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Artículos científicos

Evaluación de los aceites esenciales Thymus vulgaris, Cymbopogon flexuous y Syzygium aromaticum en la reducción del crecimiento de Aspergillus flavus en medios agrícolas

Assestment of thymus vulgaris, cymbopogon flexuous and syzygium aromaticum essential oils in the reduction of aspergillus flavus growth in agriculture media

Avaliação dos Óleos Essenciais de Thymus Vulgaris, Cymbopogon Flexuous e Syzygium Aromaticum na Redução do Crescimento de Aspergillus flavus em Meios Agrícolas

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Resumen

La mayor parte de la dieta que se consume a diario está tratada con aditivos, conservantes y otros aditivos químicos para mantener los alimentos en buen estado durante más tiempo. Hoy en día, se ha demostrado que el público en general ha mostrado cierto interés por consumir alimentos libres de aditivos químicos, con alto valor nutricional y que sean una alternativa en la prevención de enfermedades. En esta investigación se analiza el efecto antifúngico de tres aceites esenciales mediante bioensayos de inhibición del crecimiento en el micelio de *Aspergillus flavus*. Tras cinco días de incubación, se midió el área de crecimiento del micelio. El mejor efecto antifúngico se observó en el aceite esencial de *Thymus vulgaris*, con una concentración mínima inhibitoria (CMI) del 0,05 %. El aceite esencial de *Cymbopogon flexuosus* mostró una concentración mínima inhibitoria del 0,08 %, mientras que el aceite esencial de *Syzygium aromaticum* mostró el porcentaje de concentración efectiva más alto, del 0,6 %. La estimación de las concentraciones mínimas inhibitorias proporciona pruebas de que todos los aceites evaluados eran fungistáticos.

Palabras clave: *Aspergillus flavus*, aceites esenciales, diseño de experimentos.

Abstract

Most of the diet that is consumed every day is treated with additives, preservatives and other chemical additives to keep the food in good condition for a longer period of time. Today, it has been demonstrated that the general public has expressed a degree of interest. in consuming food that is free of chemical additives, has high nutritional value and is an alternative in disease prevention. In this investigation, the antifungal effect of three essential oils is analyzed using growth inhibition bioassays on the mycelia of *Aspergillus flavus*. After five days of incubation, the area of mycelial growth was measured. The best antifungal effect was observed in *Thymus vulgaris* essential oil with a minimum inhibitory concentration (MIC) of 0.05%. *Cymbopogon flexuosus* essential oil showed a minimum inhibitory concentration of 0.08%, while *Syzygium aromaticum* essential oil showed the highest effective concentration percentage of 0.6%. The estimation of minimum inhibitory concentrations provides evidence that all oils evaluated were fungistatic.

Keywords: *Aspergillus flavus*, Essential Oils, Design of Experiments.

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Resumo

A maioria dos alimentos que consumimos diariamente é tratada com aditivos, conservantes e outros ingredientes químicos para manter os alimentos em boas condições durante um intervalo de tempo mais extenso. Atualmente, as pessoas estão interessadas em consumir alimentos que não contenham aditivos químicos, que tenham um elevado valor nutricional e que sejam uma alternativa na prevenção de doenças. Nesta proposta de investigação, o efeito antifúngico de três óleos essenciais é analisado através de bioensaios de inibição do crescimento dos micélios de *Aspergillus flavus*. Depois de cinco dias de incubação, foi medida a área de crescimento micelial. O melhor efeito antifúngico foi observado no óleo essencial de *Thymus vulgaris* com uma concentração inibitória mínima (CIM) de 0,05%. O óleo essencial de *Cymbopogon flexuosus* apresentou uma concentração mínima de inibição de 0,08%, enquanto o óleo essencial de *Syzygium aromaticum* apresentou a maior concentração percentual de 0,6%. Todos os óleos avaliados foram fungistáticos.

Palavras-chave: *Aspergillus flavus*, óleos essenciais, desenho experimental.

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Introduction

In the official Mexican standard NOM-187-SSA1 / SCFI-2002 the term "aflatoxins" is defined as secondary metabolites produced by several fungal species. These chemicals possess a heterocyclic chemical structure and are classified as *bifuranocoumarins*. They have acute and chronic toxicity, as well as mutagenic and carcinogenic effects in animals and humans. They are fungi with toxic mycotoxigenic potential and belong mainly to the genus *Aspergillus, Fusarium* and *Penicillium*. Aflatoxins, produced by the *Aspergillus* genus can contaminate corn at various stages; these toxins have negative health effects at very low concentrations.

Aspergillus flavus and *Aspergillus parasiticus* are the aflatoxigenic species with agricultural and health significance and belong to the *flavi* section (Peterson et al., 2000). They are the most potent carcinogens known and are therefore the most studied and controlled mycotoxins (Martínez et al., 2013). Some these compounds inhibit fungal growth under *in vitro* conditions (Hidalgo et al., 2002). More than 280 species of plant extracts, such oils and phenolic compounds have been tested for their toxic effects, of which one hundred have shown some activity on fungal growth (Pepeljnjak et al., 2003). These compounds are

aromatic fatty liquids obtained from a plant raw material such as flowers, seeds, leaves, branches, fruits, etc. Presently, there is a notable level of interest among the population in consuming food without chemical additives and the demand for natural preservatives is increasing, while the safety aspect of chemical additives is being questioned (Chanthaphon et al., 2008). The essential oils of thyme, clove, lemon grass, among others, have shown antimicrobial activity (Velazquez et al., 2014). The effect of essential oils is favored by low temperatures, pH and oxygen levels (Burt, 2004 and Aprotosoaie et al., 2019). This could provide the necessary nutrients for the development of fungi, probably based on essential oil. These essential oils can be used as a substitute for chemical fungicides as they are safe and biodegradable. The antifungal activity of these essential oils is due to their content of eugenol and carvacrol, respectively (Klaric et al., 2006). Such oils could be safely used as preservatives in some types of foods at low concentrations (Fernández et al., 2019).

