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Plumbagin elicits evidence of apoptosis-like cell death and inhibits conidial germination and mycelial growth in ginger rot isolate *Fusarium* strain GI-FS1

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Ginger (*Zingiber officinale*) is an economically important spice crop widely cultivated for its culinary and medicinal values. However, its productivity is severely constrained by soft rot disease, causing substantial yield losses, quality, and persistent challenges in ginger cultivation. In this study, the major pathogenic fungus GI-FS1 was isolated from *Z. officinale* with typical symptoms and was identified as *Fusarium* species through morphological and molecular identification. The antifungal potential of plumbagin, a plant-derived naphthoquinone from *Plumbago rosea*, noted for antibacterial, antioxidant, and anticancer properties, was evaluated against GI-FS1. Plumbagin treatment significantly inhibited conidial germination and mycelial growth with Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values of 10 µg/mL and 15 µg/mL, respectively. Conidial germination assays, microscopy, onion peel epidermis penetration confirmed inhibition, as plumbagin-treated spores failed to germinate and penetrate epidermis. 4',6-Diamidino-2-phenylindole (DAPI) staining showed chromatin condensation and Acridine orange/ethidium bromide (AO/EB) revealed membrane disruption, coupled with 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) detected Reactive oxygen species (ROS) accumulation, signifying oxidative stress-induced cell death by plumbagin. Elevated electrolyte leakage and DNA fragmentation supported apoptosis-like mechanisms underlying fungal growth inhibition; however, further studies on apoptotic factor gene expression are required to confirm this mechanism. Scanning Electron Microscopy (SEM) analysis revealed spore shrinkage and thinner, collapsed, rough mycelia, indicating loss of cell integrity on treatment with plumbagin. Overall, these findings provide the first report on the targeted mechanisms of plumbagin in inhibiting *Fusarium* conidial germination and inducing apoptotic-like cell death. By disrupting fungal viability through oxidative stress and apoptosis-related pathways, the study highlights plumbagin's potential as a natural antifungal agent for the sustainable management of soft rot disease in ginger.

KEYWORDS

plumbagin, *Fusarium*, ginger softrot, conidial germination, apoptosis, oxidative stress

1 Introduction

Ginger (*Zingiber officinale* L.) is an economically important spice crop widely cultivated across tropical and subtropical regions, including China, India, Japan, Nigeria, Sri Lanka, and Australia (Dhanik et al., 2017). India remains the world's leading producer, contributing the largest share of global cultivation area and yield (B. Bag, 2018). Ginger productivity, however, is severely constrained by biotic stresses, particularly fungal diseases that affect both field-grown and stored rhizomes. Among these, soft rot (rhizome rot) is the most destructive disease, causing significant yield and post-harvest losses and posing a serious threat to the sustainability of ginger cultivation (Ravindran and Babu, 2016). Soft rot disease is primarily associated with moisture-dependent fungal pathogens such as *Pythium*, *Fusarium*, *Rhizoctonia*, and *Sclerotium* species (Ravindran and Babu, 2016). Infected rhizomes exhibit tissue maceration, discoloration, foul odor, and rapid decay, rendering them unsuitable for storage or market (Talley et al., 2002). *Fusarium oxysporum* is one of the most widespread and destructive plant-pathogenic fungi, with numerous formae speciales capable of infecting over 120 economically important crops. It is among the most aggressive causal agents in ginger soft rot, causing yield losses of 50–90% under favourable environmental conditions (Ravindran and Babu, 2016; Fanai et al., 2025). There is a growing concern about synthetic fungicides; researchers are exploring various eco-friendly alternatives. Plant-derived fungicides have emerged as a promising and sustainable solution. Among these, naphthoquinones constitute a structurally diverse class of secondary metabolites with well-documented antifungal properties. Their biological activity is largely attributed to their redox-active quinone moiety, which disrupts cellular redox homeostasis, induces oxidative stress, and interferes with essential metabolic processes in fungal cells (Prasath et al., 2023). Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a naturally occurring naphthoquinone predominantly isolated from *Plumbago* species, has attracted considerable attention due to its broad-spectrum antimicrobial, anticancer, and anti-inflammatory activities (Nair et al., 2016). Antifungal effects of plumbagin have been reported against multiple fungal genera, including *Aspergillus*, *Candida*, *Penicillium*, and *Fusarium* (Hassan et al., 2016).

