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Antifungal Efficacy Of Rhus Tripartita Essential Oil Against Aflatoxin- And Ochratoxin-Producing Aspergillus In Wheat

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Abstract. *Wheat is more than a staple crop in Algeria, it's a pillar of food security. Yet, across the warm silos of the western regions, something invisible and dangerous quietly spreads: Aspergillus fungi, capable of producing deadly mycotoxins. Confronted with this silent threat, we turned to Rhus tripartita, a resilient Saharan plant long valued in traditional medicine but rarely explored in food preservation contexts. We extracted its essential oil using hydro-distillation and found a modest yield–only 0.19%–but what it lacked in quantity, it made up for in biological strength. Chemical screening revealed a diversity of secondary metabolites, including phenolics and flavonoids, suggesting an innate capacity to fight microbial threats. When tested against two major mycotoxigenic species, A. flavus-parasiticus and A. ochraceus, the oil didn't just slow growth–it stopped it in its tracks, especially at higher concentrations. Minimum inhibitory concentrations (MICs) ranged between 1.15 and 1.23 µL/mL, with A. ochraceus proving more sensitive. In both diffusion and broth assays, results were clear: this oil disrupts fungal growth and limits toxin production, without the need for synthetic chemicals. The implications are significant. A plant that thrives under harsh desert conditions may hold the key to safer grain storage–and perhaps a more sustainable model for food protection in vulnerable regions. This study is not just a dataset. It's a step toward reconnecting with ancestral knowledge, rethinking how we preserve our food, and reaffirming that the answers to complex problems may sometimes lie in the roots of familiar plants.*

Keywords: *Rhus tripartita, Essential oil, Aspergillus, Aflatoxin, Ochratoxin, Antifungal activity, Mycotoxin*

1. INTRODUCTION

Mycotoxin contamination of cereals, particularly wheat, poses a major threat to food safety and public health worldwide. Aflatoxins and ochratoxins, produced predominantly by Aspergillus species, are among the most hazardous secondary metabolites, known for their hepatotoxic, nephrotoxic, and carcinogenic effects. Developing countries, including Algeria, are particularly vulnerable due to climatic conditions that favor fungal proliferation during storage and transport, and due to limited implementation of rigorous quality control systems.

In recent years, the extensive use of synthetic antifungal agents has raised significant concerns, notably the emergence of resistant fungal strains and the accumulation of chemical residues with adverse environmental impacts. These limitations have stimulated growing interest in eco-friendly alternatives derived from plant-based products. Essential oils (EOs), rich in bioactive compounds such as terpenoids and phenolics, have shown promising antifungal activity, offering a dual benefit of efficacy and biodegradability.

Despite the increasing global emphasis on natural preservatives, little is known about the occurrence of toxigenic Aspergillus strains in Algerian wheat imports and the potential use of indigenous plant species for fungal control. In this context, the present study pursues two main objectives: (1) to isolate and characterize aflatoxin- and ochratoxin-producing Aspergillus species from imported wheat samples in Western Algeria, and (2) to assess the antifungal efficacy of essential oil extracted from Rhus tripartita, a Saharan plant traditionally used in Algerian folk medicine.

Ethanolic extracts, from aerial parts of R. tripartita in the region of Bechar (south west of Algeria), showed a low inhibitory effect against Staphylococcus aureus, Escherichia coli, Enterobacter cloacae and Pseudomonas aeruginosa (Bereksi et al. 2018). While the chloroform extracts from Tunisian populations presented a strong

antibacterial activity against S. aureus and strong antifungal activity against the yeast Candida albicans (Abbasi and Hani 2011). Both chloroformic and ethanolic extracts from R. tripartita leaves in Libya showed that they have a weak antibacterial activity against P. aeruginosa and E. coli.
This investigation is, to our knowledge, the first to explore the application of R. tripartita EO as a bioactive agent against mycotoxigenic fungi in wheat, while contributing to the broader discourse on sustainable food preservation in arid regions.

2. MATERIALS AND METHODS

2.1 Essential Oil Extraction

The essential oil (EO) from Rhus tripartita leaves was extracted using hydrodistillation, a widely employed method for isolating volatile plant compounds while preserving their chemical integrity (Lahlou, 2004; Okoh et al., 2010). Fresh leaves were collected and air-dried in the shade for three days in a well-ventilated area to minimize degradation due to direct sunlight or high temperatures, which are known to affect essential oil composition (Bakkali et al., 2008). Exactly 100 g of dried plant material were immersed in 300 mL of distilled water within a 1-L round-bottom flask. The mixture was subjected to hydrodistillation for 2 hours using a Clevenger-type apparatus, in accordance with the protocol described by European Pharmacopoeia (2008). During this process, steam carried the volatile constituents through a condenser, and the resulting distillate was collected in a graduated receiver. Due to the difference in polarity and density between oil and water, the essential oil spontaneously separated from the aqueous phase (hydrosol) and was collected carefully with a Pasteur pipette. The distillates were dehydrated using anhydrous sodium sulfate (Na_2SO_4), filtered, and stored in airtight amber vials at 4°C to prevent oxidation and photodegradation prior to chemical and biological analysis (Burt, 2004; Messai&Touahria, 2021).

2.2 Essential Oil Yield Determination

The yield of essential oil (EOY) was calculated according to the standardized protocol described by the Association Française de Normalisation (AFNOR, 2000). This yield represents the efficiency of oil extraction and is expressed as a percentage relative to the mass of dry plant material. The equation used is:

$$EOY\ (\%) = (Mo / Mp) \times 100 \qquad \text{(AFNOR, 2000)}$$

where: Mo : Mass of the extracted essential oil (g), Mp : Mass of the dry plant material used (g). All extractions were performed in triplicate to ensure reproducibility, and results were expressed as mean ± standard deviation. This metric provides an important preliminary indicator of potential industrial scalability and cost-effectiveness of bioactive plant extracts (Simões et al., 2010).

