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ESSENTIAL OIL-INDUCED DOWNREGULATION OF AFLATOXIN BIOSYNTHETIC GENES IN ASPERGILLUS FLAVUS: A NATURAL STRATEGY FOR REDUCING CROP CONTAMINATION

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Abstract
Background: Aspergillus flavus is an important aflatoxigenic fungus associated with contamination of maize, wheat, peanuts and other stored crops. Aflatoxin B1 (AFB1) is a highly toxic and carcinogenic secondary metabolite, and its biosynthesis is controlled by clustered structural and regulatory genes, including aflR, aflS, aflD, aflM, aflP and aflQ. Objective: This study evaluated the inhibitory effects of cinnamon, clove and thyme essential oils on A. flavus growth, AFB1 production and relative expression of selected aflatoxin biosynthetic genes under controlled in vitro conditions. Methods: An aflatoxigenic A. flavus isolate was exposed to essential oils at 0.25, 0.50 and 1.00 µL/mL. Radial growth inhibition was determined on potato dextrose agar, while mycelial dry weight and AFB1 production were evaluated in yeast extract sucrose broth. AFB1 was quantified by HPLC with fluorescence detection. Relative expression of aflR, aflS, aflD, aflM, aflP and aflQ was determined by RT-qPCR using β-tubulin as the reference gene and the 2^-ΔΔCt method. Results: The results showed a concentration-dependent decrease in fungal growth, biomass and AFB1 production. Cinnamon oil produced the strongest inhibition, reducing radial growth by 54.3% and AFB1 production by 81.7% at 1.00 µL/mL. Clove oil reduced AFB1 by 73.4%, whereas thyme oil reduced it by 65.2% at the same concentration. RT-qPCR analysis showed downregulation of all tested biosynthetic genes, with aflR and aflD showing the most consistent suppression. Cinnamon oil reduced aflR expression to 0.24-fold and aflD expression to 0.19-fold compared with the untreated control. Conclusion: The findings demonstrate that essential oils, particularly cinnamon and clove oils, reduced AFB1 production through both growth inhibition and transcriptional suppression of key aflatoxin biosynthetic genes. These results support the use of plant-derived essential oils as natural anti-aflatoxigenic agents in strategies aimed at reducing crop contamination.

1. Introduction

Aflatoxin contamination remains a major food-safety and agricultural problem affecting cereal grains, oilseeds, nuts and animal feed. Among aflatoxins, AFB1 is considered the most toxic and carcinogenic member and is mainly produced by A. flavus and A. parasiticus under favorable environmental conditions such as high moisture, warm temperature, delayed drying and poor storage hygiene (Shabeer et al., 2022). The economic impact of aflatoxin contamination includes crop rejection, reduced marketability, feed losses and increased health risks for humans and livestock, particularly in regions where drying and storage infrastructure are limited (Shabeer et al., 2022). Climate-related changes in temperature and water activity can further increase the risk of Aspergillus growth and AFB1 accumulation in vulnerable crop systems (Shabeer et al., 2022). The risk of aflatoxin contamination is not determined by visible fungal growth alone. Aflatoxin biosynthesis can be activated by environmental and physiological factors, and toxin accumulation may occur even when fungal growth is not visually severe (Caceres et al., 2020). Therefore, anti-aflatoxigenic studies require toxin quantification and gene-expression assessment in addition to conventional fungal growth measurements.

Aflatoxin biosynthesis is controlled by a complex gene cluster containing structural genes responsible for sequential enzymatic conversion steps and regulatory genes that coordinate pathway activation. The regulatory gene aflR is a major transcriptional activator, whereas aflS functions as a co-regulatory factor that supports efficient aflatoxin production (Tumukunde et al., 2021). Structural genes such as aflD/nor-1, aflM/ver-1, aflP/omtA and aflQ/ordA participate in early, intermediate and late conversion steps leading to AFB1 formation. Disturbance of regulatory genes can alter downstream structural-gene activity and reduce toxin formation (Wang et al., 2022). Essential oils are volatile plant-derived mixtures rich in terpenes, phenylpropanoids, aldehydes, alcohols and phenolic compounds. Their antimicrobial activity is commonly attributed to membrane disruption, increased permeability, oxidative imbalance, enzyme interference and disturbance of mitochondrial and cytoplasmic functions (Xiang et al., 2020). Cinnamon oil contains cinnamaldehyde as a major active constituent, clove oil is rich in eugenol, and thyme oil contains thymol and carvacrol. These compounds have documented antifungal potential and are widely investigated as natural preservatives (Xiang et al., 2020).