Despite these reports, existing studies on *Fusarium* spp. have largely focused on mycelial growth inhibition, primarily reporting IC_{50} values for radial growth, without addressing critical early infection stages or underlying cellular death mechanisms. In particular, the effects of plumbagin on *Fusarium* isolates associated with ginger soft rot remain unexplored, and no studies have examined its impact on spore germination, the primary infection stage in ginger soft rot and the underlying mechanism in this agronomically significant pathosystem (Zhang et al., 2024). Furthermore, prior work has not distinguished between fungistatic and fungicidal activity through MIC/MFC determination, nor assessed potential specificity toward ginger-associated pathovars, including regional variants of *F. oxysporum* f. sp. *zingiberis*. Recent studies have nevertheless highlighted the agronomic feasibility of plumbagin-based interventions for ginger disease management. Pre-planting rhizome soaks with plumbagin (0.5–2 $\mu\text{g mL}^{-1}$, 30 min) have been shown to significantly reduce soft rot incidence

in controlled pot trials, while foliar and soil drench applications (5–10 $\mu\text{g mL}^{-1}$) during sprouting are compatible with fertilization-based practices (*In vitro* Antifungal Activity of *Plumbago zeylanica*, n.d.). Advances in formulation, including chitosan- and alginate-based nanoencapsulation, have further enhanced the persistence of plumbagin under environmental conditions by improving stability and controlled release (Chrastina et al., 2018). In addition, integration with plant growth-promoting rhizobacteria (PGPR), such as *Bacillus subtilis* and *Pseudomonas fluorescens*, has resulted in superior disease suppression compared with conventional fungicide treatments alone. Sustainable sourcing from *Plumbago indica* roots, which yield appreciable levels of plumbagin, further supports its economic viability for large-scale application (Mane et al., 2021). Given plumbagin's redox-active nature, it is hypothesised to exert antifungal activity through oxidative stress mediated disruption of fungal viability, thereby inhibiting spore development and inducing apoptosis-like cell death in ginger soft rot-associated *Fusarium* isolates. Such multi-target antifungal action is expected to confer fungicidal efficacy and support the potential of plumbagin as a sustainable and targeted botanical fungicide for crop disease management.

The current study offers a comprehensive evaluation of the antifungal activity of plumbagin against *Fusarium* GI-FS1, the causal agent of ginger soft rot, a highly economically damaging disease of ginger crops. By systematically investigating spore germination inhibition, mycelial growth inhibition, fungicidal activity (MIC/MFC), and intracellular reactive oxygen species accumulation, this study confirms the dual inhibitory and fungicidal properties of plumbagin in this agronomically relevant pathosystem. Moreover, this study offers the first mechanistic proof of plumbagin-induced apoptosis-like cell death in the ginger soft rot-related *Fusarium* isolate, thus extending the existing knowledge on plant-derived antifungal compounds beyond the conventional growth inhibition assays. The rising disease pressure and the increasing limitations of synthetic fungicides, and development of resistance along with environmental issues highlights major challenges in disease control. By identifying plumbagin as a multi-target, plant-derived antifungal compound with mechanistically supported properties, this study contributes to the development of environmentally responsible crop protection strategies and supports the use of natural bioactive metabolites in sustainable ginger disease management practices.

2 Materials and methods

2.1 Isolation of fungal strains

Fresh rhizomes of *Zingiber officinale* were collected from cultivated fields in Palakkad district, Kerala, India (10.72°N, 76.56°E). Mature rhizomes, developed over approximately nine months, were harvested in mid-November. Fungal isolation from infected ginger rhizomes was performed using the agar plating method with slight modifications (Overy and Frisvad, 2005). Conidia from mold-infected regions were aseptically collected using a flame-sterilized inoculation loop and streaked onto Potato Dextrose Agar (PDA). The plates were incubated in the dark at 28 °

C for 5 days and examined for characteristic fungal growth (Subhash et al., 2022). Subsequently, distinct isolates formed were sub-cultured onto PDA slants, followed by preservation at 4° C.

2.2 Identification of isolated fungi

The isolated fungal strain was identified using cultural, morphological, and molecular approaches (Shinano et al., 1995; Kumar et al., 2013). Macroscopic characteristics, including colony color and growth pattern, were assessed by visual examination after culturing the fungus on PDA plates for 5 days in the dark at 25 °C. Microscopic evaluation of mycelia, conidiophores, and conidia was performed to determine morphological features. For molecular characterization, genomic DNA was extracted from the fungal culture using the Cetyltrimethylammonium Bromide (CTAB) method (Ivors, 2015). The internal transcribed spacer (ITS) region was amplified using universal primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC). The purified PCR products were subjected to Sanger sequencing at Eurofins India Pvt. Ltd., Bangalore, India. The resulting sequence was analyzed using the NCBI nucleotide BLAST tool to determine sequence homology and confirm fungal identity (Raja et al., 2017). MEGA X software was used to construct a phylogenetic tree based on neighbor-joining (NJ), and bootstrap analysis was performed with 1000 repetitions.

2.3 Extraction of plumbagin

Roots of mature *Plumbago rosea* plants were collected in December from the campus of Amrita Vishwa Vidyapeetham, Amritapuri, Kollam, Kerala, India (9°05'42.0"N, 76°29'33.0"E). A voucher specimen was properly identified and deposited in the Sree Narayana College Herbarium (SNCH) under accession number 5158. Plumbagin was extracted from *Plumbago rosea* roots following a previously reported procedure (Raja et al., 2017; Prakash et al., 2021). Fresh roots were initially defatted with diethyl ether using a rotary shaker, and the resulting extract was concentrated under reduced pressure using a rotary evaporator maintained below 40 °C to obtain crude plumbagin. The residue was subjected to thin-layer chromatography using a solvent system of petroleum ether (70%) and ethyl acetate (30%) to monitor the extract profile. Further purification was achieved through column chromatography, where plumbagin was eluted with petroleum ether containing increasing concentrations of chloroform. The identity and purity of the isolated plumbagin were confirmed using a Shimadzu LC-20 HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a Phenomenex C18 reverse-phase column and Photodiode Array detector. The mobile phase consisted of a 1:1 ratio of water and methanol, and detection was performed at 245 nm. Plumbagin stock solutions (10 mg/mL) were prepared in 100% DMSO; final assay concentrations were maintained at ≤2.5% DMSO across all treatments.