2.3 Phytochemical Profiling

A preliminary phytochemical screening was performed to identify major classes of secondary metabolites present in the aerial parts of Rhus tripartita. The methodology followed well-established protocols described by Nemlin and Brunel (1995), Dohou et al. (2003), and Karaman et al. (2003), with slight modifications to adapt to the local laboratory conditions. A sequential exhaustive extraction was carried out using solvents of increasing polarity—petroleum ether, methanol, and distilled water—in order to ensure the solubilization of both lipophilic and hydrophilic phytoconstituents. For each extraction step, 20 g of finely powdered plant material were mixed with 60 mL of the respective solvent and subjected to continuous agitation at 200 rpm for 30 minutes at ambient temperature (25 ± 1 °C). The resulting mixtures were filtered using Whatman No. 1 filter paper, and the filtrates were concentrated under reduced pressure using a Büchi R-210 rotary evaporator at 40 °C. Extracts were stored at 4 °C until analysis.
The presence of specific secondary metabolite classes was determined through classical qualitative tests, as outlined below:

- Alkaloids: Detected using Mayer's and Wagner's reagents following the method of Harborne (1998). A yellowish-white precipitate with Mayer's reagent and a brown precipitate with Wagner's reagent indicated the presence of alkaloids.
- Anthraquinones (emodols): Evaluated using the Bornträger reaction, in which an orange-red to purple coloration upon treatment with 10% ammonium hydroxide (NH_4OH) confirmed the presence of anthraquinone derivatives (Evans, 2009).

• Coumarins: A portion of the ether extract was hydrolyzed and examined under UV light at 365 nm after treatment with 10% NH_4OH. The appearance of blue-green fluorescence confirmed the presence of coumarins (Bruneton, 1999).
• Sterols and triterpenes: Identified through the Liebermann–Burchard reaction, where the formation of a green-blue ring (sterols) or a red-purple coloration (triterpenes) at the interface of acetic anhydride/chloroform and concentrated sulfuric acid indicated a positive reaction (Khandelwal, 2008).
All qualitative tests were performed in triplicate, and appropriate positive and negative controls were used to validate the observations.

2.4 Quantitative Phytochemical Analysis

2.4.1 Total Phenolic Content (TPC)

The quantification of total phenolic content (TPC) was performed using a modified Folin–Ciocalteu colorimetric method, as described by Singleton et al. (1999). Briefly, 125 µL of the methanolic plant extract was mixed with 500 µL of distilled water and 125 µL of Folin–Ciocalteu reagent. After a 5-minute reaction period at room temperature (22 °C), 1.25 mL of 7% sodium carbonate (Na_2CO_3) solution was added. The final volume was adjusted to 3 mL with distilled water, and the mixture was incubated in darkness for 90 minutes to allow full color development. Absorbance was measured at 750 nm using a Shimadzu UV-1800 spectrophotometer. A standard calibration curve was established using gallic acid solutions (0–500 µg/mL). Results were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

2.4.2 Total Flavonoid Content (TFC)

Total flavonoid content (TFC) was estimated using the aluminum chloride ($AlCl_3$) colorimetric method, according to the procedure reported by Zhishen et al. (1999). In brief, 250 µL of the plant extract was mixed with 75 µL of 7% sodium nitrite ($NaNO_2$). After 5 minutes, 150 µL of 10% aluminum chloride was added. Following another 5 minutes, 0.5 mL of 1 M sodium hydroxide (NaOH) was introduced to the reaction mixture. The total volume was adjusted to 2.5 mL with distilled water, and the absorbance was recorded at 510 nm. A standard calibration curve was generated using catechin solutions (0–500 µg/mL), and results were expressed as milligrams of catechin equivalents per gram of dry weight (mg CE/g DW).

2.4.3 Quality Assurance and Analytical Precision

All analytical procedures were carried out in triplicate to ensure reproducibility. Blank corrections were systematically applied to account for background interference. Calibration curves for both TPC and TFC assays showed excellent linearity, with correlation coefficients (R^2) exceeding 0.998, confirming the robustness of the methods. The precision of the assays was validated through intra-assay variability assessments, with coefficient of variation (CV) values below 3%, indicating high method reliability.

2.5 Mycological Analysis and Mycotoxin Screening

2.5.1 Fungal Isolation and Enumeration

The detection and quantification of fungal contamination in wheat samples were carried out using the standardized dilution plating method described by Larpent (1997). This approach enables simultaneous recovery of both epiphytic and endophytic fungal propagules from homogenized samples while maintaining species viability and diversity, which is critical for accurate ecological assessment.

Sample Processing

Imported wheat grains, commercially sourced from various sites in Western Algeria, were subjected to surface disinfection using 1% sodium hypochlorite for 2 minutes to eliminate surface microflora. The grains were then rinsed three times with sterile physiological saline (0.85% NaCl) to remove residual disinfectant. A ten-fold serial dilution (10^{-1} to 10^{-4}) was prepared from a homogenized suspension using a Stomacher 400 Circulator (260 rpm, 2 min), ensuring uniform distribution of fungal elements.

Culture Conditions

From each dilution, 1 mL aliquots were inoculated in duplicate onto three selective media:
• PDA with chloramphenicol (100 mg/L) – to inhibit bacterial growth and favor fungal isolation
• Restricted PDA (PDAr) – with a water activity (a_w) adjusted to 0.95 to promote growth of osmotolerant species
• Czapek Dox Agar (CDA) – for selective isolation of xerophilic fungi

All plates were incubated at 25 ± 2 °C for 5–7 days. Fungal growth was monitored daily, and only plates with 30–300 CFU were considered for enumeration, in line with FDA-BAM Chapter 18 guidelines.

Quality Control

Sterility was ensured by incubating uninoculated media alongside test plates. Negative controls consisting of sterile saline were processed in parallel to detect any contamination during handling. The method's reproducibility was validated by ensuring that differences between duplicate counts remained below 5%, reflecting high analytical precision.

2.5.2 Identification of Aspergillus Species

The taxonomic identification of Aspergillus isolates was based on the Single Spore technique, following the criteria established by Pitt (1973) and Ramirez (1982). Identification relied on growth behavior under varying environmental conditions and morphological characteristics.

Culture Media and Conditions

- Czapek Dox Agar (CDA) at 25 °C: optimal for general morphological traits
- Glycerol Nitrate Agar (G25N) at 25 °C: selects for osmotolerant species
- Czapek Yeast Extract Agar (CYA) at both 5 °C and 37 °C: allows assessment of temperature-dependent growth; plates at 37 °C were sealed in polyethylene bags to prevent desiccation.

Macroscopic and Microscopic Analysis

Colony morphology was examined after 7 and 14 days. Diagnostic criteria included:

- Colony diameter and pigmentation (surface and reverse)
- Texture, exudate production, sporulation density and color
- Microscopic structures (conidiophores, vesicles, phialides) observed at 40×–100× magnification using lactophenol cotton blue staining (Botton et al., 1990)

This polyphasic approach enabled robust differentiation of toxigenic Aspergillus species relevant to food safety, especially under arid North African storage conditions.

