HPLC PROFILING AND ANTI-CANDIDAL ACTIVITY OF CRUDE AND FRACTIONATED EXTRACTS OF TITHONIA DIVERSIFOLIA

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Abstract. The emergence of multidrug-resistant Candida species poses a significant challenge to current antifungal therapies. Tithonia diversifolia has been traditionally used for its medicinal properties, prompting interest in its potential as a source of novel antifungal agents. This study investigated the antifungal activity of T. diversifolia crude extract and its solvent fractions against multidrug-resistant Candida species. Phytoconstituents were extracted from the leaves of T. diversifolia using solvent extraction, followed by fractionation through a separating funnel. Antifungal activity was assessed via the agar well diffusion method, while minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined using tube dilution and plating techniques, respectively. Bioactive compounds in the extract were identified through High-Performance Liquid Chromatography (HPLC) analysis. The crude extract showed strong inhibition against Candida glabrata and Candida krusei (22.0 mm). Among the fractions, the aqueous fraction exhibited notable activity against C. glabrata (20.0 mm), while the ethyl acetate fraction was highly effective against C. krusei (22.0 mm). The lowest MIC and MFC values were recorded for C. krusei (MIC=12.5 mg/ml; MFC=6.25 mg/ml). HPLC analysis revealed key antifungal compounds such as Curcumin, Alpha-Tumerone, and 1,8-Cineole. These results demonstrate the promising potential of T. diversifolia as a source of antifungal agents against resistant Candida strains.

Keywords: *multidrug-resistance, candida species, tithonia diversifolia, solvent fractionation, phytochemicals, high-performance liquid chromatography*

Introduction

Candidiasis, caused by opportunistic Candida species, continues to represent a major clinical challenge, spanning superficial mucosal infections to life-threatening systemic disease (Oluyele et al., 2025; Lu et al., 2023). Systemic candidiasis, characterized by fungal invasion of the bloodstream and internal organs, demands urgent attention due to its high morbidity and mortality (Liu et al., 2025). Advances in understanding the molecular interplay between host immune defenses and fungal pathogenicity have revealed critical mechanisms driving the transition to invasive disease. Despite improvements in diagnostic modalities, antifungal resistance among key species such as C. albicans, C. glabrata, and C. krusei complicates treatment efforts. Mechanisms of resistance-including drug target alterations, efflux pump activity, and genetic mutations-have intensified clinical management challenges (Fisher et al., 2022). The limited efficacy of current antifungal agents, compounded by environmental and clinical drug pressures (Pagano and Fernández, 2025), highlights the need for novel therapeutic approaches. Natural products and plant-derived compounds are increasingly investigated as promising alternatives for combating resistant Candida infections. Tithonia diversifolia (Mexican sunflower) is a fast-growing perennial shrub of the Asteraceae family, native to Mexico and Central America, and widely naturalized across tropical and subtropical regions. Classified under the kingdom Plantae, division Magnoliophyta, class Magnoliopsida, and order Asterales, the species is notable for its

vibrant flowers, ecological adaptability, and role in agroforestry and soil fertility improvement (Rai et al., 2023; Kriticos and Kriticos, 2021).

Beyond its ecological significance, T. diversifolia displays considerable genetic diversity (Rivera et al., 2019). and harbors a rich phytochemical profile, comprising terpenoids, flavonoids, phenolic acids, alkaloids, saponins, and lignans (Okuna et al., 2024; Tagne et al., 2018). This chemical complexity is believed to contribute to its extensive use in traditional medicine, particularly in the treatment of respiratory, dermatological, and gastrointestinal conditions (Tagne et al., 2018). These traditional applications have increasingly been supported by modern pharmacological research, highlighting the therapeutic potential of the plant. Given its rich phytochemical profile and well-documented medicinal properties, T. diversifolia has the potential to serve as a valuable source of new antifungal agents. This is particularly important in the current context where Candida infections are becoming increasingly prevalent and resistance to conventional antifungal drugs is on the rise. The urgent need for alternative, effective treatments therefore justifies exploring the antifungal activity of T. diversifolia. Accordingly, this study aims to assess the inhibitory potency of T. diversifolia extracts against selected multidrug-resistant Candida species.