Literature review

Essential oils derived from aromatic plants and some spices have been shown to have antimicrobial effects on yeasts, molds and bacteria (Elgayyar et al., 2001). The practice of food preservation has been in existence for multiple centuries. Throughout this time, various products have been preserved. In the contemporary era, there is a heightened demand for natural, safe, and high-quality food. This demand has prompted the development of novel technologies that ensure food safety (Graça et al., 2010). Nowadays, it is known that some essential oils have a potential use in food, being able to become an alternative to reduce or replace traditional antimicrobial agents (Velazquez et al., 2014).

Essential oils

Originally, these substances were studied from an aromatic point of view and as flavoring agents, but today it is known that essential oils and their chemical components have a use as antimicrobial and antioxidant agents (Sacchetti et al., 2005). The bactericidal activity of essential oils has been reported by several authors. This activity could be related to the specific composition of the volatile oils of each plant, the structural configuration of the components of the oils, their functional groups and possible synergistic interactions between their components (Dorman & Deans, 2000). The hydrophobicity of these substances allows them to be incorporated into the lipids of bacterial membranes, causing disturbances in their

structure and permeability, leading to the leakage of ions and other compounds (Borugă et al., 2014). For this reason, several researchers mention that the antimicrobial activity depends mainly on three characteristics: the chemical components present in the essential oil, the hydrophilic or hydrophobic character, and the type of microorganisms to be attacked (Hidalgo et al., 2002).

Mycotoxins

Mycotoxins, defined as chemical substances produced by fungi, have been demonstrated to induce disease and death in humans and animals. Mycotoxins are secondary fungal metabolites formed by a series of sequential reactions catalyzed by biochemical intermediates of the primary metabolism (Martínez et al., 2003). The production of mycotoxins is associated with the sporulation process of the fungus, which is closely related to environmental conditions and the concentration of nutrients in the environment. The diseases caused by mycotoxins are known as mycotoxicosis (Logrieco et al., 2002). In high concentrations, mycotoxins can produce acute disease syndromes, while in low concentrations they are carcinogenic, mutagenic, teratogenic and produce mitotic changes (Mitotic changes refer to the specific, orderly, and sequential structural modifications a cell undergoes to divide its replicated DNA and cytoplasm into two genetically identical daughter cells. These changes, occurring in prophase, metaphase, anaphase, and telophase, include chromosome condensation, spindle formation, and nuclear envelope breakdown to ensure equal division), (Peterson, 2000). They affect the liver, kidney, nervous, endocrine and immune systems.

Aflatoxins

Aflatoxins are mycotoxins produced by toxigenic strains of the fungi *Aspergillus flavus* and *Aspergillus parasiticus* (García et al., 2006). These substances are highly carcinogenic, causing toxicity and liver cancer. They have been detected in various crop fields, during harvest, transport and home storage. According to Acuña et al., (2005), corn is easily contaminated, aflatoxins are usually designated with letters that refer to a physical or other characteristic of the compound, for example, B1 and B2 have blue fluorescence and G1 and G2, green fluorescence when exposed to long-wave ultraviolet radiation. Toxigenic strains of *Aspergillus flavus* generally produce only aflatoxin B1 and B2, whereas toxigenic

strains of *Aspergillus parasiticus* produce aflatoxin B1, B2, G1, and G2 (Bogantes-Ledezma et al., 2004). Those responsible for public health are faced with a complex problem: mycotoxins should be eliminated from food, but since these secondary metabolites are present in certain functional foods, some exposure of the population to mycotoxins should be tolerated. The mandatory limits for aflatoxin content in foods susceptible to them have been set very close to the detection limit of the analytical methodology, based on the principle that there is no known safe level for humans; the standard issued by the U.S. Food and Drug Administration (FDA) for primary agricultural foods and their derivatives is 20 µg/kg total aflatoxins. The Mexican Official Standard (NOM-187-SSA1/SCFI- 2002), states that the limit maximum aflatoxin is 12 mg/kg in corn products such as dough, tortillas nixtamal, toasted corn nixtamalized and nixtamalized cornmeal to prepare tortillas and toasts.

Materials and methods

Essential oils

Three commercial essential oils (Piping Rock ®) were evaluated for antifungal activity: Thyme (*Thymus vulgaris*), Lemon grass (*Cymbopogon flexuosus*), and Clove (*Syzygium aromaticum*).

Inoculum Preparation

For the preparation of the *inoculum*, strains of the fungus *Aspergillus flavus* were used, preserved in Petri plates with potato dextrose agar (PDA). The agar plates seeded with the fungus were extracted with an inoculating needle handle, taking a colony isolated from the organism to transfer to a new *Petri* plate containing up to 30 mL of potato dextrose agar solution, leaving the fungus at a temperature of 28 °C for five days.