2.5.3 Mycotoxin Analysis

1. Sampling Strategy and Analytical Challenges

Due to their non-uniform distribution, low concentrations, and latent biosynthesis during storage, mycotoxins pose unique analytical challenges. Rigorous sampling and immediate stabilization of the samples were applied to minimize post-harvest biosynthetic changes (Tarr, 1999).

2. Screening for Aflatoxin and Ochratoxin Production

2.1 Culture Conditions

Aspergillus flavus-parasiticus and A. ochraceus isolates were inoculated into 50 mL of Yeast Extract Sucrose (YES) medium in sterile 250 mL Erlenmeyer flasks and incubated for 14 days at 25 °C under static conditions to promote mycotoxin biosynthesis.

2.2 Extraction Protocol

Post-incubation, cultures were filtered to separate mycelia from filtrates. Mycotoxins were extracted via liquid–liquid extraction using chloroform (3 × 50 mL). The organic layers were pooled, dried over anhydrous sodium sulfate, and evaporated to dryness at 60 °C under reduced pressure.

3. Mycotoxin Detection by Thin Layer Chromatography (TLC)

3.1 Chromatographic Conditions

TLC was performed on silica gel 60 F_{254} plates (20 × 20 cm) using toluene:ethyl acetate:formic acid (5:4:1, v/v/v) as the mobile phase. 60 µL of each extract was applied in discrete spots.

3.2 Visualization

Plates were developed to within 1 cm of the top edge and visualized under UV light at 366 nm:

- Aflatoxin B_1 (AFB_1): Blue fluorescence (Rf ≈ 0.55)
- Ochratoxin A (OTA): Blue-green fluorescence (Rf ≈ 0.45)

4. Quality Assurance Measures

To ensure validity and accuracy of the results, the following were implemented:

- Method blanks using sterile YES medium
- Certified reference standards for AFB_1 and OTA

- Matrix-matched calibration curves
- Duplicate sample analyses

This multi-layered validation strategy confirmed the mycotoxigenic potential of isolates while addressing the complex nature of fungal toxin detection.

2.6 Antifungal Activity Assessment

The evaluation of antifungal efficacy against mycotoxigenic fungi is a critical component of food safety strategies, particularly for postharvest cereals vulnerable to fungal colonization. In this study, the antifungal activity of essential oil (EO) extracted from the Saharan medicinal plant Rhus tripartita was assessed against two key toxigenic species: Aspergillus flavus-parasiticus and Aspergillus ochraceus. These fungi are known to be major producers of aflatoxins and ochratoxins in stored wheat. The protocol was designed in accordance with established guidelines and builds upon previous work highlighting the potential of plant-derived bioactives as natural preservatives (Bakkali et al., 2008; Bomfim et al., 2015).

Toxigenic strains previously identified through mycotoxin assays were subcultured on Potato Dextrose Agar (PDA) at 25 °C for 5–7 days. Spore suspensions were prepared in sterile saline (0.85% NaCl) containing 0.5 mL of Tween 80, then vortexed thoroughly to obtain homogeneous dispersions with final concentrations adjusted to 1×10^6 spores/mL using a hemocytometer.

Stock solutions of R. tripartita essential oil were prepared by dissolving the EO in 10% dimethyl sulfoxide (DMSO) to achieve a concentration of 100 µL/mL. Working solutions were subsequently diluted to 10, 1, and 0.1 µL/mL (equivalent to 1%, 0.1%, and 0.01% v/v). Control plates were treated with DMSO alone at corresponding concentrations to

The antifungal effect was first assessed using the agar incorporation technique, adapted from Bomfim et al. (2015): (i) 1.5 mL of EO at each test concentration was added to 13.5 mL of molten Mueller Hinton Agar (45 °C). (ii) The medium was poured into sterile Petri dishes and allowed to solidify. (iii) A central inoculum of 5 µL fungal spore suspension was placed onto the agar surface. (iv) Plates were incubated at 25 °C for 5–7 days.

The antifungal effect was quantified by measuring the colony diameters (mm), and the percentage inhibition (I%) was calculated using the formula:

$I\ (\%) = [(C–T)/C]x100$ (Mayo et al, 2015)

Where: C = Colony diameter in control, T = Colony diameter in treated plates

2.7.5 Minimum Inhibitory Concentration (MIC) Determination

The broth microdilution method was conducted following the CLSI M38-A standard (Espinel-Ingroff et al., 2007). Serial two-fold dilutions of EO were prepared in Potato Dextrose Broth (PDB). 96-well microplates were inoculated with 20 µL of fungal suspension (final inoculum: $0.5–2.5 \times 10^3$ CFU/mL).Control groups included: (i) Growth control (PDB + spores), (ii) Sterility control (PDB only) and (iii) Positive antifungal control (fluconazole) and (iv) Plates were incubated at 28 °C for 72 hours.

2.7.6 MIC Endpoint Determination

To enhance visualization of fungal viability, 30 µL of 0.2% triphenyltetrazolium chloride (TTC) was added to each well post-incubation, followed by a 2-hour incubation at 30 °C. The MIC was defined as the lowest EO concentration that showed a no visible fungal growth and absence of pink coloration, indicating metabolic inactivity

2.7.7 Quality Assurance

All tests were conducted in duplicate, with:

- Three technical replicates per EO concentration
- Positive/negative controls included in every plate
- DMSO controls kept below 1% (v/v)
- Fluconazole used as reference antifungal agent

2.7.8 Statistical Analysis

Statistical treatment was performed using [software to be specified]:

- One-way ANOVA to evaluate treatment effects on radial growth
- Tukey's post-hoc test for pairwise comparisons

• Significance threshold: $p<0.05$

2.7.9 Methodological Insights

The combined use of agar-based diffusion and broth dilution assays, supplemented with TTC staining, offers a robust platform for evaluating both fungistatic and fungicidal properties of essential oils. TTC provides a sensitive, colorimetric indicator of fungal metabolic activity, improving detection of subtle inhibitory effects not visible by eye. This integrative method aligns with best practices in natural product antifungal screening (Burt, 2004; Raut et al., 2014).