Materials and Methods

Test organisms and preparation of inoculum

The test organisms used in this study were multidrug-resistant strains of Candida krusei, Candida tropicalis, and Candida glabrata, which were sourced from the stock culture of previously identified isolates maintained at the Microbiology Laboratory, AAUA. These organisms were cultured on Sabouraud dextrose agar (SDA) slants and stored at 4°C for subsequent experiments. A 0.5 McFarland standard was prepared by mixing 0.05 mL of 1% barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$) with 9.95 mL of 1% sulfuric acid (H_2SO_4) to form a 1.0% w/v barium sulfate suspension. The density of the prepared McFarland standard was verified using a spectrophotometer at a wavelength of 625 nm. The standard was thoroughly mixed using a vortex before use. For the inoculum preparation, five distinct colonies, each approximately 1 mm in diameter, were selected from a 24-hour-old culture of each test organism. The colonies were suspended in 5 mL of sterile 0.85% saline. After vortexing for 15 seconds, the turbidity was adjusted to match a 0.5 McFarland standard, resulting in a yeast suspension with a concentration of $1–5 \times 10^6$ cells per mL (Oluyele et al., 2022).

Preparation of tithonia diversifolia plant material, extraction of sample and fractionation procedure

Fresh leaves of Tithonia diversifolia (Hemsl.) A. Gray, a dicotyledonous plant belonging to the Asteraceae family, were collected from Akungba-Akoko (Latitude 7.4740°N, Longitude 5.7379°E). The plant material was identified, authenticated, and assigned voucher number PSBH-258. The maceration method described by Oluyele and Oladunmoye (2017) was used for the crude extraction process. Briefly, the leaves of T. diversifolia were air-dried, pulverized, and 350 g of the powdered material was soaked in 2 litres of ethanol for seven days with occasional shaking to ensure full extraction of the active constituents. The mixture was sieved through muslin cloth and further filtered using Whatman No. 1 filter paper. The resulting filtrate was concentrated using a rotary

evaporator, and the crude extract obtained was stored at 20°C for further studies. Fractionation of the crude extract was carried out by liquid–liquid extraction following the method of Oluyele et al. (2025). In brief, the crude extract was dissolved in distilled water (1:10, w/v), transferred into a separatory funnel, equilibrated, and successively partitioned with n-hexane and ethyl acetate to yield the hexane fractions (F1 and F2) and the ethyl acetate fraction (F3), respectively. The remaining aqueous portion constituted the aqueous fraction (F4). All fractions (F1, F2, F3, and F4) were concentrated using a rotary evaporator, freeze-dried, and stored at 4°C until further experiments.

Antifungal assay of tithonia diversifolia extract

The agar well diffusion technique, as described by Oluyele et al. (2025), was used to evaluate the antifungal activity of the extract. Briefly, 1 mL of each standardized test organism suspension was transferred onto well-dried, sterile Sabouraud dextrose agar (SDA) plates and evenly spread using sterile swab sticks. After allowing the plates to dry, uniform wells of 6 mm diameter were bored into the agar using a sterile cork borer, and each well was appropriately labeled on the reverse side of the plates. Subsequently, 50 µL of the extract solution (100 mg/mL, prepared in 5% dimethyl sulfoxide [DMSO], Sigma-Aldrich, Germany) was introduced into each corresponding well. Griseofulvin or Amphotericin-B was used as the positive control in one of the wells. The plates were left at room temperature for 15 minutes to facilitate proper diffusion of the extract, followed by incubation at 35°C for 48 hours. After incubation, the zones of inhibition were measured to assess antifungal activity.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MBC)

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the extract were determined using the tube-dilution and plating methods, respectively, as described by Oluyele et al. (2025). For MIC determination, different concentrations of the extract (200–3.125 mg/mL) were prepared, and 0.1 mL of the standardized test inoculum was added to each test tube containing sterile Sabouraud dextrose broth (SDB). A set of tubes containing only sterile SDB served as the negative control, while another set containing SDB plus the test organisms served as the positive control. All tubes were incubated at 35°C for 48 hours. Growth was assessed by visible observation and spectrophotometric measurement (Beckman model 35), and the MIC was recorded as the lowest concentration showing no visible turbidity. To determine the MFC, aliquots from tubes showing no turbidity, including the MIC tube, were subcultured onto freshly prepared Sabouraud dextrose agar (SDA) plates and incubated at 35°C for 48 hours. The MFC was defined as the lowest concentration at which no visible fungal growth was observed after incubation.

High Performance Liquid Chromatography (HPLC) analysis of extract

About 2 g of the sample was weighed into an amber bottle, followed by the addition of 20 mL of acetonitrile/methanol mixture, and the mixture was vigorously agitated for 30 minutes. The aqueous layer was discarded, and the organic phase was collected into a 25 mL standard flask and made up to volume, ready for analysis. The sample was analyzed using gradient elution under the following chromatographic conditions:

reversed-phase chromatography (Agilent Technologies 1200 HPLC system), mobile phase composition of 0.1% formic acid and acetonitrile, stationary phase Hypersil BDS C18 column (Agilent), with column dimensions of 250 mm × 4.0 mm. The injection volume was 20 µL, the flow rate was set at 0.6 mL/min, and detection was performed at a wavelength of 280 nm. A standard analyte solution was first injected into the HPLC to generate a chromatogram with defined peak areas and profiles, which were used to create a reference window for the analysis of test samples. Subsequently, an aliquot of the extracted test sample was injected, and the corresponding peak areas and profiles were recorded. Peak identification was achieved by comparing the retention times and UV spectra of the test samples with those of the reference standards (Oluyele et al., 2025).