Preparation of PDA

In order to achieve the targeted concentration of 0.5% essential oil, a previously disinfected vertical laminar flow hood is utilized, along with a micropipette, to meticulously measure and transfer approximately 1.5 mL of essential oil into a graduated centrifuge tube. Subsequently, the prepared PDA solution was added until the total volume reaches 30 mL. The mixture was then thoroughly stirred, transferred to Petri dishes, and allowed to gel. This procedure was repeated for various types of essential oils and different concentrations in this

initial experiment. To enhance the experiment's reliability, it was replicated on three separate occasions.

Once the *Petri* dishes have been prepared with different essential oils and different concentrations, the *Aspergillus flavus* fungus was inoculated. This is achieved with the assistance of a timer, which allows for the generation of inoculums with an area of approximately a dimeter of 0.44 cm^2.

The *Petri* dishes were sealed with parafilm tape and stored in a room maintained at a temperature of 28°C. The growth area was measured at 24-hour intervals using the Image Tool Software® until 120 hours had elapsed. Additionally, a control experiment was conducted, which did not contain any type of oil. Note: The strains of Aspergillus flavus were provided by CINVESTAV (Center for Research and Advanced Studies of the National Polytechnic Institute, Mexico).

Statistical analysis

A statistical analysis was conducted on the mycelial growth results. This involved the use of analysis of variance (ANOVA), a general linear model (GLM), and a comparison of means using the Dunnett test, all of which were performed using the Minitab 18® statistical program.

Results

In the following section of this report, the results of the study are presented and discussed. In all cases, an alpha error of 0.05 was considered. The measurements were of mycelial growth in cm^2 (Area). The evaluation of homoscedasticity and independence in all analyses yielded satisfactory results. The normality was not satisfactory thus the data were ranked to assure normality.

As an initial step in the experimental process, it was essential to ascertain the minimum concentration levels of the selected essential oils. This was done in order to establish a reference point with respect to the minimum concentration at which the *Aspergillus flavus* growth process begins. This is a crucial point of reference in subsequent experimental processes, as it allows for the determination of the minimum concentration required for fungal inhibition.

The growth of the fungus Aspergillus Flavus was evaluated in vitro with essential oils of clove, lemon grass, and thyme at concentrations of 0.5%, 1.0%, and 2.0%. The ranges were calculated and evaluated in an ANOVA with the assistance of Minitab 18® using the data obtained from the growth areas. The results are presented in Table 1. The blocks, which represent the three levels of repetition in the experiment, comprise the three different types of essential oil and varying concentrations at three levels, as well as the five levels of time. These blocks are then subjected to analysis, the arrange included:

- Essential oil, three levels (clove, lemon grass, and thyme)
- Concentration, three levels (0.5%, 1.0%, and 2.0%)
- Time, five levels (24, 48, 72, 96 and 120 hours)
- Blocks, The experiment was replicated 3 times, each replicate is represented as a block.

Table 1. Analysis of Variance, General Experiment

Source	DF	Adj SS	Adj MS	F- Value	*p*- Value
Replicate	2	1.7	0.87	0.00	0.996
Essential oil	2	15187.5	7593.75	30.75	0.000
Concentration	2	15187.5	7593.75	30.75	0.000
Time	4	28.5	7.12	0.03	0.998
Error	124	30619.3	246.93		
Total	134	61024.5			

Source: Authors' elaboration

The calculated p-value for the replicates and time exceeded 0.05, indicating that these variables are not statistically significant; this is not the case for the concentration and type of essential oil. The experiments were conducted separately for each essential oil (thyme, clove, and lemon grass) with time in hours and the percentage of essential oil concentration as factors. Replicates are not of interest in the study; however, they have been included as a means of strengthening the experiment, given that they are expected to be insignificant.

The Degrees of Freedom (DF), Sum of Squares (SS) and Middle Squares (MS) for the essential oil and the concentration resulted exactly the same, this is an unfortunate

coincidence (it must be considered that the data was ranked due to lack of normality). There is no error in the experimental proceeding nor calculations.

Clove essential oil experiment

The objective of this experiment is to examine the effects of clove essential oil on mycelial growth of *A. flavus*.

Subsequently, tests were conducted with concentrations of 0.3%, 0.4%, 0.5%, and 0.6% of essential clove oil for 120 hours, with growth area readings taken every 24 hours. This was done to ascertain whether the essential oil of clove exhibited growth at a concentration of 0.5%. An ANOVA was performed on the values of the growth areas (Table 2), using a general linear model. The factors included in the analysis were the blocks (the three replicates), the concentration of essential oil (0.3%, 0.4%, 0.5%, 0.6% and 0.0%, as control), and time in hours (24, 48, 72, 96 and 120 hours):

- Two factors: Concentration in five levels and Time in five levels
- Three blocks: each run replicated three times.

The results of the analysis provide the ranges of the growth areas in cm^2. The results are presented in accordance with the foregoing.

Table 2. ANOVA, General Linear Model for the Growth Area Ranges for Clove Essential Oil

Source	DF	Adj SS	Adj MS	F- Value	*p*- Value
Replicate	2	45.5	22.75	1.07	0.348
Concentration	4	28512.8	7128.21	336.05	0.000
Time	4	5085.6	1271.41	59.94	0.000
Error	64	1357.5	21.21		
Total	74	35001.5			

Source: Authors' elaboration

The p-value for the replicates is 0.348 greater than 0.05, indicating that they are not statistically significant. However, this does not apply to the concentration and time variables.

The coefficient of determination R^2 is 96.12%, with an adjusted R^2 of 95.52%, indicating strong model fit.

