigation on mechanism of antifungal activity of eugenol against *Trichophyton rubrum*. Medical Mycology 51(5): 507–513. https://doi.org/10.3109/13693786.2012.742966.

Diario Oficial de la Federación de México. 2012. Aditivos y coadyuvantes en alimentos, bebidas y suplementos alimenticios, su uso y disposiciones sanitarias. https://dof.gob.mx/nota_detalle.php?codigo=5437267&fecha=16/05/2016#gsc.tab=0 (consulta, enero 2024).

Díaz-García E, Valenzuela-Quintanar, A I, Sánchez-Estrada A, González-Mendoza D, Tiznado-Hernández M E, Islas-Rubio A R and Troncoso-Rojas R. 2024. Phenolic Compounds Synthesized by Trichoderma longibrachiatum Native to Semi-Arid Areas Show Antifungal Activity against Phytopathogenic Fungi of Horticultural Interest. Microbiology Research, 15(3): 1425–1440. https://doi.org/https://doi.org/10.3390/microbiolres15030096

Flores Farías R, Martínez Bustos F, Salinas Moreno Y and Ríos E. 2002. Characterization of commercial nixtamalized maize flours. Agrociencia 36: 557–567. https://www.redalyc.org/pdf/302/30236507.pdf

García Leaños ML, Aguirre Gómez JA, Narro Sánchez J, Cortés Baheza E and Rivera Reyes JG. 2007. Silo hermético para el control de plagas de granos almacenados en Guanajuato, México. Agricultura Técnica En México 33(3), 231–239. https://www.scielo.org.mx/scielo.php?script=sci_arttext&pid=S0568-25172007000300002

González-Mendoza D, Argumedo-Delira R, Morales-Trejo A, Pulido-Herrera A, Cervantes-Díaz L, Grimaldo-Juarez O and Alarcón A. 2010. A rapid method for isolation of total DNA from pathogenic filamentous plant fungi. Genetics and Molecular Research 9(1): 162–166. https://pubmed.ncbi.nlm.nih.gov/20198572/

Guerra FL, Lopes W, Cazarolli JC, Lobato M, Masuero AB, Dal Molin DCC, Bento FM, Schrank A and Vainstein MH. 2019. Biodeterioration of mortar coating in historical buildings : microclimatic characterization , material , and fungal community. Building and Environment 155: 195–209. https://doi.org/doi.org/10.1016/j.buildenv.2019.03.017

Gupta L, Sen P, Bhattacharya AK, and Vijayaraghavan P. 2022. Isoeugenol affects expression pattern of conidial hydrophobin gene RodA and transcriptional regulators MedA and SomA responsible for adherence and biofilm formation in *Aspergillus fumigatus*. Archives of Microbiology 204(4): 214. https://doi.org/10.1007/s00203-022-02817-w

Heer A, Guleria S and Razdan VK. 2017. Chemical composition, antioxidant and antimicrobial activities and characterization of bioactive compounds from essential oil of Cinnamomum tamala grown in north-western Himalaya. Journal of Plant Biochemistry and Biotechnology 26(2): 191–198. https://doi.org/10.1007/s13562-016-0381-7

Heredia-Sandoval NG, Santiaguin-Padilla AJ, Granados-Nevarez MC, Scheuren-Acevedo SM, Islas-Rubio AR, Mazorra-Manzano MA, García-Sánchez G and Ramírez-Suarez C. 2021. Supplementation of corn tortilla with freeze-dried jumbo squid muscle flour : physicochemical properties and microbiological stability during storage. Biotecnia, 23(2), 9. https://doi.org/10.18633/biotecnia.v23i2.1420

Hernández Cerda EM, Ordoñez Díaz JM y Giménez de Azcárate J. 2018. Análisis comparativo de dos sistemas de clasificación bioclimática aplicados en México. Investigaciones Geográficas 95. https://doi.org/https://doi.org/10.14350/rig.57451

Hua H, Xing F, Selvaraj JN, Wang Y, Zhao Y, Zhou L, Liu X and Liu Y. 2014. Inhibitory effect of essential oils on *Aspergillus ochraceus* growth and ochratoxin A production. PloS One 9(9): 1–11. https://doi.org/https://doi.org/10.1371/journal.pone.0108285

Ibarra Valenzuela AP. 2019. Evaluación de la actividad antimicrobiana de dos envases activos con aceite esencial de azahar(*Citrus aurantium*). En *Centro de Investigación en Alimentos y Desarrollo, A.C.* https://ciad.repositorioinstitucional.mx/jspui/browse?type=author&value=ANA+PATRICIA+IBARRA+VALENZUELA

INEGI, Insitituto Nacional de Estadística y Geografía. 2020. Censo de población y vivienda. https://www.inegi.org.mx/contenidos/saladeprensa/boletines/2021/EstSociodemo/ResultCenso2020_Nal.pdf.