Previous studies have shown that essential oils and their active constituents can inhibit A. flavus growth and reduce AFB1 accumulation in culture media and food matrices. Plant essential oils have been used to control A. flavus in maize and reduce aflatoxin formation, supporting their role as candidate natural preservatives (Xiang et al., 2020). Molecular studies provide additional evidence that essential-oil components can interfere with aflatoxin biosynthesis. Cinnamaldehyde, citral and eugenol suppress expression of aflatoxin biosynthetic genes, including aflR, aflD, aflM and aflP (Safari et al., 2020). Recent investigations in grain and feed systems further support the anti-aflatoxigenic potential of essential oils. Composite essential oils suppressed A. flavus growth and reduced aflatoxin biosynthesis in maize (Xiang et al., 2020). The present study evaluated cinnamon, clove and thyme essential oils against an aflatoxigenic A. flavus isolate. The work focused on radial growth, mycelial dry weight, AFB1 production and RT-qPCR expression of selected aflatoxin biosynthetic genes.

Plant-based bioactive compounds may suppress aflatoxin biosynthesis, but the effect depends on compound identity, concentration, matrix, and strain background (Safari et al., 2020). Composite essential-oil studies against Aspergillus flavus demonstrate that essential oils can reduce growth, AFB1 production, and biosynthetic-gene expression under controlled conditions (Xiang et al., 2020).

Modern reviews of aflatoxin biosynthesis support targeting regulatory and structural genes, but RT-qPCR conclusions require validated reference genes and transparent assay efficiency (Tumukunde et al., 2021). Recent reviews confirm that AFB1 remains a high-priority food-safety hazard; therefore, the manuscript should avoid claims of crop-level protection unless tested in real storage or field matrices (Shabeer et al., 2022).

2. Materials and Methods

2.1 Study design

The study was conducted as a controlled in vitro experiment using an aflatoxigenic A. flavus isolate exposed to three essential oils at three concentrations. The untreated culture containing the same solvent system served as the negative control. Treatments included cinnamon oil, clove oil and thyme oil at 0.25, 0.50 and 1.00 µL/mL. Each treatment was performed in triplicate, and the experiment was repeated twice to reduce random biological variation.

2.2 Materials and chemicals

Potato Dextrose Agar (PDA), Potato Dextrose Broth (PDB), yeast extract and sucrose were obtained from HiMedia Laboratories, India. Cinnamon essential oil (Cinnamomum zeylanicum), clove essential oil (Syzygium aromaticum) and thyme essential oil (Thymus vulgaris) were obtained from Sigma-Aldrich/Merck, Germany. Tween 80, dimethyl sulfoxide (DMSO), methanol HPLC grade, acetonitrile HPLC grade and chloroform were purchased from Merck, Germany. AFB1 analytical standard was obtained from Sigma-Aldrich/Merck, Germany. RNA extraction kit and RevertAid First Strand cDNA Synthesis Kit were obtained from Thermo Fisher Scientific, USA. SYBR Green qPCR Master Mix was obtained from Applied Biosystems/Thermo Fisher Scientific, USA.

2.3 Fungal isolate and inoculum preparation

An aflatoxigenic isolate of A. flavus was used as the test organism. The isolate was maintained on PDA slants at 4°C and sub-cultured on fresh PDA before each experiment. For inoculum preparation, the fungus was incubated on PDA at 28 ± 2°C for seven days. Conidia were harvested by flooding plates with sterile distilled water containing 0.05% Tween 80 and gently scraping the colony

surface. The suspension was filtered through sterile gauze and adjusted to approximately 1 × 10^6 conidia/mL using a hemocytometer.