In order to ascertain which concentrations of clove oil are effective in inhibiting the growth of the fungus *Aspergillus flavus*, a Dunnett test is employed, comparing the results to a control experiment whit an untreated control. The analysis of variance is performed on the areas in order to obtain the Mean Square Error (MSE), for this purpose, interactions are included in the model (see Table 3).

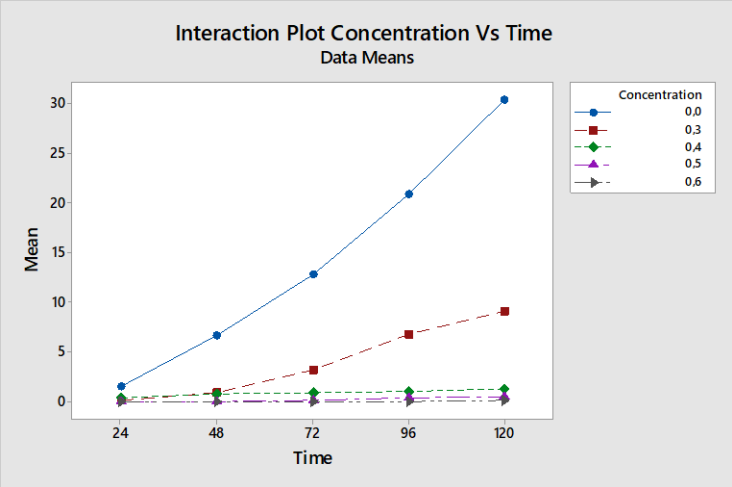
Table 3. ANOVA, General Linear Model; Clove Essential Oil

Source	DF	Adj SS	Adj MS	F- Value	*p*- Value
Replicate	2	0.88	0.440	3.16	0.056
Concentration	4	2239.87	559.967	4018.92	0.000
Time	4	605.21	151.303	1085.91	0.000
Replicate * Concentration	8	2.49	0.311	2.23	0.051
Replicate *Time	8	1.01	0.126	0.90	0.525
Concentration * Time	16	1165.15	72.822	522.65	0.000
Error	32	4.46	0.139		
Total	74	4019.07			

Source: Authors' elaboration

The p-value for the interaction between repetitions and time, as well as repetitions and concentration, is greater than 0.05, indicating that the interaction is not significant. However, this is not the case for the remaining interactions. Figure 1 depicts the interaction between different concentrations and time. It can be observed that as the concentration increases, the growth of the fungus decreases.

Figure 1. Interaction between the Different Concentrations of Clove Essential Oil against Time



Source: Authors' elaboration.

The mean values for each treatment of the growth areas (percentage of essential oil) are calculated. These averages, along with the mean square error (MSE) and degrees of freedom of Table 3 and Dunnett ranges, are used to perform the Dunnett test. In this test, assuming that treatment 1 is the control, the following values are calculated: a = 5 (a is the number of treatments), a-1 = 4, f = 32 (f are the degrees of freedom of error). The value d 0.05 (4,32) = 2.246 is then found by interpolating the values from the table provided by Montgomery (2012) and using a level of significance of 0.05. Consequently, the critical difference is calculated as follows:

$$d_{0.05}(4,32)\sqrt{\left(\frac{2\,MSE}{n}\right)} = (2.246)\sqrt{\frac{(2)(0.139)}{5}} = 0.5295$$

Accordingly, a treatment should be regarded as significantly different from the control if the discrepancy exceeds 0.5295 or is less than –0.5295. The calculations use the average percentage concentration per level and the average control growth reading (14.4273), which is used in all comparisons. The observed differences are as follows:

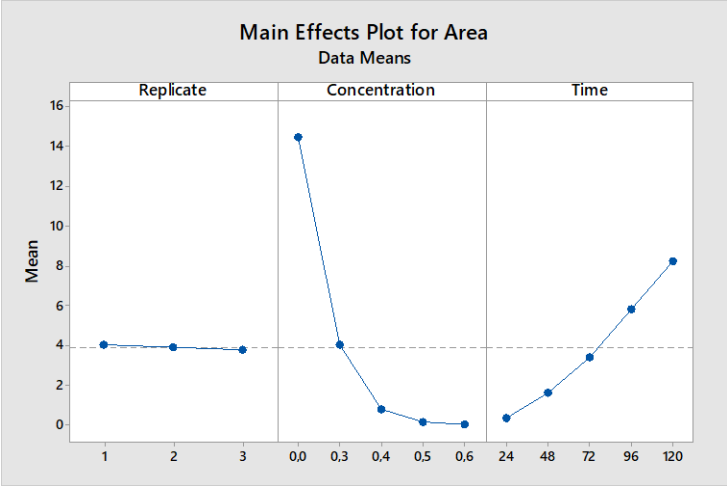
Table 4. Dunnett Comparison Clove *Versus* Control

Comparison	Treatments	Difference	Absolute Difference	Criteria	Desicion
Clove 0.6% Vs Control:	$\bar{y}_2 - \bar{y}_1 =$	0.0120-14.4273 = -14.4153	14.4153	0.5295	Significative
Clove 0.5% Vs Control:	$\bar{y}_3 - \bar{y}_1 =$	0.1693-14.4273 = -14.2580	14.2580	0.5295	Significative
Clove 0.4% Vs Control:	$\bar{y}_4 - \bar{y}_1 =$	0.8360-14.4273 = -13.5913	13.5913	0.5295	Significative
Clove 0.3% Vs Control:	$\bar{y}_5 - \bar{y}_1 =$	3.6420-14.4273 = -10.7853	10.7853	0.5295	Significative

Source: Authors' elaboration

As Shown on table 4, the results demonstrate that at all evaluated concentrations, there is a notable distinction when compared to the control. Consequently, it can be inferred that when assessing the effects of clove essential oil at concentrations of 0.3%, 0.4%, 0.5%, and 0.6%, there is a discernible inhibition of the *Aspergillus flavus* fungus. Consider here that the design used is a fixed effects model, that is, of the selected levels, the best one is observed (the lowest is best), but it is not necessarily the optimal one (MIC).