2.4 Antifungal activity of Plumbagin

2.4.1 Radial growth assay

The antifungal effect of plumbagin on mycelial growth was evaluated using a radial growth inhibition assay (Koka et al., 2021).

Plumbagin was incorporated into PDA at concentrations ranging from 5 to 100 µg/mL. A 5 mm mycelial plug from an actively growing *Fusarium* GI-FS1 culture was placed at the center of each plate. Carbendazim was included as a positive control. All treatments were performed in triplicate. The plates were incubated at 28 °C for 5 days, after which the radial growth diameter was measured to determine the extent of growth inhibition.

Conidial germination was assessed by evaluating the effect of plumbagin on the growth of *Fusarium* GI-FS1 spores (Slawecki et al., 2002). PDA plates containing plumbagin at concentrations ranging from 5 to 100 µg/mL were spot-inoculated with 5 µL of a spore suspension (1×10^6 spores/mL). DMSO served as the solvent control, and carbendazim (5 µg/mL) was used as the positive control. All treatments were conducted in triplicate. The plates were incubated at 28 °C for 5 days, and fungal growth was quantified by measuring the diameter of the resulting colonies.

2.4.2 Minimum inhibitory concentration and minimum fungicidal concentration

MIC of plumbagin against GI-FS1 was determined using the broth microdilution method in 48-well microtiter plates (Dellavalle et al., 2010). Serial dilutions of plumbagin (5–100 µg/mL) were prepared, with carbendazim included as the positive control, DMSO as the solvent control, and untreated potato dextrose broth (PDB) as the negative control. Each well contained 100 µL of broth inoculated with a spore suspension (1×10^6 spores/mL). Following a 30-minute sedimentation period at 27 °C, the initial absorbance was measured at 595 nm, and a second reading was taken after 48 hours of incubation. MIC was defined as the lowest concentration of plumbagin that produced ≥90% inhibition of fungal growth, calculated using the formula:

$$[(\Delta C - \Delta T) / \Delta C] \times 100$$

where, ΔC - the corrected absorbance of the control micro culture at 595 nm.

ΔT - the corrected absorbance of the test micro culture.

The corrected absorbance value equal to the absorbance at 595 nm of the culture measured after 48h minus the absorbance at 595 nm measured after 30 min.

For MFC determination, the microtiter plates were incubated for an additional 24 hours (total 72 hours). Subsequently, 10 µL from each well showing no visible growth was plated onto PDA and incubated for 48 hours to confirm fungal viability (Paola Díaz Dellavalle et al., 2010).

2.4.3 Conidial germination assay

Conidial germination was evaluated in PDB supplemented with graded concentrations of plumbagin (5, 10, 15, 25, 50, 75, and 100 µg/mL). Carbendazim was used as the positive control, and DMSO served as the solvent control. The assay was performed in a 96-well microtiter plate. Each well was filled with 100 µL of PDB and inoculated with 10 µL of a GI-FS1 spore suspension (1×10^6 spores/mL), followed by the addition of the respective concentrations of plumbagin, carbendazim, or DMSO. After 24

hours of incubation, conidial germination was examined microscopically to assess the inhibitory effect of plumbagin on spore germination (Rongai et al., 2012).

2.4.4 Mycelial growth inhibition assay

Fusarium GI-FS1 conidia were allowed to germinate in PDB at 28 °C on a rotary shaker (200 rpm) for 24 hours. The germinated cultures were then exposed to different concentrations of plumbagin, with carbendazim as the positive control and DMSO as the solvent control, for 2 and 24 hours. Following treatment, the conidial suspensions were centrifuged at 10,000 rpm for 5 minutes at 4 °C, washed twice with sterile distilled water, and resuspended in sterile distilled water. Aliquots of the treated conidia were plated onto PDA and incubated at 28 °C for 2 days to assess mycelial growth inhibition.

2.5 Scanning electron microscopy

The effect of plumbagin on GI-FS1 spores and mycelia was examined SEM. Fungal samples were fixed in 2.5% glutaraldehyde in phosphate-buffered saline for 12 hours at 4 °C, followed by a 20-minute wash with sterile distilled water. Samples were dehydrated through a graded ethanol series (30%, 50%, 70%, 80%, 90%, and 95%) for 10 minutes each, then treated with 100% ethanol for 30 minutes, and air-dried. This gradual dehydration minimized structural shrinkage while preserving surface morphology.

