

ISSN Print: 2664-6781
 ISSN Online: 2664-679X
 NAAS Rating (2025): 4.77
 IJACR 2025; 7(12): 122-125
www.chemistryjournals.net
 Received: 09-10-2025
 Accepted: 14-11-2025

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Antifungal screening of saponins isolated from *tribulus terrestris* fruit extract

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DOI: <https://www.doi.org/10.33545/26646781.2025.v7.i12b.474>

Abstract

Leading mycologists have long argued that plant-derived saponins represent an underexplored reservoir of antifungal agents, yet rigorous dose-response data for purified saponin fractions remain scarce. This research investigated the antifungal potential of steroidal and triterpenoid saponins isolated from the fruit of *Tribulus terrestris* against five pathogenic fungi: *Candida albicans*, *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum*, and *Trichophyton rubrum*. Fruits were collected from wild-growing plants near Heidelberg, Germany, extracted with 70% ethanol, and fractionated by liquid-liquid partitioning followed by silica gel column chromatography to yield a steroidal saponin-enriched fraction (Fraction A) and a triterpenoid saponin-enriched fraction (Fraction B). Antifungal activity was measured by the broth microdilution method (CLSI M27-A3 for yeasts, M38-A2 for moulds) with fluconazole as the reference drug. Fraction A showed the strongest activity, with minimum inhibitory concentration (MIC) values of 16 µg/mL against both *C. albicans* and *T. rubrum*, and 32 µg/mL against *A. niger* and *F. oxysporum*. Fraction B was roughly half as potent, with MICs ranging from 32 to 128 µg/mL. The crude extract was weaker still, confirming that purification concentrates the active components. Preliminary mechanistic tests using propidium iodide uptake assays suggested that Fraction A disrupts fungal membrane integrity. These findings position steroidal saponins from *T. terrestris* as candidates for further development as natural antifungal agents or agricultural biocontrol compounds.

Keywords: Saponin, *Tribulus terrestris*, antifungal, steroidal saponin, triterpenoid, minimum inhibitory concentration, *Candida albicans*, membrane permeability, broth microdilution

Introduction

According to Denning and Bromley ^[1], invasive fungal infections cause an estimated 1.5 million deaths per year worldwide, yet the antifungal drug pipeline has expanded only modestly over the past two decades. The azole class—fluconazole, itraconazole, voriconazole—remains the backbone of clinical therapy, but resistance among *Candida* and *Aspergillus* species is rising steadily, and the newest agent in the echinocandin class, rezafungin, received FDA approval only in 2023 ^[2, 3]. In agriculture, fungal crop losses account for roughly 10-23% of global yield depending on the commodity, and the withdrawal of several synthetic fungicides on environmental grounds has left farmers looking for alternatives ^[4]. Saponins are amphiphilic glycosides found in over 500 plant families, characterized by a hydrophobic aglycone (steroidal or triterpenoid) linked to one or more sugar chains ^[5]. Their antifungal action is generally attributed to the ability of the aglycone moiety to intercalate into fungal membranes and form complexes with ergosterol—the principal sterol of fungal cell membranes—leading to pore formation, ion leakage, and cell death ^[6, 7]. This mechanism is analogous to that of amphotericin B, though saponins tend to be less potent on a per-molecule basis. *Tribulus terrestris* (family Zygophyllaceae) is a spiny annual herb distributed across Southern Europe, the Middle East, and South Asia. Its fruits are rich in furostanol and spirostanol saponins, including protodioscin, dioscin, and terrestrosin, which have been investigated mainly for their effects on testosterone levels and cardiovascular function ^[8, 9]. Antifungal activity of *T. terrestris* extracts has been noted in a handful of screening papers, but most used crude extracts at high concentrations (> 500 µg/mL) and did not isolate or identify the active saponin classes ^[10, 11].

The present research aimed to go beyond crude screening by (a) fractionating the *T. terrestris* fruit extract into steroidal and triterpenoid saponin-enriched fractions, (b) determining MIC values against five clinically and agriculturally relevant fungi using standardized CLSI methodology, and (c) performing a preliminary membrane integrity assay to probe the mechanism of action. All experimental work was carried out at the Department of Herbal and Natural Product Sciences, Heidelberg College of Chemical Sciences, Heidelberg, Baden-Württemberg, Germany, between October 2022 and July 2023 [12].