Figure 2. Clove Essential Oil Main Effects Plot.



Source: Authors' elaboration.

As illustrated in Figure 2, the graph of the main effects, the lower the oil concentration, the greater the growth area and the greater the elapsed time, thus indicating an increased possibility of growth area.

Lemon grass experiment

The objective of the experiment was to investigate the effects of lemon grass on fungal growth.

In the general experiment, no growth of fungi was observed in the lemon grass essential oil at concentrations of 2%, 1%, and 0.5%. Consequently, the decision was made to lower the concentrations to 0.05%, 0.06%, 0.07%, 0.08%, 0.09% and 0.0% as the control. These were then analyzed again under the same conditions as the other experiments. The experiment was conducted three times, with each repetition representing a block of the experiment. The concentration was maintained at six levels, and the time was set every 24 hours, for five days. These variables were then subjected to an ANOVA and a general linear model, as detailed in Table 5. In the ANOVA analysis, interactions were considered.

Table 5. ANOVA, General Linear Model for Lemon Grass Essential Oil.

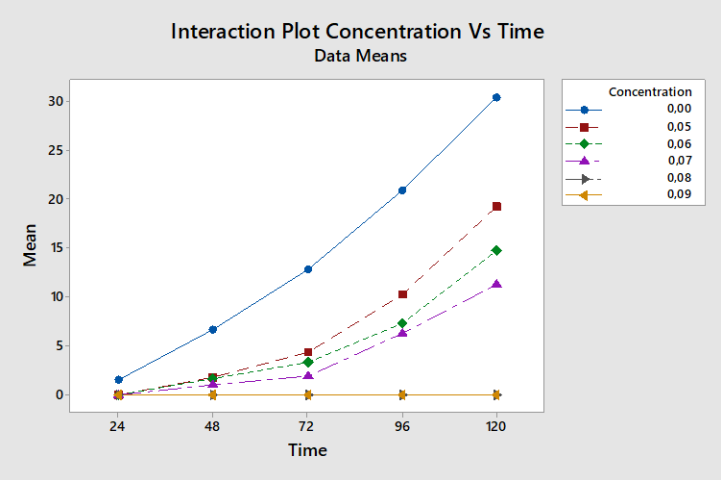
Source	DF	Adj SS	Adj MS	F- Value	*p*- Value
Replicate	2	0.98	0.490	4.27	0.021
Concentration	5	2163.62	432.724	3766.60	0.000
Time	4	1767.52	441.881	3846.30	0.000
Replicate *Concentration	10	2.48	0.248	2.16	0.042
Replicate *Time	8	1.07	0.134	1.17	0.343
Concentration*Time	20	1233.21	61.661	536.72	0.000
Error	40	4.60	0.115		
Total	89	5173.48			

Source: Authors' elaboration.

The p-value calculated for the concentration, blocks, time, and some interactions is less than 0.05, indicating that they have a significant effect on the growth of the fungus. However, this is not the case for the interaction between the blocks and time, for which the p-value is 0.343. The coefficient of determination R^2 is 99.91%, with an adjusted R^2 of 99.80%, indicating a strong model fit. Figure 3 depicts the interaction between different

concentrations and time, demonstrating that as the concentration of lemon grass essential oil decreases, the growth of the fungus increases.

Figure 3. Interaction between the Different Concentrations of Lemon Grass Essential Oil Against Time.



Source: Authors' elaboration.

The Dunnett test is employed to ascertain whether the concentrations of lemon grass essential oil can impede the growth of the *Aspergillus flavus* fungus. The mean growth areas for each concentration of essential oil are calculated. The mean square of the error and the degrees of freedom of the errors, as presented in Table 6, are used together with the Dunnett ranks to obtain the following results:

$$d_{0.05}(5,40)\sqrt{\left(\frac{2\,MSE}{n}\right)} = (2.310)\sqrt{\frac{(2)(0.115)}{6}} = 0.4522$$

Therefore, a treatment should be considered significantly different from the control if the difference is greater than 0.4522 or less than -0.4522. The observed differences are:

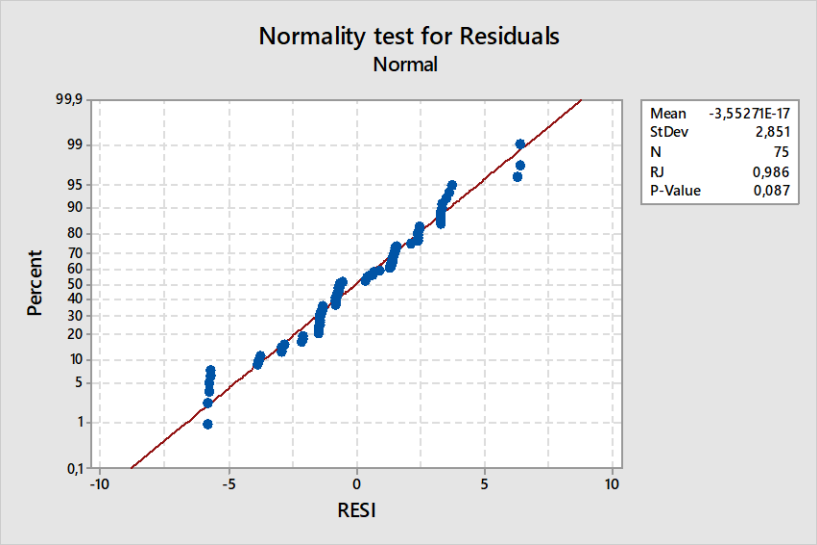
Table 6. Dunnett Comparisons Lemon Grass *Versus* Control.