The dried samples were mounted onto SEM stubs using double-sided adhesive tape and coated with a gold-palladium layer under vacuum to enhance conductivity. Fungal spores and mycelia were then examined and imaged using a scanning electron microscope to observe morphological alterations following plumbagin treatment (Hu et al., 2024).

2.6 Penetration assay

The effect of plumbagin on the penetration ability of *Fusarium* GI-FS1 was assessed using onion (*Allium cepa*) epidermis sections (An et al., 2016; Han et al., 2020). Small epidermal strips were placed on glass slides and inoculated with a spore suspension (2×10^6 spores/mL). Plumbagin (15 µg/mL) was applied 24 hours prior to fungal inoculation. DMSO served as the solvent control, and carbendazim was used as the positive control. Inoculated samples were incubated in a moist chamber at 28 °C in darkness. 12 and 24 hours post-inoculation, the epidermal strips were stained with 0.05% lactophenol cotton blue for 10 minutes (Windram et al., 2012).

2.7 Cell viability assay using dual fluorescent staining

Cell viability of GI-FS1 hyphae was assessed using dual staining with fluorescein diacetate (FDA, green fluorescence) and propidium iodide (PI, red fluorescence). 2×10^6 spores/mL of *F. oxysporum* were inoculated into PDB medium and incubated at 28 °C for 3 days in the dark, followed by treatment with plumbagin at concentrations of 5, 10, 15, 20, 25, 50, 75, and 100 µg/mL for

24 hours, with carbendazim and DMSO included as positive and solvent controls, respectively. Following treatment, hyphal samples were harvested, washed five times with PBS (10 mM, pH 7.4), and resuspended in PBS containing 10 µM PI and 30 µM FDA. Samples were vortexed and incubated in the dark at 25 °C for 20 minutes. Excess dye was removed by five additional washes with PBS, and samples were resuspended in 2 mL sterile distilled water and observed for fluorescence (Hu et al., 2024).

2.8 Assessment of electrolyte leakage

Electrolyte leakage from GI-FS1 hyphae was evaluated to assess membrane integrity. Spores (2×10^6 spores/mL) were inoculated in PDA at 28 °C in darkness for 72 hours. Following incubation, hyphae were treated with plumbagin at concentrations of 5, 10, 15, 20, 25, 50, 75, and 100 µg/mL for 3 hours. Carbendazim and DMSO served as positive and solvent controls, respectively. After treatment, the cultures were centrifuged, and the electrical conductivity of the supernatant was measured using a conductivity meter (Hu et al., 2024).

2.9 Reactive oxygen species detection

Intracellular accumulation of reactive oxygen species (ROS) in GI-FS1 hyphae following plumbagin treatment was assessed using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Ngo et al., 2019). Hyphae were cultured for 72 hours and then treated with plumbagin at concentrations of 5, 10, 15, 20, 25, 50, 75, and 100 µg/mL for an additional 24 hours. Carbendazim and DMSO were included as positive and solvent controls, respectively.

After treatment, the hyphae were centrifuged at $5,000 \times g$ for 8 minutes and resuspended in 10 mM phosphate-buffered saline (PBS, pH 7.4). Samples were incubated with 10 µM DCFH-DA in the dark at 25 °C for 30 minutes. Following staining, the hyphae were washed five times with PBS to remove excess dye and resuspended in 1 mL sterile double-distilled water. Fluorescence imaging was performed using a microscope under both bright-field and fluorescence modes (Hu et al., 2024).

2.10 DNA laddering assay

DNA fragmentation in GI-FS1 was assessed using a DNA laddering assay. Spores (2×10^6 spores/mL) were inoculated in PDA at 28 °C for 72 hours (control). After incubation, the mycelium was treated with plumbagin at 15 µg/mL for 24 hours. Treated and control mycelia were harvested by centrifugation at 10,000 rpm for 10 minutes at 4 °C and filtered using blotting paper. The mycelial pellet was crushed into a paste using a mortar and pestle and stored at -80 °C until DNA extraction. Approximately 0.5 g of mycelium was transferred to a fresh tube, and 450 µL of 2% CTAB buffer - 2% (w/v) cetyltrimethylammonium bromide (CTAB), 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl was added. The mixture was centrifuged at 11,000 rpm for 10 minutes at 4 °C. The supernatant was extracted twice with an equal volume of chloroform: isoamyl alcohol (24:1), centrifuging at 11,000 rpm for 10 minutes at 4 °C each time. DNA was precipitated by adding two-thirds volume of ice-cold isopropanol and

incubating at -20°C overnight. The DNA pellet was collected by centrifugation at 11,000 rpm for 5 minutes at 4°C , washed with 450 μL of 80% ethanol, air-dried, and resuspended in 30 μL Milli-Q water. Genomic DNA was stored at -20°C until use (Ivors, 2015). Finally, 10 μg of DNA from each sample was analyzed by electrophoresis on a 2% agarose gel to assess fragmentation (Lee et al., 2012).