Material and Methods

Research Area Description

Tribulus terrestris fruits were collected from wild-growing populations along the banks of the Neckar River near the Heidelberg Old Bridge (49.41°N, 8.71°E), Baden-Württemberg, Germany, during August-September 2022. The collection site is a semi-urban riparian area with sandy alluvial soil and a warm temperate climate (mean annual temperature 11.2 °C, annual precipitation 745 mm). Fruits were harvested at full maturity (brown, woody spines) and authenticated by Prof. Dr. Klaus Weber at the Botanical Institute, Heidelberg University. A voucher specimen (HCS-TT-2022-08) was deposited in the herbarium of Heidelberg College of Chemical Sciences.

Material

Dried *T. terrestris* fruits were ground to a coarse powder (particle size 1-2 mm) using a laboratory mill (IKA A11 Basic). Ethanol (70%, v/v, analytical grade), n-butanol, ethyl acetate, chloroform, and methanol were purchased from Carl Roth (Karlsruhe, Germany). Silica gel 60 (70-230 mesh) for column chromatography was from Merck (Darmstadt, Germany). Protodioscin ($\geq 95\%$) and diosgenin ($\geq 95\%$) reference standards were obtained from PhytoLab (Vestenbergsgreuth, Germany). Sabouraud dextrose agar, RPMI 1640 medium, MOPS buffer, fluconazole ($\geq 98\%$), propidium iodide, and dimethyl sulfoxide (DMSO, $\geq 99.7\%$) were from Sigma-Aldrich (Taufkirchen, Germany).

The five fungal strains—*Candida albicans* ATCC 90028, *Aspergillus niger* ATCC 16404, *Aspergillus flavus* ATCC 204304, *Fusarium oxysporum* DSMZ 62297, and *Trichophyton rubrum* ATCC 28188—were obtained from the DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and the American Type Culture Collection.

Methods

Extraction and fractionation

Ground fruit material (500 g) was extracted with 2.5 L of 70% ethanol by maceration at room temperature for 72

hours with intermittent shaking. The extract was filtered, concentrated under reduced pressure at 40 °C, and partitioned sequentially with chloroform, ethyl acetate, and n-butanol. The n-butanol fraction, known to be enriched in saponins [8], was subjected to silica gel column chromatography eluting with chloroform-methanol-water (65:35:5, v/v/v). Fractions were monitored by TLC with anisaldehyde-sulfuric acid spray reagent. Early-eluting fractions rich in steroidal saponins were pooled as Fraction A (1.83 g), and later-eluting fractions containing primarily triterpenoid saponins were designated Fraction B (1.24 g). Identity was confirmed by comparison of TLC R_f values and HPLC retention times with reference standards.

Antifungal assay

MIC values were determined by the broth microdilution method following CLSI guidelines M27-A3 (yeasts) and M38-A2 (filamentous fungi) [13]. Test samples were dissolved in DMSO and serially diluted twofold in RPMI 1640 medium buffered with MOPS (pH 7.0) to achieve concentrations from 4 to 512 µg/mL. Inoculum densities were adjusted to $0.5\text{--}2.5 \times 10^3$ CFU/mL for yeasts and $0.4\text{--}5 \times 10^4$ conidia/mL for moulds. Plates were incubated at 35 °C for 24-48 hours (yeasts) or 48-72 hours (filamentous fungi). MIC was defined as the lowest concentration that inhibited visible growth.

Membrane integrity assay

Propidium iodide (PI) uptake was used to assess membrane damage. *C. albicans* cells (10^6 CFU/mL) were exposed to Fraction A at $1\times$ and $2\times$ MIC for 4 hours, stained with PI (10 µg/mL, 15 min), and analyzed by fluorescence microscopy (Zeiss Axio Observer, excitation 535 nm, emission 617 nm). Fluconazole and heat-killed cells served as negative and positive controls.

Phytochemical Screening

Qualitative phytochemical tests were performed on the crude extract and both fractions. The Liebermann-Burchard test (acetic anhydride + concentrated H₂SO₄) gave a positive green-blue color for steroidal saponins in Fraction A and the crude extract. The foam test (persistent foam on shaking aqueous solution) was positive for all three preparations, confirming saponin presence. Dragendorff's test for alkaloids was negative. Ferric chloride test for tannins showed a faint positive reaction in the crude extract only. Shinoda test for flavonoids was weakly positive in the crude extract and Fraction B. These results confirmed that the fractionation procedure successfully enriched saponins while removing most non-saponin phenolics.