Comparison	Treatments	Difference	Absolute Difference	Criteria	Desicion
Lemon grass 0.09 % Vs Control:	$\bar{y}_2 - \bar{y}_1 =$	0.0000-14.4273 = -14.4273	14.4273	0.4522	Significative
Lemon grass 0.08 % Vs Control:	$\bar{y}_3 - \bar{y}_1 =$	0.0000-14.4273 = -14.4273	14.4273	0.4522	Significative
Lemon grass 0.07 % Vs Control:	$\bar{y}_4 - \bar{y}_1 =$	4.0780-14.4273 = -10.3493	10.3493	0.4522	Significative
Lemon grass 0.06 % Vs Control:	$\bar{y}_5 - \bar{y}_1 =$	5.4240-14.4273 = -9.0033	9.0033	0.4522	Significative
Lemon grass 0.05 % Vs Control:	$\bar{y}_6 - \bar{y}_1 =$	7.1266-14.4273 = -7.3007	7.3007	0.4522	Significative

The results on table 6 indicate that, in all the evaluated concentrations, there is a significant difference when compared to the control. Therefore, it can be concluded that the concentrations of 0.09%, 0.08%, 0.07%, 0.06% and 0.05% have a certain degree of inhibitory effect on the *Aspergillus flavus* fungus (Growth at 0.09% and 0.08% was 0.0000 in both cases).

In order to estimate the coefficients of a regression model by least squares, it is sufficient to assume linearity. The normality of the data is also based on the assumptions of normality and homoscedasticity. Therefore, residues from the growth areas of the lemon grass essential oil were generated to ensure that the aforementioned conditions were met. In the analysis of the residues, it was determined that the i-th residual and i = 1, ..., n originated from a normal population with a mean of 0 and variance σ^2 of 1 through the use of Ryan Joiner test, similar to Shapiro-Wilk, which is a chi-square test (see Figure 4).

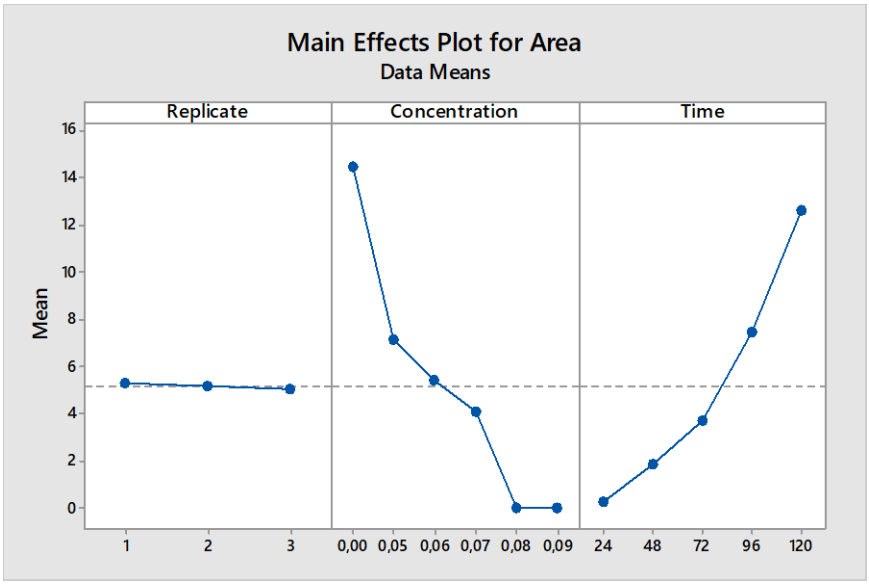
Figure 4. Lemon Grass Normality Test.



Source: Authors' elaboration.

The calculated p-value (the probability of committing an alpha error) of Figure 4 is 0.087, which is greater than 0.05, indicating that the data is normal. Figure 5 shows the graph of main effects. In the replicates, there was no variation; considering the average per level, the minimum concentration of inhibition for the fungus *Aspergillus flavus* with lemon grass essential oil is 0.08%, since the aim is to minimize the use of essential oil.

Figure 5. Lemon Grass Main Effects Graph.



Source: Authors' elaboration.

Thyme experiment

In the general experiment, concentrations of 0.5%, 1.0% and 2.0% of thyme essential oil were employed. At these concentrations, no growth of the *Aspergillus flavus* fungus was observed. Consequently, as a second step, the concentrations were reduced to 0.05%, 0.1% and 0.2%. However, there was no fungal growth. Therefore, on a third step, the concentrations were reduced to 0.02%, 0.03%, 0.04% and 0.05% of thyme essential oil. These lower concentrations were tested under the same conditions of temperature and time, and the experiment was performed three times. An ANOVA was conducted on the growth areas obtained from the experiment, using a general linear model for the essential oil of thyme. The results are presented in Table 7, which analyses the concentration of essential oil of thyme at four levels (plus control), the time at five levels and the blocks at three levels (replicates).