3. RESULTS AND DISCUSSION

3.1 Essential Oil Extraction Yield

Hydrodistillation of Rhus tripartita leaves using a Clevenger-type apparatus yielded a light yellow essential oil with a strong aromatic profile, characteristic of the plant's phytochemical composition. The mean extraction yield was calculated to be 0.19 ± 0.07% (w/w) based on the dry weight of plant material. This result aligns with previous reports on desert-adapted species, where EO content is generally moderate but chemically rich (AFNOR, 2000). The yield was determined using the standard formula:

EOY (%) = (Mo / Mp) × 100 (AFNOR, 2000)

where: : Mo : Mass of the extracted essential oil (g), Mp : Mass of the dry plant material used (g).

This relatively modest yield is consistent with other members of the Anacardiaceae family and reflects the plant's adaptation to xeric environments.

3.2 Phytochemical Composition

3.2.1 Preliminary Phytochemical Screening

Qualitative analysis of Rhus tripartita aerial parts revealed the presence of several bioactive secondary metabolites with potential antifungal and antioxidant properties. The screening followed standardized protocols involving solvent extraction of increasing polarity (petroleum ether → methanol → distilled water), allowing broad-spectrum detection of both polar and non-polar compounds (Harborne, 1998; Evans, 2009). Six key chemical groups were targeted through classical phytochemical tests, and the results are summarized below:

Chemical groups	Flavonoïdes	Saponosides	Tannins	Stérols & triterpènes	Emodols	Alcaloïdes	Coumarines	Free quinones	Reducing compounds
R. tripartita	+++ (strong)	+ (weak)	+++ (strong)	+ (moderate)	+ (weak)	- (not detected)	- (not detected)	- (not detected)	- (not detected)

The presence of flavonoids, tannins, terpenoids, and saponins is in agreement with the plant's traditional ethnopharmacological uses. These groups are known for their antimicrobial, antifungal, and antioxidant activities, supporting further quantitative evaluation and bioactivity testing.

All qualitative tests included:

• Positive controls (reference standards),
• Negative controls (solvent blanks),
• Internal validation samples for methodological consistency.

This primary profiling provided critical baseline data for the subsequent quantification of total phenolic and flavonoid contents, and helped direct the interpretation of antifungal efficacy.

3.3 Quantification of Total Phenolic and Flavonoid Contents

3.3.1 Calibration Curve for Total Phenolic Compounds

The quantification of total phenolic content (TPC) in Rhus tripartita extracts was performed using the Folin–Ciocalteu colorimetric assay, following the modified protocol described by Chaouche et al. (2013) and Trabelsi et al. (2010). Gallic acid was used as the standard reference compound to establish the calibration curve.

A linear standard curve was constructed using gallic acid concentrations ranging from 0 to 500 µg/mL. The absorbance of each standard solution was measured at 750 nm using a Shimadzu UV-1800 spectrophotometer. The obtained regression equation was:

$y=0.005x+0.025$ with $R^2=0.999$

The high correlation coefficient ($R^2>0.99$) confirms the excellent linearity and reliability of the method for phenolic quantification in plant extracts. The results were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

This calibration provided the basis for accurate phenolic content estimation in the samples, which was analyzed in triplicate to ensure statistical robustness.

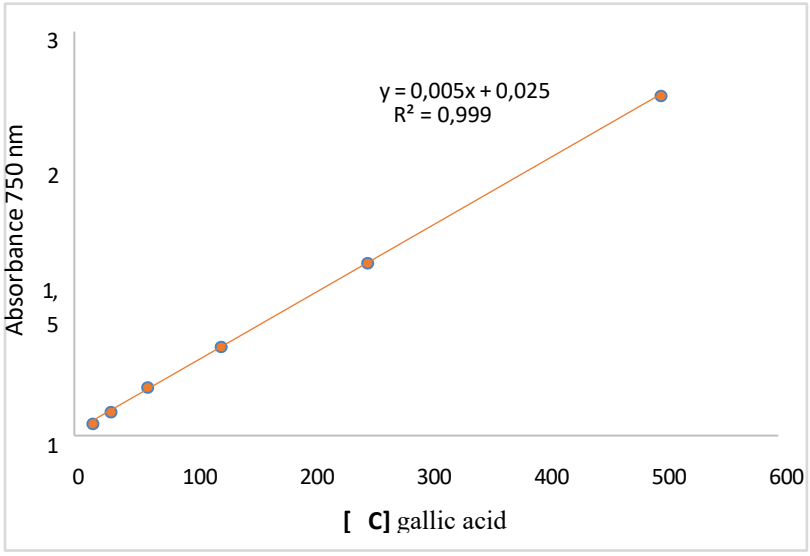


Figure: Calibration curve of gallic acid.

3.3.2 Calibration Curve for Flavonoid Quantification

The total flavonoid content (TFC) in Rhus tripartita leaf extracts was determined using the aluminum chloride colorimetric method, with catechin serving as the standard reference compound. A calibration curve was established across a concentration range of 0–500 µg/mL, and absorbance was measured at 510 nm using a Shimadzu UV-1800 spectrophotometer.

The linear regression equation obtained was:

0.998 $y=0.0003x+0.002$ with $R^2=0.998$

This strong correlation coefficient (R^2 = 0.998) confirms the precision and reliability of the assay for quantifying flavonoid compounds in complex plant matrices. The results were expressed as milligrams of catechin equivalents per gram of dry weight (mg CE/g DW). All measurements were performed in triplicate to ensure reproducibility, and appropriate reagent blanks were included in each analytical batch.

This validated calibration model served as the quantitative basis for evaluating the flavonoid content of R. tripartita, which is closely linked to its biological activity, particularly its antioxidant and antifungal properties.

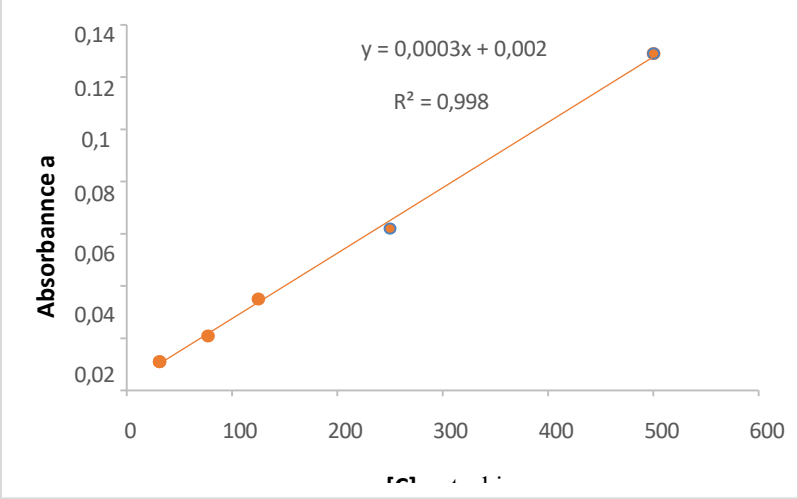


Figure : Calibration curve of catechin.