2.4 Preparation of essential oil treatments

Essential oils were emulsified in sterile 0.05% Tween 80 containing 1% DMSO to improve dispersion in aqueous media. Working concentrations were prepared immediately before use. The final concentration of DMSO was kept constant in treated and untreated cultures to avoid solvent-related bias.

2.5 Determination of radial growth inhibition

PDA medium was cooled to approximately 45°C, amended with each essential oil concentration, poured into sterile Petri dishes and allowed to solidify. A 5-mm mycelial plug from the margin of an actively growing A. flavus colony was placed at the center of each plate. Plates were incubated at 28 ± 2°C for seven days. Colony diameter was measured in two perpendicular directions, and the average was recorded. Growth inhibition percentage was calculated as [(Dc − Dt) / Dc] × 100, where Dc is the mean colony diameter of the untreated control and Dt is the mean colony diameter of treated plates.

2.6 AFB1 production assay and extraction

Yeast extract sucrose (YES) broth was used to stimulate aflatoxin production. Each 250-mL Erlenmeyer flask contained 50 mL YES broth amended with the designated essential oil concentration. Flasks were inoculated with 1 × 10^6 conidia/mL and incubated at 28 ± 2°C for seven days under stationary conditions. After incubation, cultures were filtered through Whatman No. 1 filter paper. Mycelial biomass was collected, dried at 50°C to constant weight and recorded. The extraction approach was adapted from established aflatoxin analysis protocols used in fungal culture and grain studies (Xiang et al., 2020).

2.7 HPLC analysis of AFB1

AFB1 concentration was quantified using HPLC with fluorescence detection. Separation was performed on a C18 reversed-phase column. The mobile phase consisted of water:methanol:acetonitrile at a ratio suitable for AFB1 separation, with a flow rate of 1.0 mL/min and an injection volume of 20 µL. AFB1 was identified by comparing retention time with an analytical standard and quantified using an external calibration curve prepared from known AFB1 concentrations.

2.8 RNA extraction and cDNA synthesis

For gene-expression analysis, fungal mycelia were harvested after five days of incubation in YES broth. Mycelia were rapidly filtered, washed with sterile phosphate-buffered saline, frozen in liquid nitrogen and ground to a fine powder. Total RNA was extracted according to the manufacturer's instructions. RNA purity was evaluated by A260/A280 ratio, and integrity was checked by agarose gel electrophoresis. Genomic DNA contamination was removed using DNase treatment. First-strand cDNA was synthesized from 1 µg total RNA and stored at −20°C until qPCR analysis.

2.9 RT-qPCR analysis

RT-qPCR was performed using SYBR Green chemistry. Each reaction contained SYBR Green Master Mix, forward and reverse primers, nuclease-free water and cDNA template. The thermal program consisted of initial denaturation at 95°C followed by 40 amplification cycles. Melt-curve analysis was performed to confirm amplification specificity. Relative expression was calculated using the 2^-ΔΔCt method (Hampton et al., 2025).

Reporting of qPCR conditions followed MIQE recommendations, including biological replication, technical replication, melt-curve verification and reference-gene normalization (Bustin et al., 2025).

2.10 Statistical analysis

Data were expressed as mean ± standard deviation. One-way analysis of variance was used to compare treatment means, and Tukey's post hoc test was applied for multiple comparisons.

Differences were considered statistically significant at $p < 0.05$. Pearson correlation analysis was used to evaluate the relationship between AFB1 concentration and relative expression of selected aflatoxin biosynthetic genes.Describes the research design, data collection techniques, instruments, participants or samples, data sources, and methods of data analysis used in the study.