2.11 Apoptosis detection by acridine orange/ethidium bromide and DAPI

Apoptotic morphological changes in GI-FS1 hyphae were assessed using dual acridine orange/ethidium bromide (AO/EB) staining and DAPI staining (Pajaniradje et al., 2014; Agus et al., 2018). AO/EB staining, cells were washed with PBS (pH 7.4), and 100 μL of cell suspension was mixed with 5 μL of AO/EB solution (60 $\mu\text{g}/\text{mL}$ AO and 100 $\mu\text{g}/\text{mL}$ EB in PBS). After 5 minutes of incubation at room temperature, cells were washed with PBS and examined under a fluorescence microscope. Acridine orange penetrates all cells, staining nuclei green, whereas ethidium bromide is selectively taken up by cells with compromised membranes, producing orange-red fluorescence.

For DAPI staining, cells were fixed with 3.7% formaldehyde for 1 hour, washed with PBS, and stained with 1 $\mu\text{g}/\text{mL}$ DAPI to assess chromatin condensation and apoptotic DNA fragmentation. Stained samples were visualized under a fluorescence microscope to confirm apoptotic features.

2.12 Computational molecular docking studies

The objective of the computational work was to identify the affinity of plumbagin towards the enzymes (Lanosterol 14α -demethylase-CYP51, Cytochrome C, Topoisomerase I & II). These enzymes were selected based on experimental data.

The docking was performed in Autodock vina (Mir et al., 2022), the docking was performed on Plumbagin's active site with the 4 enzymes. The required structural data was retrieved through Homology modelling using Swiss Model (Waterhouse et al., 2018). Standard protein and ligand preparations were done like removing H_2O and hetero atoms, repairing missing atoms, adding polar hydrogen and kollman charges. Then the results were visualized using PyMOL, to identify the specific amino acid interactions BIOVIA Discovery Studio Visualizer (Mir et al., 2022) was used.

2.14 Leaf disc assay

Phytotoxic effects of plumbagin were evaluated using a leaf disc assay (Shinano et al., 1995). Healthy and fully expanded ginger leaves were selected, and uniform leaf discs were prepared using a sterile cork borer. The discs were immediately transferred into sterile 24-well culture plates under aseptic conditions. Each well received 1 mL of plumbagin solution prepared at concentrations of 5, 10, 15, 20, and 25 $\mu\text{g}/\text{mL}$. A solvent control containing 1% (v/v) dimethyl sulfoxide (DMSO) was included as a control. The plates were incubated under light conditions at room temperature for

48–72 hours. Following incubation, the leaf discs were visually examined for phytotoxic symptoms such as chlorosis and necrosis. For quantitative pigment analysis, chlorophyll was extracted by immersing the leaf discs in 100% DMSO and incubating until complete pigment extraction was achieved. The absorbance at 665nm (A_{665}) and absorbance at 648nm (A_{648}) of the extracted pigment solution was measured using a 96-well microplate reader to estimate chlorophyll a and chlorophyll b content using the formula:

$$\text{Chlorophyll a} = 14.85 A_{665} - 5.14 A_{648}$$

$$\text{Chlorophyll b} = 25.48 A_{648} - 7.36 A_{665}$$

$$\text{Total Chlorophyll} = (20.21 \times A_{648}) + (7.63 \times A_{665})$$

2.14 GenBank accession numbers

The ITS sequence of GI-FS1 used in this study was deposited in the GenBank database under the accession number MN598647.

2.15 Statistical analysis

GraphPad Prism 8.2 (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis. All experiments were conducted with three replicates, and the experiments were repeated thrice. The results of the experiments were expressed in terms of mean values and standard deviations. Statistical comparison was carried out using one-way ANOVA (analysis of variance) Dunnett's multiple comparison test at $P < 0.05$, paired t test at $P < 0.05$, and two-way ANOVA at $P < 0.0001$ was used to indicate statistical significance.

3 Results

3.1 Identification of fungal strain

3.1.1 Microscopic and macroscopic characters

The fungal strain isolated from diseased ginger was identified using cultural, morphological, and molecular approaches. On Potato Dextrose Agar (PDA), the fungus produced slow-growing, white cottony colonies with red pigmentation on the bottom surface of the agar (Supplementary Figures 1A, B). Microscopic examination revealed hyaline, septate hyphae with two types of conidia: sickle-shaped macroconidia and oval microconidia (Supplementary Figures 1C, D). These morphological features are consistent with the known characteristics of *Fusarium* species.

3.1.2 Molecular characterization

PCR amplification of the ITS region produced a fragment of approximately 500 bp, consistent with the expected ITS amplicon size for *Fusarium* species. The PCR products were sequenced for species-level identification, and the resulting sequence was analyzed using the NCBI BLAST tool. The BLAST results and phylogenetic

analysis showed 99.45% sequence identity with *Fusarium* confirming that the isolate belongs to the *Fusarium* species complex (Figure 1). The ITS sequence was deposited in the GenBank database under the accession number MN598647. Together with the cultural and microscopic characteristics, the molecular data conclusively identified the isolate as *Fusarium* sp.