Table 7. Analysis of Variance, General Linear Model for Thyme Essential Oil.

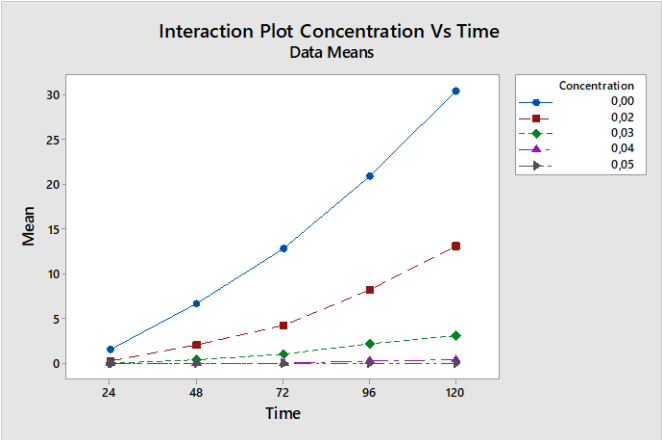
Source	DF	Adj SS	Adj MS	F- Value	*p*- Value
Replicate	2	0.51	0.256	1.75	0.190
Concentration	4	2234.45	558.612	3816.08	0.000
Time	4	783.14	195.784	1337.47	0.000
Replicate*Concentration	8	2.81	0.351	2.40	0.037
Replicate*Time	8	0.94	0.117	0.80	0.605
Concentration*Time	16	1143.90	71.494	488.40	0.000
Error	32	4.68	0.146		
Total	74	4170.43			

Source: Authors' elaboration.

The calculated p-value for the blocks and the interaction between the blocks and the time are 0.190 and 0.605, respectively. These values indicate that the blocks (Replicates) and the replicate*time interaction are irrelevant for the experiment; however, they are not so for the concentrations and the time and the other interactions. A satisfactory linear adjustment can be made on the basis of the coefficient of determination R^2 (99.89%) and the adjusted R^2

(99.74%). Figure 6 illustrates the interaction between the various concentrations and time. It can be observed that as the concentration of thyme essential oil decreases, the growth of the fungus increases.

Figure 6. Interactions between Different Concentrations of Thyme Essential Oil Against



Source: Authors' elaboration

The objective of Dunnett's test is to ascertain whether the concentrations of Thyme essential oil employed impede the growth of the *Aspergillus flavus* fungus. The mean growth areas for each concentration of essential oil are calculated from Table 5. By employing the mean square of the error and the degrees of freedom pertaining to the treatments and error, as delineated in Table 5, in conjunction with the Dunnett ranks, we arrive at the following:

$$d_{0.05}(4,32)\sqrt{\left(\frac{2\,MSE}{n}\right)} = (2.246)\sqrt{\frac{(2)(0.146)}{5}} = 0.5427$$

It can be concluded that a treatment should be considered significantly different from the control if the observed difference is greater than 0.5427 or less than -0.5427. The differences observed are as shown on table 8:

Table 8. Dunnett Comparison Thyme *Versus* Control.

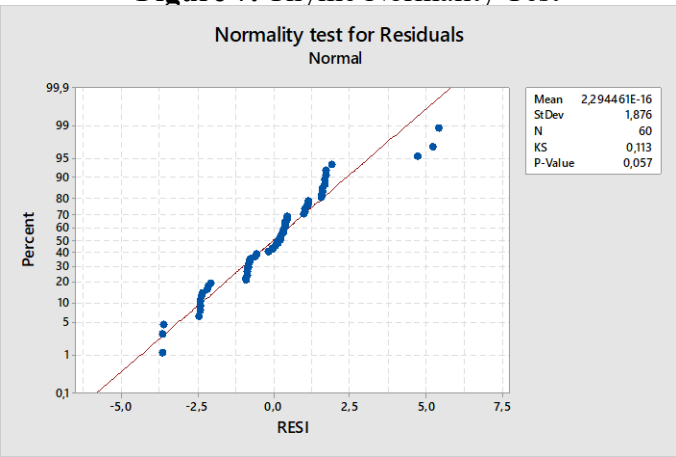
Comparison	Treatments	Difference	Absolute Difference	Criteria	Desicion
Thyme 0.05 % Vs Control:	$\bar{y}_2 - \bar{y}_1 =$	0.0000-14.4273 = -14.4273	14.4273	0.5427	Significative
Thyme 0.04 % Vs Control:	$\bar{y}_3 - \bar{y}_1 =$	0.1116-14.4273 = -14.3157	14.42315	0.5427	Significative
Thyme 0.03 % Vs Control:	$\bar{y}_4 - \bar{y}_1 =$	1.3140-14.4273 = -13.1133	13.1133	0.5427	Significative
Thyme 0.02 % Vs Control:	$\bar{y}_5 - \bar{y}_1 =$	5.5233-14.4273 = - 8.9040	8.9040	0.5427	Significative

Source: Authors' elaboration.

This indicates that in all the concentrations evaluated, there is a significant difference when compared to the control. Therefore, it can be concluded that when evaluating the concentrations of 0.05%, 0.04%, 0.03% and 0.02%, there is a certain degree of inhibition of the *Aspergillus flavus* fungus.