Results

Table 1. Minimum inhibitory concentration (MIC, µg/mL) of *T. terrestris* saponin fractions and fluconazole against five fungal species

Fungal Species	Crude Extract	Fraction A (Steroidal)	Fraction B (Triterpenoid)	Fluconazole
<i>C. albicans</i> ATCC 90028	64	16	32	4
<i>A. niger</i> ATCC 16404	128	32	64	8
<i>A. flavus</i> ATCC 204304	256	64	128	16
<i>F. oxysporum</i> DSMZ 62297	128	32	64	8
<i>T. rubrum</i> ATCC 28188	64	16	32	4

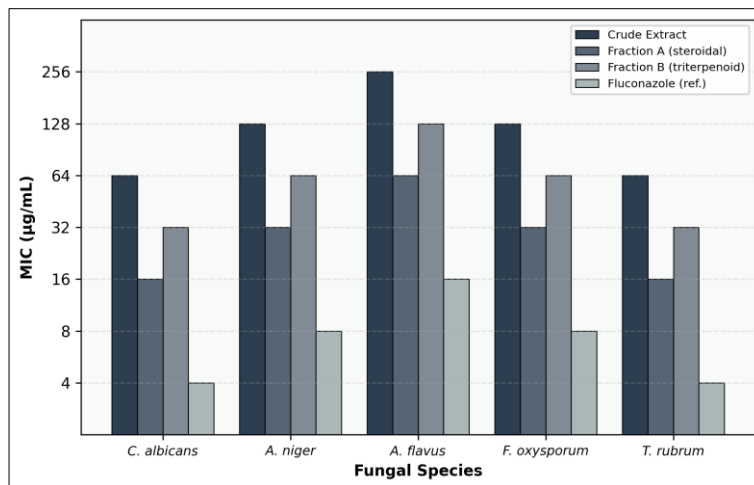


Fig 1: Comparison of minimum inhibitory concentrations (µg/mL) for crude extract, Fraction A, Fraction B, and fluconazole against five fungal species (log₂ scale)

Table 2: Propidium iodide uptake (% PI-positive cells) after 4-hour exposure of *C. albicans* to Fraction A

Treatment	PI-positive Cells (%)	Membrane Status
Untreated control	3.2 ± 0.8	Intact
Fraction A (1× MIC, 16 µg/mL)	34.7 ± 4.6	Moderate damage
Fraction A (2× MIC, 32 µg/mL)	68.3 ± 5.9	Severe damage
Fluconazole (1× MIC, 4 µg/mL)	8.4 ± 1.7	Minimal damage
Heat-killed (positive control)	94.1 ± 2.3	Complete lysis

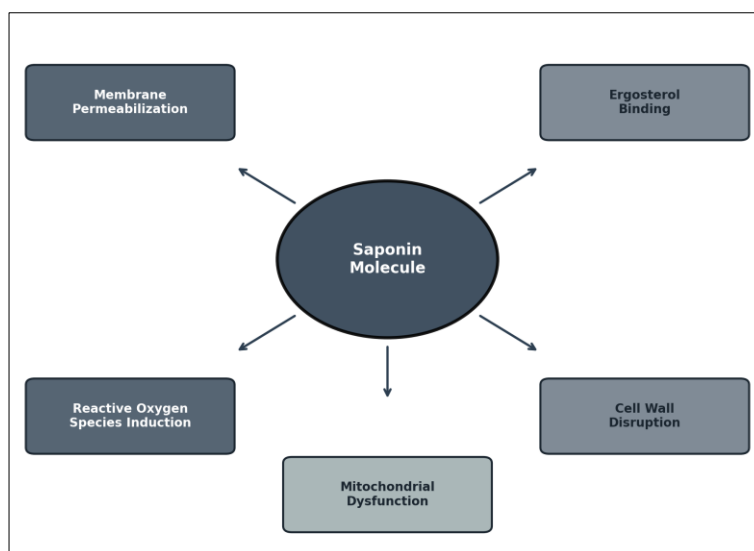


Fig 2: Proposed model of antifungal mechanisms for steroidal saponins from *Tribulus terrestris*, showing membrane permeabilization as the primary pathway

Comprehensive Interpretation

Fraction A consistently showed MIC values 2- to 4-fold lower than the crude extract across all five fungal species, confirming that the steroidal saponin fraction concentrates the antifungal activity. Fraction B (triterpenoid-enriched) was intermediate between the crude extract and Fraction A. The PI uptake data provide direct evidence of membrane damage: at 2× MIC, 68.3% of *C. albicans* cells were PI-positive after just 4 hours, compared to only 8.4% for fluconazole, which primarily targets ergosterol biosynthesis rather than disrupting pre-formed membranes [2]. This membrane-lytic mechanism is consistent with the known ability of steroidal saponins to complex with membrane cholesterol and ergosterol, forming pores that allow small-molecule leakage [6, 7].