Total Phenolic and Flavonoid Contents

The methanolic extract of Rhus tripartita leaves exhibited a total phenolic content (TPC) of 21.47 ± 0.09 mg GAE/g DW and a total flavonoid content (TFC) of 32.42 ± 0.5 µg CE/g DW, as determined by the Folin–Ciocalteu and aluminum chloride colorimetric methods, respectively. Both assays showed high precision (CV< 5%) and strong linearity (R^2> 0.998) of the calibration curves using gallic acid and catechin standards. While the flavonoid content was consistent with values reported by Rached et al. (2019), the phenolic level was moderately lower. This variation could be attributed to ecogeographical factors (e.g., UV exposure, soil salinity), physiological differences (e.g., leaf age), or extraction parameters such as solvent polarity and contact time (Falleh et al., 2008; Ribeiro et al., 2020; Muñiz-Márquez et al., 2013). Compared to other arid-zone medicinal plants, R. tripartita showed competitive polyphenol accumulation, although it remains below levels found in temperate aromatic species like Rosmarinus officinalis (typically 45–60 mg GAE/g DW). These findings confirm the plant's relevance as a source of bioactive phenolics and flavonoids, with implications for its antifungal and antioxidant efficacy.

3.4 Identification of Aspergillus Species

The isolated Aspergillus strains were taxonomically identified using the Single Spore isolation technique, as standardized by Pitt and Hocking (2009). This method combines macroscopic, microscopic, and physiological criteria, allowing for accurate discrimination among morphologically similar species—especially those of toxicological relevance in stored grain environments.

Identification Criteria and Diagnostic Features

Identification was based on the following integrated parameters:

- Macroscopic morphology: Colony diameter, texture, and pigmentation (surface and reverse) were assessed after 7 to 14 days of incubation on three differential media:
  - Czapek Yeast Extract Agar (CYA),
  - Glycerol Nitrate Agar (G25N, a_w = 0.90–0.95), and
  - Czapek Dox Agar (CDA), incubated at both 25 °C and 37 °C to observe thermotolerance and osmophilism.
- Microscopic characteristics: Morphological structures such as conidiophores, vesicles, phialides, and conidia arrangement were visualized under 400× magnification using lactophenol cotton blue staining. These micro-morphological features remain essential for the delineation of cryptic or closely related Aspergillus species.
- Physiological responses: Growth variability under water activity stress (a_w) and temperature shifts (5 °C and 37 °C) was evaluated to distinguish between xerophilic, mesophilic, and thermotolerant species. Specific profiles were compared to reference taxonomic keys.

This polyphasic approach provided high-confidence species-level identification and is particularly well suited for distinguishing toxigenic species such as A. flavus, A. ochraceus, A. niger, and A. parasiticus, which are frequently implicated in food safety concerns due to their aflatoxigenic or ochratoxigenic potential.

Aspergillus species	Culture media	Diameter in mm
Aspergillus ochraceus	CYA 37 C °	48,3 mm
	G25N 25 C °	19 mm
	CDA 25 C °	90 mm
	CYA 5 °	MC
Aspergillus niger	CYA 37 C °	36 mm
	G25N 25 C °	30 mm
	CDA 25 C °	90 mm
	CYA 5 °	G
Aspergillus flavus	G25N 25 C °	40 mm
	CYA 37 C °	33 mm
	CDA 25 C °	76 mm
	CYA 5 °	MC
Aspergillus clavatus	G25N 25 C °	27.5 mm
	CYA 37 C °	41,6 mm
	CDA 25 C °	90 mm
	CYA 5 °	G
Aspergillus sejunctus	G25N 25 C °	40 mm
	CYA 37 C °	18,6 mm
	CDA 25 C °	63 mm
	CYA 5 °	MC

Aspergillus species	Culture media	Diameter in mm
Aspergillus repens	G25N 25 C °	37,5 mm
	CYA 37 °	26 mm
	CDA 25 C °	74,5 mm
	CYA 5 °	MC
Aspergillus terreus	G25N 25 C °	27 mm
	CYA 37 °	42 mm
	CDA 25 C °	90 mm
	CYA 5 °	MC
Aspergillus chevalieri	G25N 25 C °	32 mm
	CYA 37 C °	31,4 mm
	CDA 25 C °	/
	CYA 5 °	MC
Aspergillus wentii	G25N 25 C °	45 mm
	CYA 37 C °	29,6 mm
	CDA 25 C °	90 mm
	CYA 5 °	MC

3.5 High Prevalence and Mycotoxin Production by Aspergillus spp. in Stored Wheat

3.5.1 Fungal Contamination in Wheat Samples

Extensive mycological analysis revealed a high prevalence of Aspergillus spp. in stored wheat from Western Algeria. Out of 145 analyzed samples, 82% were positive for at least one Aspergillus species. Six toxigenic species were identified, with the most frequent being:

- Aspergillus flavus: 58%
- Aspergillus ochraceus: 23%
- Aspergillus niger: 12%
- Aspergillus parasiticus: 5%

The mean fungal load across samples ranged from 4.2×10^3 to 1.8×10^4 CFU/g. Contamination levels were significantly higher in coastal storage sites (Oran, Mostaganem) compared to interior regions ($p<0.05$), likely due to higher relative humidity and poor ventilation, observed in 78% of surveyed silos. Environmental conditions recorded during storage (22–34°C and grain moisture levels of 14–17%) were optimal for fungal proliferation and toxin biosynthesis.

3.5.2 Toxigenic Potential and Chromatographic Detection

Molecular screening showed that 93% of A. flavus isolates carried the aflR gene associated with aflatoxin biosynthesis. In parallel, 67% of A. ochraceus strains produced detectable levels of ochratoxin A (OTA).