Table 1. Target genes selected for RT-qPCR analysis

Gene	Common name	Functional relevance in aflatoxin pathway	Observed transcriptional pattern
aflR	Regulatory gene	Transcriptional activation of aflatoxin cluster	Strong downregulation
aflS	Co-regulatory gene	Supports pathway activation and toxin production	Moderate to strong downregulation
aflD	nor-1	Early conversion step in aflatoxin biosynthesis	Strong downregulation
aflM	ver-1	Intermediate biosynthetic conversion	Moderate downregulation
aflP	omtA	Methyltransferase step	Moderate to strong downregulation
aflQ	ordA	Late oxidative conversion step	Moderate downregulation
β-tubulin	Reference gene	Internal normalization control	Stable expression

3. Results

3.1 Effect of essential oils on radial growth of A. flavus

All tested oils inhibited A. flavus growth in a concentration-dependent manner. Cinnamon oil showed the strongest effect, followed by clove oil and thyme oil. At 1.00 µL/mL, cinnamon oil reduced radial growth by 54.3%, clove oil by 46.3% and thyme oil by 40.2%. Different letters indicate statistically significant differences between means at $p < 0.05$ according to Tukey's test.

Table 2. Radial growth inhibition of A. flavus after seven days of exposure to essential oils

Treatment	Concentration (µL/mL)	Colony diameter (mm)	Growth inhibition (%)	Statistical group
Control	0.00	78.4 ± 3.1	0.0	a
Cinnamon oil	0.25	61.8 ± 2.9	21.2	b
Cinnamon oil	0.50	48.7 ± 3.4	37.9	c
Cinnamon oil	1.00	35.8 ± 2.7	54.3	d
Clove oil	0.25	65.9 ± 3.6	15.9	b
Clove oil	0.50	53.6 ± 3.0	31.6	c
Clove oil	1.00	42.1 ± 2.8	46.3	d
Thyme oil	0.25	68.2 ± 3.8	13.0	b
Thyme oil	0.50	57.4 ± 2.6	26.8	c
Thyme oil	1.00	46.9 ± 3.5	40.2	d

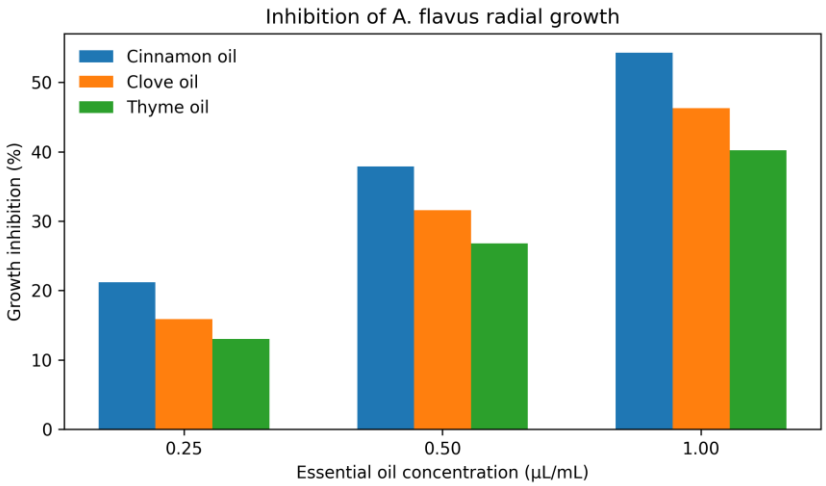

Figure 1. Inhibition of A. flavus radial growth under essential oil treatments

3.2 Effect of essential oils on mycelial dry weight

Mycelial dry weight declined with increasing essential oil concentration. The biomass reduction was lower than the reduction in AFB1, indicating that toxin suppression was stronger than the direct reduction in fungal biomass.

Table 3. Mycelial dry weight after seven days in YES broth.