3.2 Plumbagin extraction

High performance liquid chromatography (HPLC) analysis was carried out on both the column-purified sample and the standard plumbagin using a reverse-phase C18 column. The mobile phase was equipped with methanol and water in equal proportions, delivered at a flow rate of 1 mL/min. The purified sample exhibited a sharp peak at a retention time of 4.70 minutes, which closely matched the retention time of the standard plumbagin at 4.67 minutes (Figure 2). This close concordance in retention time strongly supports the identification of the isolated compound as plumbagin. Furthermore, the chromatogram revealed that the

isolated sample possessed a purity exceeding 90%, thereby confirming the effectiveness of the purification process.

3.3 Antifungal assays

3.3.1 MIC and MFC

The MIC of plumbagin against GI-FS1 was determined using the broth microdilution method. Complete inhibition of fungal growth was observed at 10 µg/mL, which was considered the MIC. Lower concentrations (0.5–5 µg/mL) showed minimal inhibitory effects, whereas inhibition sharply increased at 10 µg/mL and remained consistent at higher concentrations (Figure 3A).

The MFC is defined as the lowest concentration of a compound at which no visible fungal growth occurs. Plumbagin exhibited fungicidal activity against GI-FS1 at concentrations ≥15 µg/mL, establishing its MFC at 15 µg/mL. Lower concentrations (5 and 10 µg/mL) allowed fungal growth. DMSO and untreated control showed visible mycelial regrowth (Figure 3B).

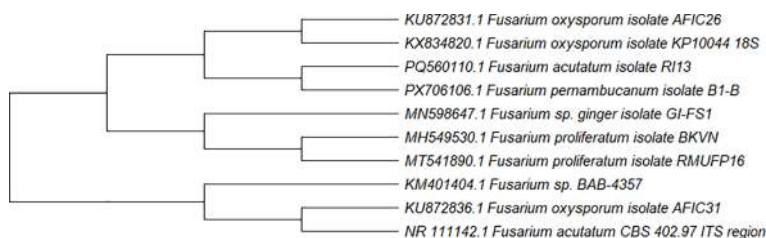


FIGURE 1

Phylogenetic tree of *Fusarium* sp. GI-FS1 (query MN598647) gene sequences by maximum likelihood method.

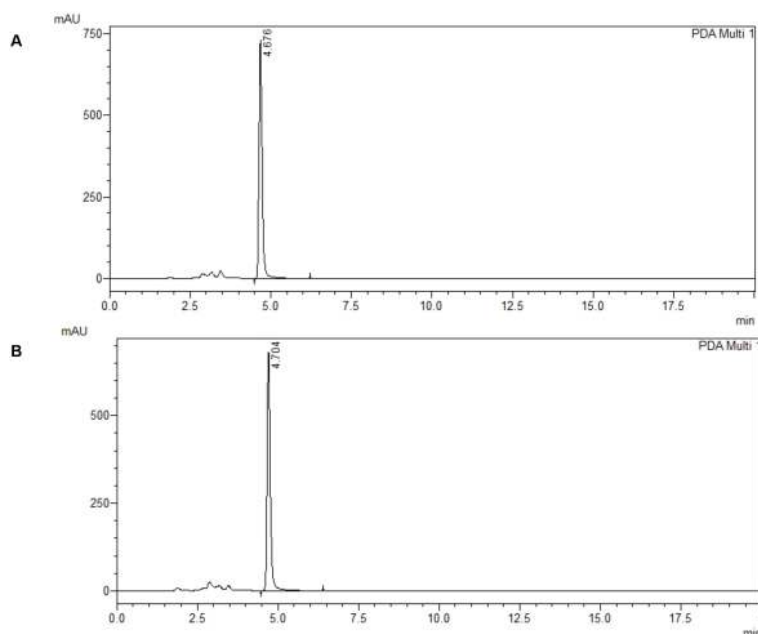


FIGURE 2

HPLC chromatogram of plumbagin. (A) Standard plumbagin. (B) Plumbagin isolated from *Plumbago rosea* using column chromatography, showing 90% purity.