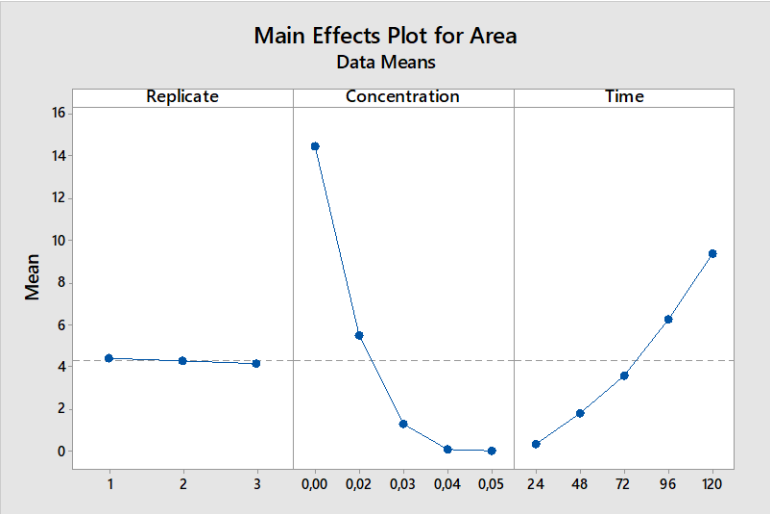
The residuals were plotted to ascertain whether the data exhibited a normal distribution. The resulting graph is presented in Figure 7. The p-value was found to be slightly greater than 0.05, indicating that the data were normally distributed. Figure 8 illustrates the main effects graph, which demonstrates that as time increases, microbial growth also rises. Notably, the lowest concentration of thyme essential oil that prevented the growth of the *Aspergillus flavus* fungus remained consistent at 0.05% at all time lapses.

Figure 7. Thyme Normality Test



Source: Authors' elaboration.

Figure 8. Thyme Main Effects Plot.



Source: Authors' elaboration.

In conclusion, the minimum inhibitory concentrations for essential oils are presented in Table 9. It is imperative to reiterate that the experimental design employed in this study corresponds to a fixed effects arrangement. This design involves the use of a successive approximation method, which involves the reduction of concentration levels in pursuit of the optimal response. The final selected level corresponds to the option that offers the greatest benefit (i.e., the lowest the best) among the levels studied, although it may not necessarily represent an overall optimum. Furthermore, the most significant difference is sought, so although an inhibitory effect is observed at other levels, the one that represents the greatest difference from the control has been selected.

Table 9. Minimum Inhibitory Concentrations of Essential Oils.

Essential oil	Minimum Concentrations Inhibitory
Clove	0.6%
Lemon Grass	0.08%
Thyme	0.05%

Source: Authors' elaboration.

Discussion

A significant number of studies has been dedicated to the research of the mechanisms of action of these essential oils. This research includes studies conducted by Nazzaro et al., (2017), Tabassum and Vidyasagar (2013), El-Shenawy et al., (2015), López-Romero et al., (2015), and Aparecida et al., (2015). These studies explore the mechanisms of action at the membrane level, growth, and morphology of inhibited cells. A plethora of studies have been conducted in the domain of ophthalmological diseases, this reference is important because it shows the variety and breadth that this research can have; the use of essential oils is not exclusive to the food industry. (Cannas et al., 2015); in addition, research has been undertaken utilizing such oils derived from fruits (Velásquez et al., 2014); the extraction and analysis of bioactivity in its application for food preservation has been investigated (Tongnuanchan and Benjakul, 2004).

In other cases, the inhibition of the development of pathogens in meat intended for human consumption has been explored (Bošković et al., 2013).

To the best of our knowledge this area has been extensively researched; however, no studies were identified that evaluate the effectiveness of the oils studied specifically in corn products (maize/corn, grains, tortillas, nixtamalized products, etc.), as searched in Springer, Scopus, Google Scholar, with literature reviewed from 2007 to date, using the keywords in this report. This is a pertinent contribution given the pervasive consumption of corn products in numerous Latin American countries and parts of North America.

Some important aspects have not been addressed in the reviewed literature, such as the shelf life of these oils (chemical stability of active compounds, loss of antifungal activity or organoleptic changes) and their susceptibility to external factors such as light, temperature and humidity. It is crucial that these aspects are included in future studies given that the literature reviewed extensively documents that these factors have a significant impact on the growth of these fungi.

Conclusions

This study reveals significant differences that were observed among oils derived from clove, thyme, and lemon grass against the growth of the fungus *Aspergillus flavus*. The lowest concentration showing significant inhibition identified was 0.05% of the essential oil of thyme, while the essential oils of clove and lemon grass required higher concentrations to achieve inhibition. Therefore, based on the findings, this oil can be considered as a potential alternative for the control of post-harvest spoilage diseases caused by *Aspergillus flavus* in maize/corn products. However, further studies are required to elucidate the mode of action and morphological alterations caused by this oil on *Aspergillus flavus*, as well as to ascertain the potential negative effects on the product. *In vitro* inhibition suggests potential, but requires validation *in situ/in vivo* or in real food matrices. Since the tests were *in vitro* in PDA, application in real foods requires further studies.

Future research lines

The results suggest that the methodology Design of Experiments is an effective approach for the advancement of new technologies and the expansion of knowledge in the understanding of the inhibitory effect of these oils on the *Aspergillus flavus* fungus. The present study demonstrated the potential for inhibiting mold growth in corn products through the use of essential oils. However, further analysis is necessary to assess the impact on flavor and product consistency (organoleptic and physicochemical properties). Another variable that warrants consideration is the type of packaging utilized, with the objective of evaluating whether plastic, glass, paper and other materials influence the growth of these fungi due to their possible permeability to oxygen and water vapor.

Conflicts of Interest: The authors declare no conflicts of interest.

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