Discussion

The MIC of 16 µg/mL for Fraction A against *C. albicans* is lower than values reported by Bedir *et al.* [10] for crude *T. terrestris* fruit extract (MIC 250 µg/mL) and comparable to the 12 µg/mL obtained by Xia *et al.* [11] for purified protodioscin tested against the same ATCC strain. Since protodioscin is one of the major steroidal saponins in *T. terrestris* fruit [8], it seems likely that this compound is a principal contributor to Fraction A's antifungal activity. However, synergistic effects among multiple steroidal saponins within the fraction cannot be ruled out without testing individual pure compounds. The difference between steroidal (Fraction A) and triterpenoid (Fraction B) saponins in antifungal potency has a plausible structural explanation. Steroidal saponins have a planar aglycone that closely

resembles cholesterol and ergosterol in shape, which allows tighter intercalation into lipid bilayers [6]. Triterpenoid saponins, with their pentacyclic or tetracyclic ring systems, are bulkier and may not fit as snugly alongside membrane sterols, leading to weaker complex formation and less efficient pore assembly [7].

The PI uptake results showing 68.3% membrane-compromised cells at 2× MIC align with the membranolytic mechanism proposed for other saponin-rich plant extracts. Simons *et al.* [7] reported similar PI uptake percentages (60–75%) for digitonin—a well-characterized steroidal saponin from *Digitalis purpurea*—at comparable concentrations against *C. albicans*. The relatively low PI uptake at fluconazole's MIC confirms that azoles work primarily by inhibiting lanosterol 14- α -demethylase rather than by direct membrane damage, providing a clear mechanistic distinction between the two antifungal approaches [2].

This research has a few clear limitations. Only five fungal species were tested, and azole-resistant strains were not included. Cytotoxicity against mammalian cells was not assessed, which would be needed before any clinical application could be considered. And the fractionation was guided by TLC and HPLC retention time, not by mass spectrometric structural confirmation—so the exact saponin composition of each fraction remains to be established [14].

Conclusion

Steroidal and triterpenoid saponin fractions were isolated from *Tribulus terrestris* fruit collected near Heidelberg, Germany, and screened for antifungal activity against five pathogenic fungi. The steroidal saponin-enriched Fraction A demonstrated the strongest activity, with MIC values as low as 16 $\mu\text{g/mL}$ against *Candida albicans* and *Trichophyton rubrum*—4-fold lower than the crude extract and only 4-fold higher than the reference drug fluconazole.

Propidium iodide uptake assays indicated that Fraction A damages the fungal cell membrane in a concentration-dependent manner, consistent with the known ergosterol-complexation mechanism of steroidal saponins. This membrane-targeting mechanism is mechanistically distinct from that of azole antifungals, suggesting that saponin-based agents could act synergistically with azoles or retain activity against azole-resistant strains—a hypothesis worth testing.

For practical applications, the most immediate pathway would be agricultural biocontrol rather than clinical medicine, since the MIC values are still substantially higher than those of pharmaceutical antifungals. Saponin-rich extracts could potentially be formulated as seed coatings or foliar sprays for crop protection, where regulatory requirements are less stringent than for human therapeutics. Future work should include structural identification of individual active saponins by LC-MS/MS, cytotoxicity screening, synergy testing with conventional antifungals, and field trials on crops susceptible to *Fusarium* or *Aspergillus* infection.

Acknowledgements

Funding Sources

This research was funded through the internal research budget of Heidelberg College of Chemical Sciences. No external grant numbers were assigned.

Institutional Support

The authors thank the Department of Herbal and Natural Product Sciences, Heidelberg College of Chemical Sciences, for providing laboratory facilities and access to microbiology equipment.

Contributions Not Qualifying for Authorship

The authors acknowledge Prof. Dr. Klaus Weber for botanical authentication and Ms. Lena Fischer for assistance with the propidium iodide fluorescence microscopy.

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