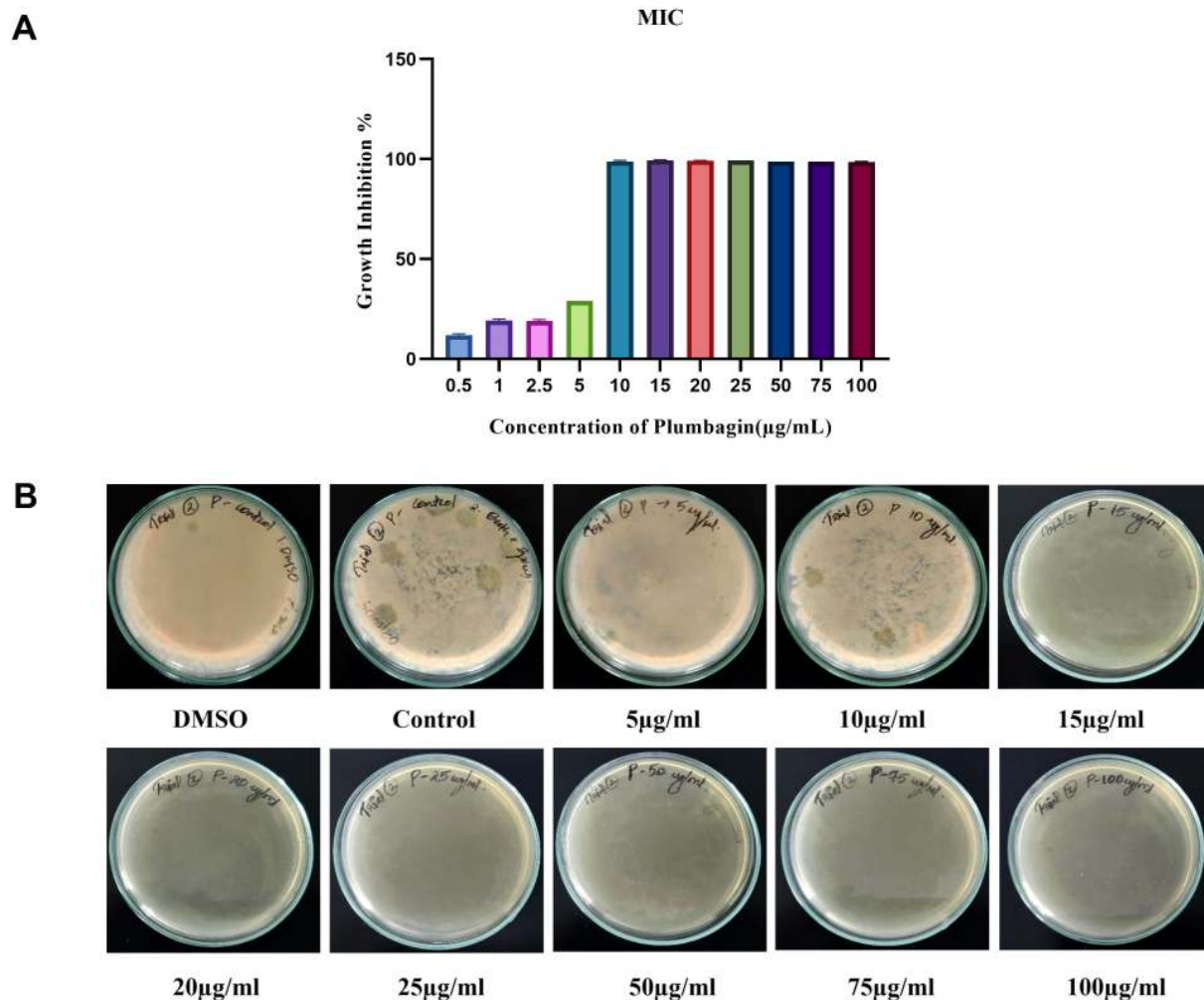


FIGURE 3

(A) MIC determination of plumbagin against GI-FS1. Plumbagin inhibited fungal growth in a concentration-dependent manner, with complete inhibition at 10 $\mu\text{g/mL}$. Data are presented as percent inhibition relative to the untreated control. (B) Minimum Fungicidal Concentration (MFC) of plumbagin against GI-FS1. Plumbagin exhibited complete fungicidal activity at concentrations ≥ 15 $\mu\text{g/mL}$. While DMSO and untreated controls showed visible mycelial regrowth, confirming the fungicidal efficacy of the treatments.

3.3.2 Radial growth assay

The effect of plumbagin on GI-FS1 conidial germination (Figure 4) and mycelial growth (Figure 5) was evaluated using a radial growth assay. PDA plates were supplemented with varying concentrations of plumbagin (5–100 $\mu\text{g/mL}$), inoculated with fungal spores and mycelial agar blocks for conidial germination and mycelial growth, respectively. Carbendazim was used as control, and DMSO served as solvent control. Conidial germination was observed only at lower plumbagin concentrations (5 and 10 $\mu\text{g/mL}$) and was completely inhibited at concentrations ≥ 15 $\mu\text{g/mL}$. Mycelial growth was dose-dependently suppressed, with higher plumbagin concentrations resulting in progressively greater inhibition of radial expansion.

3.3.3 Conidial germination assay

Plumbagin significantly reduced conidial germination of GI-FS1, with inhibition from 5 $\mu\text{g/mL}$ and complete inhibition at

concentrations ≥ 15 $\mu\text{g/mL}$ (Figure 6). At these higher concentrations, conidia remained ungerminated, indicating that plumbagin interferes with the early stages of fungal development. Carbendazim also inhibited germination, whereas DMSO and untreated controls displayed normal germ tube formation and hyphal elongation. These results highlight the strong antifungal potential of plumbagin, particularly due to its ability to block conidial germination at early stages without causing structural deformations, suggesting a mechanism of action that differs from Carbendazim.