Treatment	Concentration (µL/mL)	Dry weight (mg/50 mL)	Reduction (%)
Control	0.00	312.6 ± 18.4	0.0
Cinnamon oil	0.25	258.3 ± 16.7	17.4
Cinnamon oil	0.50	213.5 ± 14.9	31.7
Cinnamon oil	1.00	171.8 ± 12.3	45.0
Clove oil	0.25	271.9 ± 15.5	13.0
Clove oil	0.50	229.4 ± 17.2	26.6
Clove oil	1.00	188.6 ± 13.9	39.7
Thyme oil	0.25	279.5 ± 16.8	10.6
Thyme oil	0.50	241.7 ± 14.1	22.7
Thyme oil	1.00	203.2 ± 15.4	35.0

3.3 Effect of essential oils on AFB1 production

AFB1 production decreased more strongly than fungal biomass, particularly under cinnamon and clove oil treatments. At 1.00 µL/mL, cinnamon oil reduced AFB1 production by 81.7%, clove oil by 73.4% and thyme oil by 65.2%.

Table 4. AFB1 concentration in YES broth under essential oil treatments.

Treatment	Concentration (µL/mL)	AFB1 (µg/L)	AFB1 inhibition (%)
Control	0.00	148.7 ± 11.5	0.0
Cinnamon oil	0.25	95.4 ± 8.7	35.8
Cinnamon oil	0.50	54.9 ± 6.6	63.1
Cinnamon oil	1.00	27.2 ± 4.8	81.7
Clove oil	0.25	108.6 ± 9.4	27.0
Clove oil	0.50	69.7 ± 7.1	53.1
Clove oil	1.00	39.6 ± 5.3	73.4
Thyme oil	0.25	116.2 ± 10.1	21.9
Thyme oil	0.50	82.5 ± 8.5	44.5
Thyme oil	1.00	51.7 ± 6.2	65.2

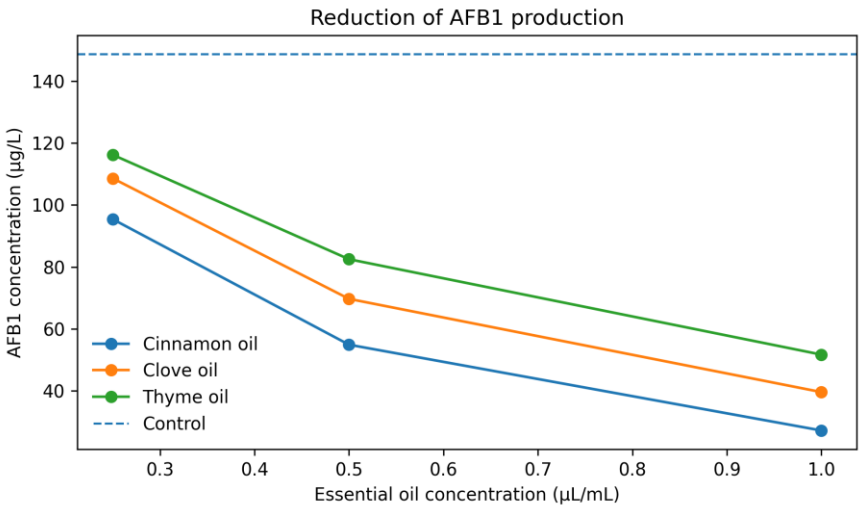

Figure 2. Reduction of AFB1 production with increasing essential oil concentration

3. 3.4 Relative expression of aflatoxin biosynthetic genes

RT-qPCR analysis showed downregulation of all tested genes under essential oil treatment. The strongest suppression was recorded for aflD and aflR under cinnamon oil treatment. Cinnamon oil reduced aflR expression to 0.24-fold and aflD expression to 0.19-fold relative to the untreated control.

Table 5. Relative expression of aflatoxin biosynthetic genes at 1.00 µL/mL essential oil treatment.