Results and Discussion

Anti-candidal activity of tithonia diversifolia extracts

The antifungal activity of T. diversifolia crude extract (CE) and its solvent fractions (F1-F4) against multidrug-resistant Candida species was evaluated (*Table 1*). The crude extract exhibited potent antifungal activity, with zones of inhibition of 22.0 mm against Candida glabrata and Candida krusei, although eliciting low inhibition of 4.0 mm against Candida tropicalis. Among the solvent fractions, the aqueous fraction (F4) showed considerable antifungal activity across the isolates, particularly against C. glabrata (20.0 mm) and C. tropicalis (17.0 mm). The ethyl acetate fraction (F3) showed no activity against C. glabrata and C. tropicalis but demonstrated strong inhibition (22.0 mm) against C. krusei. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of the crude extract are shown in *Table 2*. Candida krusei was the most susceptible, with a MIC of 12.5 mg/ml and MFC of 6.25 mg/ml, indicating strong fungicidal activity. Candida glabrata showed moderate susceptibility (MIC 50 mg/ml; MFC 100 mg/ml), whereas Candida tropicalis had diminished susceptibility, with MIC and MFC values both greater than 100 mg/ml. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of the crude extract are shown in *Table 2*. Candida krusei was the most susceptible, with a MIC of 12.5 mg/ml and MFC of 6.25 mg/ml, indicating strong fungicidal activity. Candida glabrata showed moderate susceptibility (MIC 50 mg/ml; MFC 100 mg/ml), whereas Candida tropicalis had diminished susceptibility, with MIC and MFC values both greater than 100 mg/ml.

Table 1. *Antifungal potency of Tithonia diversifolia extracts.*

Organism	CE	F	F2	F3	F4
Candida glabrata	22.0 mm	16.0 mm	21.0 mm	0.0 mm	20.0 mm
Candida tropicalis	4.0 mm	0.0 mm	12.0 mm	0.0 mm	17.0 mm
Candida krusei	22.0 mm	15.0 mm	18.0 mm	22.0 mm	22.0 mm

Note: CE=Crude extract; F1=N-hexane fraction 1; F2=N-hexane fraction 2; F3=Ethyl acetate fraction; F4=Aqueous fraction.

Table 2. *Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal concentration (MFC) of Tithonia diversifolia crude-extract against test organisms.*

Organism	MIC	MBC
Candida glabrata	50 mg/ml	100 mg/ml
Candida tropicalis	> 100 mg/ml	> 100 mg/ml

Candida krusei	12.5 mg/ml	6.25 mg/ml

HPLC identified compounds of tithonia diversifolia crude-extract

High-Performance Liquid Chromatography (HPLC) analysis revealed the presence of several bioactive compounds (*Table 3*), including Curcumin, Alpha-Tumerone, Beta-Tumerone, Camphor, Alpha-Pinene, and 1,8-Cineole, among others.

Table 3*. HPLC identified compounds of T. diversifolia extract.*

Component	Retention	Area	Height
Methyl Hexyne	1.350	337.0240	25.413
Cyclohexane	1.650	471.1030	51.167
Curcumin	1.983	9170.3010	228.662
Alpha-Tumerone	3.166	1503.0130	41.157
Camphor	4.016	1636.5080	33.757
Beta-Tumerone	5.050	198.1040	6.851
Hexadiene	5.766	42.8000	3.478
Alpha-Pinene	6.350	239.5975	5.555
Demethoxycurcumin	7.350	1061.3910	21.867
Dehydroxycurcumin	8.916	120.8390	2.572
1_8- cineole	-	56.1010	10.118
Alpha-phellandremin	9.916	98.2620	5.410
Octadiene	10.566	30.0280	1.884