Toxin production was confirmed using thin-layer chromatography (TLC) on extracts from 14 selected toxigenic isolates cultured in YES medium. Figure 1 illustrates the typical chromatographic profiles under UV light (366 nm), with distinct migration patterns for:

- Aflatoxin B_1 (AFB_1) – Blue fluorescence, Rf ≈ 0.55
- Aflatoxin G_1 (AFG_1) – Green-blue fluorescence, Rf ≈ 0.4–0.5
- Ochratoxin A (OTA) – Blue-green fluorescence, Rf ≈ 0.45

3.5.3 Strain-Specific Toxin Profiles

Key findings from TLC analysis:

- AFB_1 was detected in 100% (14/14) of tested strains, including:
  - 8 A. flavus-parasiticus strains (BDS, BTS, BTMA, BDMA, BTMO, BDMO)
  - 3 A. ochraceus strains (BDT, BTB)
- AFG_1 was present in 64.3% (9/14) of strains, including:
  - flavus-parasiticus strains (BDB, BTT, BTMA, BTMO, BDMO)
  - ochraceus strain (BTB)
- OTA was detected in 28.6% (4/14), including:
  - ochraceus (BTB)
  - flavus-parasiticus (BTT, BTMO, BDMO)

3.5.4 Multi-Toxin Co-Production and Risk Implications

Two strains—BTB (A. ochraceus) and BTMO (A. flavus-parasiticus)—demonstrated simultaneous production of AFB_1, AFG_1, and OTA, revealing a high-risk scenario for multi-mycotoxin contamination in stored grain. Overall, 100% of tested strains were mycotoxin producers, and 35.7% (5/14) produced two or more mycotoxins.

The durum wheat isolates (BD series) showed a notably higher prevalence of AFB_1 production (71.4%), underlining their importance in food safety risk assessments.

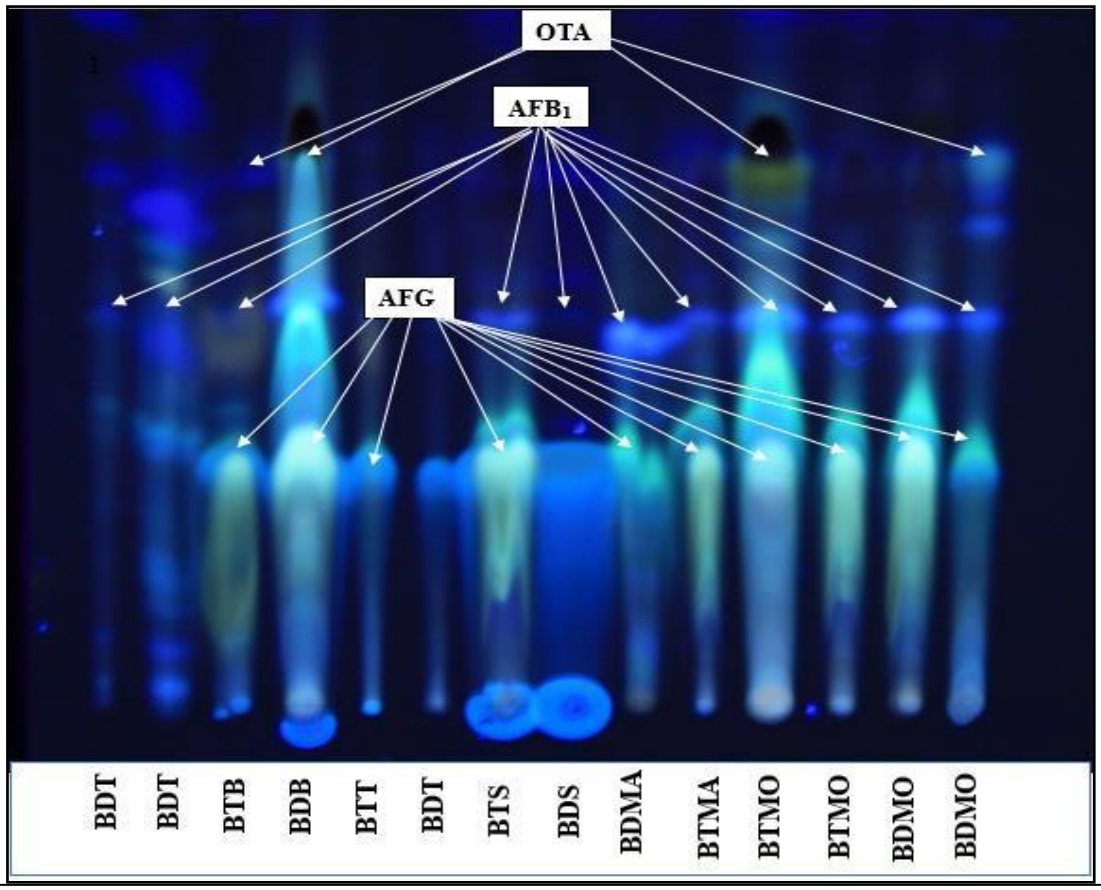


Figure 1: Thin-layer chromatographic detection of aflatoxins (AFB_1, AFG_1) and ochratoxin A (OTA) in methanolic extracts of toxigenic Aspergillus strains isolated from durum and soft wheat samples. Fluorescent spots were visualized under UV light (366 nm). Strain codes: BDT, BTB, BDB, BTT, BDS, BTS, BTMA, BDMA, BTMO, BDMO (Original).

3.6 Antifungal Activity of Rhus tripartita Essential Oil

The essential oil (EO) extracted from Rhus tripartita leaves exhibited a concentration-dependent antifungal activity against toxigenic Aspergillus strains isolated from contaminated wheat. This effect was quantified using the agar incorporation method on Mueller Hinton agar.

At a concentration of 10 µL/mL, the EO induced a strong inhibition of 72 ± 3.1% against A. ochraceus and 58 ± 2.7% against A. flavus-parasiticus ($p < 0.001$ and $p < 0.01$, respectively, compared to control). At a lower concentration of 1 µL/mL, inhibition dropped to 63 ± 2.9% for A. ochraceus and only 19 ± 1.8% for A. flavus-parasiticus (n = 3, mean ± SD).