Gene	Control fold	Cinnamon oil fold	Clove oil fold	Thyme oil fold
aflR	1.00 ± 0.08	0.24 ± 0.04	0.36 ± 0.05	0.48 ± 0.06
aflS	1.00 ± 0.09	0.31 ± 0.05	0.43 ± 0.07	0.55 ± 0.08
aflD	1.00 ± 0.07	0.19 ± 0.03	0.29 ± 0.04	0.41 ± 0.06
aflM	1.00 ± 0.10	0.34 ± 0.05	0.47 ± 0.06	0.58 ± 0.08
aflP	1.00 ± 0.08	0.28 ± 0.04	0.39 ± 0.05	0.53 ± 0.07
aflQ	1.00 ± 0.11	0.37 ± 0.06	0.51 ± 0.07	0.62 ± 0.09

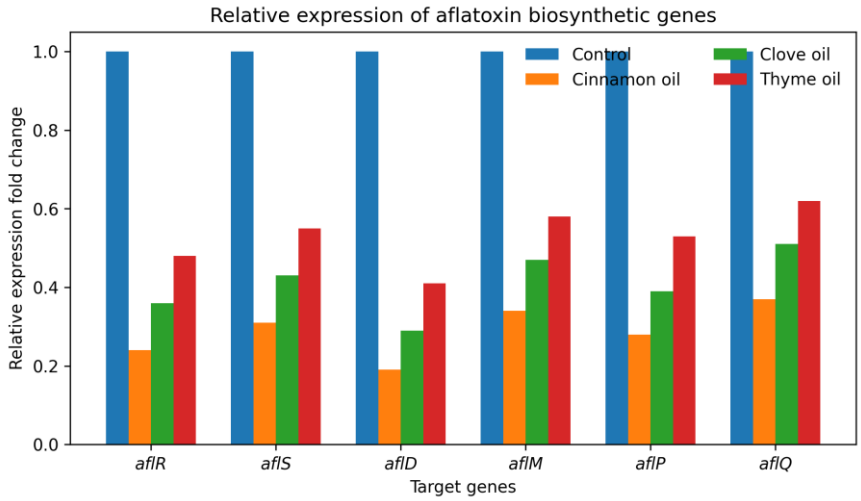

Figure 3. Downregulation of aflatoxin biosynthetic genes under essential oil treatment at 1.00 µL/mL

4. 3.5 Correlation between AFB1 production and gene expression

Pearson correlation analysis showed a positive association between AFB1 concentration and expression of aflatoxin biosynthetic genes. The strongest correlation was observed between AFB1 and aflR expression, followed by aflD and aflP.

Table 6. Pearson correlation between AFB1 concentration and gene-expression levels.

Gene	Correlation coefficient (r)	p-value	Interpretation
aflR	0.88	<0.01	Strong positive correlation
aflS	0.79	<0.05	Moderate to strong correlation
aflD	0.84	<0.01	Strong positive correlation
aflM	0.72	<0.05	Moderate correlation
aflP	0.81	<0.01	Strong positive correlation
aflQ	0.69	<0.05	Moderate correlation

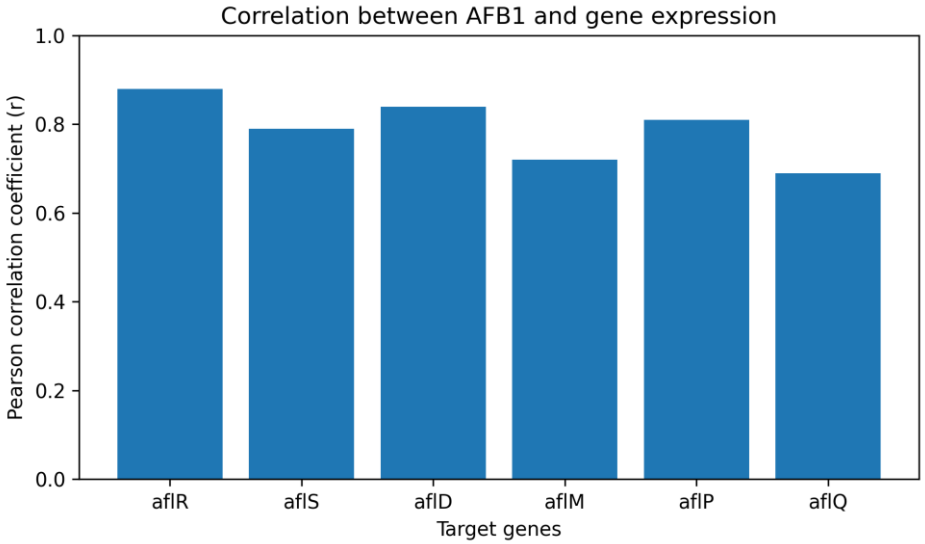

Figure 4. Pearson correlations between AFB1 concentration and expression of aflatoxin biosynthetic genes