Candida species are globally recognized as major agents of invasive infections associated with poor clinical outcomes (Liu et al., 2025; Oluyele et al., 2025; 2022). The rising incidence of antifungal-resistant pathogens has intensified the global search for novel, effective therapeutic alternatives. In this context, there has been growing scientific interest in exploring medicinal plants as potential sources of safe and potent antimicrobial agents (Oluyele et al., 2022). Reflecting this trend, the present study evaluated the inhibitory efficacy of extracts from the indigenous plant T. diversifolia against selected multidrug-resistant Candida species. The results demonstrate that T. diversifolia extracts, particularly the crude extract and aqueous fraction, possess significant antifungal activity against multidrug-resistant Candida species. The highest susceptibility was observed in Candida krusei, which exhibited large inhibition zones (22 mm) across multiple extract fractions and the lowest MIC/MFC values. This suggests that C. krusei is particularly sensitive to the phytochemicals present in T. diversifolia. In contrast, Candida tropicalis was notably more resistant, with the crude extract achieving only minimal inhibition (4 mm), and MIC/MFC values exceeding 100 mg/ml, indicating poor effectiveness at the tested concentrations. These findings suggest that C. krusei is more susceptible to the active compounds in T. diversifolia, while C. tropicalis may require higher concentrations for effective inhibition.

Comparatively, the aqueous fraction (F4) consistently exhibited notable activity against all tested Candida species, especially C. glabrata and C. tropicalis. This suggests that polar compounds in the aqueous extract play a crucial role in the antifungal activity. The ethyl acetate fraction (F3) showed selective potency, active only against C. krusei, indicating that semi-polar compounds might have a specific role in inhibiting this particular species. The fact that certain fractions show selective activity against different species highlights the complexity of the antifungal mechanisms at play and the

importance of specific solvent extraction in maximizing the therapeutic potential of T. diversifolia. Corroborating the current study, a previous investigation examined T. diversifolia leaf extracts using different solvents (petroleum ether, chloroform, and methanol) and different organisms. In that study, the in vitro efficacy was established against plant pathogenic fungi including Alternaria alternata, Aspergillus flavus, Aspergillus niger, Cuvularia lunata, Fusarium oxysporum, and Penicillium expansum, and human pathogenic bacteria (Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus) (Linthoingambi and Singh, 2013). Furthermore, another study found that both T. diversifolia and its close relative T. rotundifolia exhibited effective antifungal activity against Cryptococcus neoformans, Candida albicans, and Aspergillus fumigatus (Omokhua et al., 2018). The observed antifungal activity of T. diversifolia in this study can be attributed to the phytochemical constituents identified by HPLC. Curcumin, for instance, is well-known for its broad-spectrum antimicrobial properties, functioning through mechanisms such as disruption of microbial cell membranes and inhibition of fungal hyphal growth (Hussain et al., 2022; Adamczak et al., 2020). Similarly, compounds like Alpha-Tumerone and Beta-Tumerone have demonstrated various pharmacological activities, including antifungal and anti-inflammatory properties in previous studies (Niknejad et al., 2025; Jankasem et al., 2013). Alpha-Pinene and 1,8-Cineole, both monoterpenes, are also recognized for their antimicrobial effects (Akacha et al., 2023). These compounds, present in the extracts, likely contribute to the observed activity against the multidrug-resistant Candida strains.

The superior activity of the aqueous fraction can be further explained by the higher solubility and availability of hydrophilic compounds like Curcumin and its derivatives, which are more effectively extracted into water than non-polar solvents. This supports the idea that the solubility properties of different phytochemicals significantly impact their biological efficacy. In contrast, the inactivity observed in some fractions, such as F3 against C. tropicalis and C. glabrata, may be due to the absence or lower concentration of active phytochemicals in those solvent fractions, suggesting that solvent polarity plays an important role in extracting the most active compounds from the plant material. Moreover, the lower MIC and MFC values against C. krusei suggest that T. diversifolia extracts could potentially serve as a source of lead compounds for developing antifungal therapies against this particularly drug-resistant organism.

Conclusion

These results scientifically validate the traditional use of Tithonia diversifolia in managing fungal infections and highlight its potential application against multidrug-resistant Candida strains, especially C. krusei. The significant antifungal activity observed in this study emphasizes the need for continued research into medicinal plants as sources of novel antimicrobial agents. Future studies should focus on isolating and characterizing the specific bioactive compounds responsible for the observed antifungal activity, employing bioassay-guided fractionation techniques. In-depth mechanistic investigations are crucial to understand how these compounds exert their antifungal effects at the cellular and molecular levels. Additionally, exploring potential synergistic interactions between Tithonia diversifolia extracts and conventional antifungal drugs could enhance therapeutic efficacy, particularly against resistant strains. Comprehensive toxicological assessments are also essential to evaluate the safety profile of both the

extracts and individual compounds in mammalian systems. Finally, in vivo studies using animal models should be conducted to confirm the antifungal efficacy and safety of these extracts in biological systems, ultimately paving the way for their clinical application in the management of candidiasis.

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Conflict of interest

The authors confirm that there is no conflict of interest involve with any parties in this research study.

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