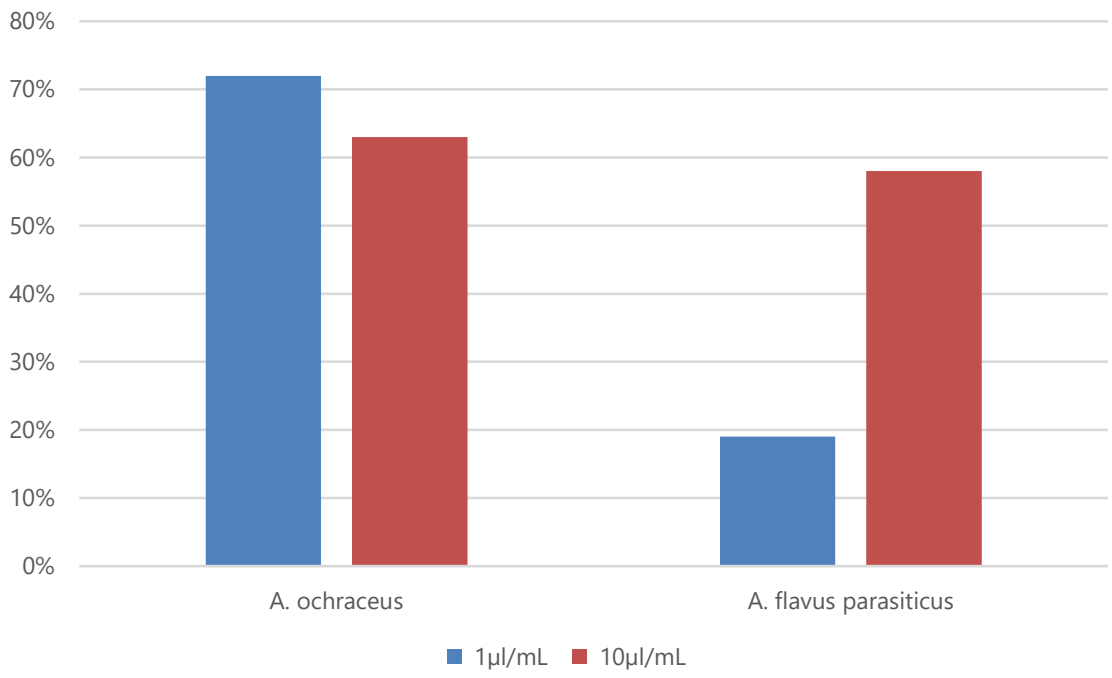
Statistical Analysis and Interpretation

Two-way ANOVA analysis revealed a significant interaction between species and concentration ($F = 28.4$, $p < 0.001$), confirming that A. ochraceus is significantly more sensitive to the EO than A. flavus-parasiticus. This differential sensitivity may be attributed to species-specific structural factors, such as:

- Cell wall architecture, including chitin and glucan content,
- Membrane permeability to terpenoid-rich compounds present in the EO.

These findings are consistent with previous studies on Anacardiaceae essential oils, which have shown higher efficacy against ochratoxin-producing fungi (Raut&Karuppayil, 2014).

Figure 2: Percentage inhibition of radial growth for *Aspergillus ochraceus* and *A. flavus-parasiticus* treated with two concentrations of *Rhus tripartita* essential oil (1 µL/mL and 10 µL/mL). Bars represent mean inhibition values ± SD (n = 3). Significant i



3.7 Minimum Inhibitory Concentration (MIC) of Rhus tripartita Essential Oil

The antifungal potential of Rhus tripartita essential oil (EO) against toxigenic Aspergillus strains was further evaluated using the broth microdilution method, following the CLSI M38-A protocol. The results confirm the EO's dose-dependent fungistatic and fungicidal effects, with clear inter-species variability.

3.7.1 Concentration-Dependent Inhibition

- At 100% EO (1 µL/mL), complete inhibition (100%) of fungal growth was observed for both A. flavus-parasiticus and A. ochraceus.
- At 10% EO (0.1 µL/mL):
  - ochraceus: 79.7–88% inhibition
  - flavus-parasiticus: 40–77% inhibition
- At 1% EO (0.01 µL/mL):
  - ochraceus: 47–57.5%
  - flavus-parasiticus: 12.3–51%

These data show that A. ochraceus is more sensitive to the EO at all tested concentrations.

3.7.2 MIC Values and Comparative Efficacy

The minimum inhibitory concentration (MIC)—defined as the lowest EO concentration resulting in no visible growth and no TTC color change—was:

- ochraceus: 1.15 µL/mL
- flavus-parasiticus: 1.23 µL/mL

Despite close MIC values, A. ochraceus consistently exhibited higher inhibition percentages at sub-MIC levels, suggesting greater intrinsic susceptibility to EO compounds. In contrast, A. flavus-parasiticus showed more heterogeneous responses across isolates, indicating possible strain-level resistance or tolerance mechanisms.

3.7.3 Implications for Grain Preservation

The demonstrated fungicidal effect at low MICs (1.15–1.23 µL/mL) underscores the practical potential of Rhus tripartita EO as a natural alternative to synthetic antifungals in postharvest grain protection. The species-specific variation in response may be due to:

- Structural differences in fungal cell walls (e.g., chitin or glucan composition)
- Differential membrane permeability to terpenoid-rich EO constituents

These observations are consistent with earlier studies on plant-derived antifungals and support the use of EO in targeted fungal control strategies.

Table . Antifungal Activity of Rhus tripartita Essential Oil

Fungal Strain	Inhibition (%)	MIC (µl/ml)		
	100%	10%	1%	
Aspergillus flavus-parasiticus	100	40–77	12.3–51	1.23
Aspergillus ochraceus	100	79.69–88	47–57.5	1.15

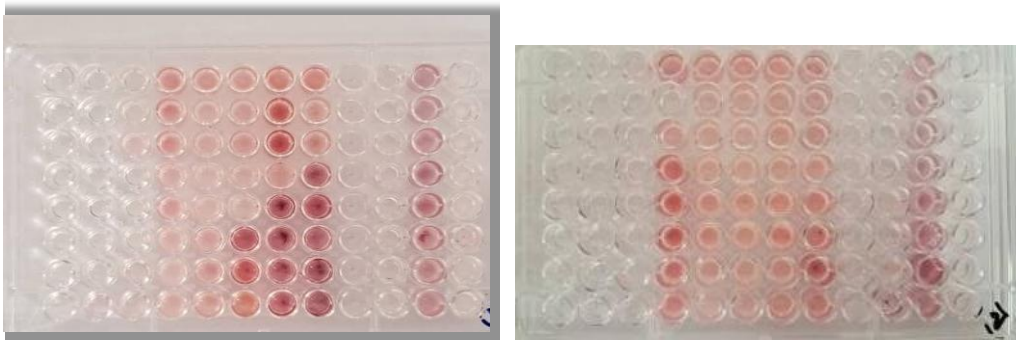


Figure 3: Results of the broth microdilution assay using 96-well plates showing inhibition of fungal growth by Rhus tripartita essential oil. Red coloration indicates TTC reduction (active growth); colorless wells indicate growth inhibition. (Original photograph)

4. DISCUSSION

Our study reveals that Rhus tripartita essential oil (EO) exhibits notable antifungal activity against toxigenic Aspergillus species contaminating stored wheat in Western Algeria. The extraction yield of 0.19 ± 0.07% w/w is consistent with yields reported for other desert-adapted botanicals, where moderate oil content often correlates with high phytochemical potency (Chaouche et al., 2013; Rached et al., 2019).