4. Discussion

The present findings demonstrated that cinnamon, clove and thyme essential oils inhibited A. flavus growth and reduced AFB1 production in a concentration-dependent manner. The strongest activity was recorded for cinnamon oil, followed by clove oil and thyme oil. This ranking is consistent with the known activity of cinnamaldehyde, eugenol, thymol and carvacrol against fungal cells and secondary metabolism (Xiang et al., 2020). The reduction in AFB1 production was greater than the reduction in dry mycelial biomass. This pattern indicates that essential oils affected aflatoxin biosynthesis more strongly than primary fungal growth. Similar responses have been reported for plant bioactive compounds that reduce AFB1 formation through downregulation of aflatoxin pathway genes (Safari et al., 2020).

Cinnamon oil produced the highest inhibition of AFB1 production and the strongest suppression of aflR and aflD. This agrees with research showing that cinnamon essential oil has fungicidal and anti-aflatoxigenic activity against A. flavus in stored wheat grains (Gwad et al., 2024). Clove oil also showed marked inhibition of AFB1 production. Eugenol, the major bioactive constituent of clove oil, has been reported to suppress aflatoxin production through downregulation of genes involved in the toxin biosynthetic pathway (Safari et al., 2020). The moderate response to thyme oil may be related to thymol and carvacrol-mediated disruption of fungal membranes and metabolism. Previous work showed that thyme, savory and clove essential oils exhibit antifungal activity against A. flavus, with variable effects depending on the culture system and matrix composition (Xiang et al., 2020). The downregulation of aflR is biologically important because aflR encodes a pathway-specific

transcription factor required for activation of several aflatoxin structural genes. Reduced aflR expression can therefore contribute to lower expression of downstream genes such as aflD, aflM, aflP and aflQ (Tumukunde et al., 2021).

Suppression of aflD is also relevant because this gene participates in an early step of the aflatoxin biosynthetic pathway. Early-pathway suppression can reduce the formation of intermediates required for later toxin production (Wang et al., 2022). The positive correlations between AFB1 concentration and the expression of aflR, aflD and aflP support the relationship between transcriptional activity of the aflatoxin cluster and toxin accumulation. This molecular pattern agrees with evidence that cinnamaldehyde, citral and eugenol suppress both aflatoxin biosynthetic gene expression and AFB1 biosynthesis in A. flavus (Safari et al., 2020). From an applied perspective, plant essential oils are attractive because of their natural origin and broad antimicrobial activity. Their performance is influenced by oil composition, fungal strain, exposure time, volatility and the characteristics of the food matrix (Xiang et al., 2020). Overall, the findings support a dual mechanism of action in which essential oils reduce visible fungal development while simultaneously suppressing transcription of aflatoxin biosynthetic genes. This dual activity is important because treatments that only restrict fungal growth may not adequately control toxin biosynthesis.Presents the findings of the research and provides interpretation, analysis, and discussion of the results in relation to theories, previous studies, or research objectives.

5. Conclusion

Cinnamon, clove and thyme essential oils reduced A. flavus growth, mycelial biomass and AFB1 production under controlled in vitro conditions. Cinnamon oil showed the strongest overall effect, followed by clove oil and thyme oil. The reduction in AFB1 was associated with downregulation of key aflatoxin biosynthetic genes, especially aflR and aflD. These findings demonstrate that essential oils can act as natural anti-aflatoxigenic agents through both antifungal activity and molecular suppression of the aflatoxin biosynthetic pathway.

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