Jarkvist J, Brockow K y Gülen T. 2020. Low frequency of IgE-mediated food hypersensitivity in mastocytosis. The Journal of Allergy and Clinical Immunology: In Practice 8(9): 3093–3101. https://doi.org/https://doi.org/10.1016/j.jaip.2020.05.044

Kim W B, Park C, Cho S Y, Chun,H S and Lee D G. 2020. Development of multiplex real-time PCR for rapid identification and quantitative analysis of Aspergillus species. Plos One, 15(3): e0229561. https://doi.org/10.1371/journal.pone.0229561

López-Morales F, Aragón-García A, Pérez-Torres B C, Vásquez-Carrillo G, Castillo-Hernández, D and Aragón Sánchez M. 2023. Identificación de hongos extraídos de tortillas de diferentes razas de maíz (Zea mays L.). Ecosistemas y Recursos Agropecuarios: 10(3). https://doi.org/10.19136/era.a10n3.3453

López Ortiz D. 2016. *Evaluación de la actividad antimicrobiana de extractos vegetales en la vida de anaquel de tortillas de maíz*. Universidad Tecnológica de Tehuacán.

Martínez Padrón HY, Hernández Delgado S, Reyes Méndez CA y Vázquez Carillo G. 2013. El género *Aspergillus* y sus micotoxinas en maíz en México: problemática y perspectivas. Revista Méxicana de Fitopatología 31(2): 126–146. https://www.redalyc.org/pdf/612/61231509005.pdf

Mirza Alizadeh A, Golzan SA, Mahdavi A, Dakhili S, Torki Z and Hosseini H. 2022. Recent advances on the efficacy of essential oils on mycotoxin secretion and their mode of action. Critical Reviews in Food Science and Nutrition 62(17): 4726–4751. https://doi.org/https://doi.org/10.1080/10408398.2021.1878102

NCBI, National Center for Biotechology Information. 2023. Information. https://blast.ncbi.nlm.nih.gov/Blast.cgi#sort_mark (consulta, junio 2022).

NCSS, Number Cruncher Statistical Systems. 2023. Software NCSS. https://doi.org/https://www.ncss.com/software/ncss/

Nguyen TTT, Jeon YJ, Mun HY, Goh J, Chung N and Lee HB. 2020. Isolation and characterization of four unrecorded *Mucor* species in Korea. Mycobiology 48(1): 29–36. https://doi.org/10.1080/12298093.2019.1703373

Parvitra. 2024. *Aceite esencial de clavo*. http://www.parvitra.com/img/Materias/Materia16/file_safety_sheet16.pdf

Pepper AN, Sriaroon P and Glaum MC. 2020. Additives and preservatives : Role in food allergy. Journal of Food Allergy USA 1: 119–123. https://doi.org/10.2500/jfa.2020.2.200014

de Pereira Pererira F, Mendes JM and de Oliveira Lima E. 2013. Investigation on mechanism of antifungal activity of eugenol against *Trichophyton rubrum*. Medical Mycology 51(5): 507-513. https://doi.org/10.3109/13693786.2012.742966

Prajapati J, Rao P, Poojara L, Acharya D, Patel SK, Goswami D and Rawal RM. 2023. A comprehensive in vitro and in silico assessment on inhibition of cyp51b and ergosterol biosynthesis by eugenol in *Rhizopus oryzae*. Current Microbiology 80(1): 1–13. https://doi.org/10.1007/s00284-022-03108-9

Rangel-Muñoz EJ, Valdivia-Flores AG, Moreno-Rico O, Hernández-Delgado S, Cruz-Vázquez C,de Luna-Lópeza MC, Quezada-Tristán T, Ortiz-Martínez R y Máyek-Pérez N. 2020. Caracterización de *Aspergillus flavus* y cuantificación de aflatoxinas en pienso y leche cruda de vacas en Aguascalientes , México Introducción. Revista Mexicana de Ciencias Pecuarias 11(2): 435–454. https://doi.org/https://doi.org/10.22319/rmcp.v11i2.5686

Rooney LW and Serna-Saldivar SO. 2016. Tortillas. In Encyclopedia of Food Grains: 90–96. Elsevier. https://doi.org/10.1016/B978-0-12-394437-5.00124-8

Roth. 2024. Eugenol e isoeugenol. https://www.carlroth.com/medias/SDB-8662-ES-ES.pdf?context=bWFzdGVyfHNlY3Vya-XR5RGF0YXNoZWV0c3wyNzAyOTJ8YXBwbGljYXRpb24vcGRmfHNlY3VyaXR5RGF0YXNoZWV0cy9oMWUvaGMzLzkwNDIwOTU1MzgyMDYucGRmfGUxZTI2MDExOTIzNzQyYWQyMzQ1OWRlYzU1Y2I3ZWU5MTdjYmIyYjU1O-TJlNjAxNTRjYzNk

Secretaría de Salud de México. 1994. Norma oficial mexicana NOM-110-SSA1-1994. Preparación y dilución de muestras de alimentos para su análisis microbiológico. http://www.salud.gob.mx/unidades/cdi/nom/110ssa14.html.