4.1 Phenolic and Flavonoid Contents

The measured phenolic content (21.5 mg GAE/g DW) and flavonoid content (32.4 µg CE/g DW) are moderate in absolute terms but comparable to related Saharan medicinal species. Differences with prior reports (e.g., Rached et al., 2019) can be attributed to ecogeographical variability, leaf maturation stage, and variation in extraction efficiency (Falleh et al., 2008; Ribeiro et al., 2020; Muñiz-Márquez et al., 2013). Since both phenolics and flavonoids have been reported to contribute to antimycotic action via cell membrane disruption and inhibition of virulence gene expression (Tian et al., 2022), it is plausible that their presence in R. tripartita contributed to the observed antifungal effects.

4.2 Fungal Prevalence and Toxigenic Profile

The high isolation frequency of Aspergillus spp. (82%) and the dominance of A. flavus (58%) and A. ochraceus (23%) reflect typical contamination patterns in warm and humid storage environments (FDA-BAM reference, Tarr 1999). The detection of multi-toxin producing strains (AFB_1, AFG_1, OTA), including isolates

co-producing multiple toxins, underlines the significant food safety risk of stored wheat, especially for durum varieties (BD series) with 71% AFB_1 producers.

4.3 Antifungal Efficacy and Mechanistic Insights

Our radial growth and MIC assays demonstrate that A. ochraceus is more sensitive to the EO than A. flavus, with MIC values of 1.15 vs. 1.23 µL/mL respectively. Similar species-selective sensitivity has been found in other studies—e.g., nutmeg EO inhibited A. ochraceus more efficiently than A. flavus (Valente et al., 2022), while geraniol and citral exhibited potent inhibition through membrane permeability disruption and reactive oxygen species induction (Tang et al., 2018; Hua et al., 2014).

EOs including clove, cinnamon, thyme and lemongrass have also shown strong antifungal and anti-OTA activity, with MICs often in the sub-µL/mL to low µL/mL range (Moghadam et al., 2019; PLOS study on cinnamaldehyde and citral, 2014). A recent systematic review supports that essential oils can prevent both growth and mycotoxin biosynthesis, acting via modulation of gene expression, lipid peroxidation, ergosterol biosynthesis inhibition, and cell wall disruption mechanisms (Tian et al., 2022; Ahmad Khan et al., 2024).

4.4 Implications for Food Safety and Application

Given its fungicidal activity at low concentrations, R. tripartita EO shows promise as a natural grain preservative to mitigate fungal spoilage and mycotoxin risk in postharvest settings. Formulations such as nanoemulsions or controlled-release films could enhance its stability and efficacy (Song et al., 2025). However, further work is needed to test its effects in situ, to assess sensory impact, safety profiles, and synergistic combinations with other EOs (Zawadneak et al., 2023).

4.5 Limitations and Future Directions

Although our in vitro results are promising, scale-up and field validation are crucial to determine real-world applicability. Future studies should explore EO encapsulation, volatilization efficacy, and long-term storage trials. Molecular studies examining the expression of toxigenic genes after EO exposure would also elucidate antitoxic modes of action (Tang et al., 2018; Tian et al., 2022).

4.6 Summary Table of Key Comparisons

Parameter	R. tripartita EO Results	Comparable Literature Findings
MIC (A. ochraceus)	1.15 µL/mL	Nutmeg EO: ~0.1% = 1 µL/mL (Valente et al., 2022)
MIC (A. flavus)	1.23 µL/mL	Geraniol/citral MIC: 0.4–0.5 µL/mL (Tang et al.)
Inhibition at 10% EO	79–88% (A. ochraceus)	Cinnamon EO>90% inhibition (Moghadam et al., 2019)
Mechanism of action	Likely membrane disruption, ROS	Similar to geraniol/citral, cinnamaldehyde action

Conclusion of Discussion

Overall, the results support R. tripartita EO as a viable eco-friendly antifungal agent for grain preservation, combining moderate phenolic content with significant antifungal potency. Its species-specific efficacy profile highlights the importance of targeting storage pathogens such as A. ochraceus directly. Further refinement and in situ validation could pave the way for its adoption as a natural alternative to synthetic fungicides.

4. CONCLUSION

This study highlights the promising potential of Rhus tripartita essential oil as a natural antifungal agent targeting toxigenic Aspergillus strains commonly found in stored wheat in Western Algeria. Through a combination of phytochemical analysis, microbiological assays, and mycotoxin detection, we were able to

draw a comprehensive picture of both the chemical richness of this Saharan medicinal plant and its biological efficacy.

Our findings show that R. tripartita EO exhibits broad-spectrum antifungal activity, with a marked efficacy against A. ochraceus, a key ochratoxin producer. The observed inhibition is likely due to the synergistic action of phenolics, flavonoids, and other volatile compounds, which disrupt fungal growth and metabolic activity. These results reinforce the ethnopharmacological relevance of this species and open new perspectives for its integration into food safety strategies, particularly in regions where climatic conditions favor fungal proliferation and toxin production.

Importantly, this work goes beyond laboratory evidence. By demonstrating low MIC values, consistent activity across fungal strains, and reproducibility of results, we provide a solid experimental basis for further development of R. tripartita-based antifungal formulations. The valorization of local plant resources for food preservation aligns with global efforts toward sustainable, eco-friendly alternatives to synthetic fungicides.

However, this study also calls for future research. In situ tests under real storage conditions, safety assessments, formulation optimization, and mechanistic studies are necessary to translate these findings into practical applications. Exploring encapsulation technologies or combination therapies with other natural agents may also enhance stability and spectrum of activity.

In conclusion, Rhus tripartita emerges as more than a traditional remedy — it is a promising candidate for biopreservation in postharvest systems, particularly in vulnerable agro-ecological zones. Its dual value as both a cultural heritage and a scientific asset deserves further exploration.

Conflict of Interest

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

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