Secretaría de Salud de México. 2002. Norma oficial mexicana NOM-187-SSA1/SCFI-2002. Productos y servicios. Masa, tortillas, tostadas y harinas preparadas para su elaboración y establecimientos donde se procesan. Especificaciones sanitarias. Información comercial. *Métodos de prueba.* http://www.salud.gob.mx/unidades/cdi/nom/187ssa1scfi02.html

Secretaría de Salud de México. 2009. Norma oficial mexicana NOM-247-SSA1-2008. Productos y servicios. Cereales y sus productos. Cereales, harinas de cereales, sémolas o semolinas. Alimentos a base de: cereales, semillas comestibles, de harinas, sémolas o semolinas o sus mezclas. Productos de. https://dof.gob.mx/nota_detalle.php?codigo=5100356&fecha=27/07/2009#gsc.tab=0

Serna-Saldivar SO. 2015. History of corn and wheat tortillas. Tortillas: wheat flour and corn product*s*. 1–28. https://doi.org/10.1016/B978-1-891127-88-5.50001-3

Sigma-Aldrich. 2023a. Aceite esencial de clavo. https://www.sigmaaldrich.com/MX/es/search/aceite-de-clavo?focus=products&page=1&perpage=30&sort=relevance&term=ACEITE DE CLAVO&type=product

Sigma-Aldrich. 2023b. Eugenol. https://www.sigmaaldrich.com/MX/es/product/aldrich/e51791?utm_source=google&utm_medium=cpc&utm_campaign=10640610204&utm_content=113553655908&gclid=CjwKCAiA5L2tBhBTEiwAdSxJX252Q7QqtXZny3R4b06wSz2uGubFIfMZ8Xf-3KPyLPeMRnrEyDnluxoCPXkQAvD_BwE

Sigma-Aldrich. 2023c. Isoeugenol. https://www.sigmaaldrich.com/MX/es/product/aldrich/i17206

Soro AB, Oliveira M, Donnell CPO and Tiwari BK. 2021. Ultrasonics Sonochemistry Ultrasound assisted modulation of yeast growth and inactivation kinetics. Ultrasonics Sonochemistry 80. https://doi.org/10.1016/j.ultsonch.2021.105819

Standards Clinical and Laboratory Institute. 2018. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. https://clsi.org/standards/products/microbiology/documents/m38/

Tarhan İ. 2021. A robust method for simultaneous quantification of eugenol, eugenyl acetate, and β-caryophyllene in clove essential oil by vibrational spectroscopy. Phytochemistry 191. https://doi.org/10.1016/j.phytochem.2021.112928

Ullah H, Simpson TJ, Alam S, Fatima K and Perveen S. 2010. Mould incidence and mycotoxin contamination in maize kernels from Swat Valley , North West Frontier Province of Pakistan. Food and Chemical Toxicology 48: 1111–1116. https://doi.org/10.1016/j.fct.2010.02.004.

Wall-Martínez HA, Ramírez-Martínez A, Wesolek N, Brabet C, Durand N, Rodríguez- Jimenes GC, García-Alvarado MA, Salgado-Cervantes MA, Robles-Olvera VJ and Roudot AC. 2019. Risk assessment of exposure to mycotoxins (aflatoxins and fumonisins) through corn tortilla intake in Veracruz City (México). Food Additives and Contaminants: Part A 36(6): 929–939. https://doi.org/10.1080/19440049.2019.1588997

Yin G, Zhao H, Pennerman KK, Ii WMJ, Fu M, Bu L, Guo A and Bennett JW. 2021. Genomic analyses of *Penicillium* species have revealed patulin and citrinin gene clusters and novel loci involved in oxylipin production. Journal of Fungi 7(9): 1–16. https://doi.org/https://doi.org/10.3390/jof7090743

Zwietering MH, Jongenburger I, Rombouts FM and Van 't RK. 1990. Modeling of the bacterial growth curve. Applied and Environmental Microbiology 56(6): 1875–1881. https://doi.org/10.1128/aem.56.6.1875-1881.1990