3.3.4 Mycelial growth inhibition assay

Plumbagin effectively suppressed GI-FS1 mycelial growth in a dose-dependent manner (Figure 7). Concentrations above 25 $\mu\text{g/mL}$ caused a significant reduction in fungal growth, and at 50 $\mu\text{g/mL}$ and higher, growth was nearly or completely inhibited after 24 hours. In comparison, carbendazim showed no inhibition. DMSO and untreated controls exhibited normal growth,

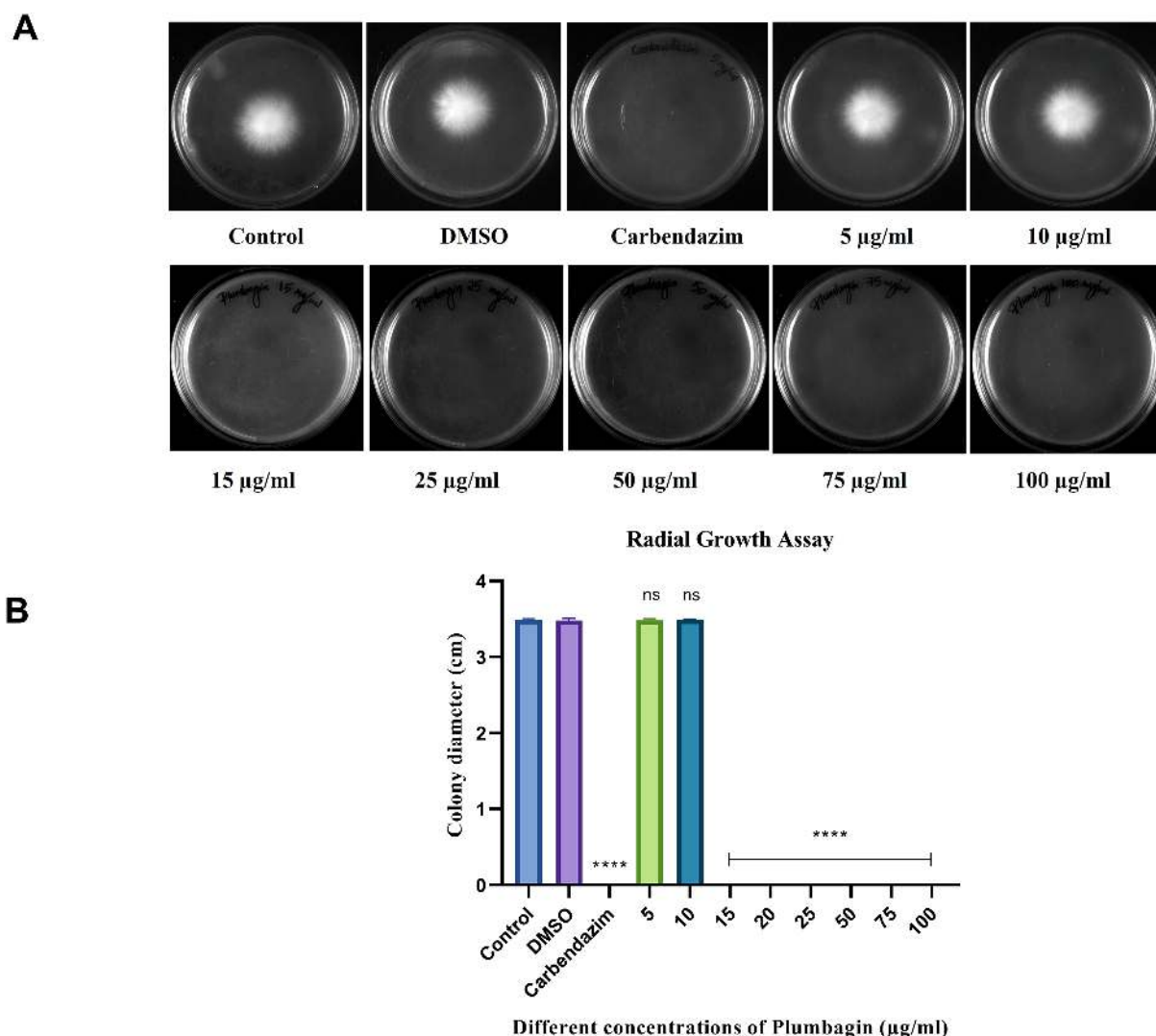


FIGURE 4

Radial growth assay showing the antifungal effect of Plumbagin on GI-FS1 conidial germination on solid agar media. (A) Plumbagin exhibited dose-dependent antifungal activity against GI-FS1 on PDA plates. Complete growth inhibition was observed at concentrations ≥ 15 $\mu\text{g/mL}$. While Carbendazim (positive control) inhibited growth, untreated control and DMSO (solvent control) showed no effect. (B) The bar graph represents the colony diameter (cm), confirming growth inhibition at higher Plumbagin concentrations. Data are expressed as mean $\pm$ SD from three independent experiments consisting of three biological replicates ($P < 0.05$, ANOVA, Dunnett's multiple comparison test). '****' denotes highly significant differences ($P < 0.0001$); 'ns' indicates non-significant differences ($P > 0.05$) based on ANOVA followed by Dunnett's multiple comparison test.

confirming that the antifungal effect was specific to plumbagin. Overall, plumbagin demonstrated stronger antifungal activity than carbendazim under the tested conditions.

3.4 Scanning electron microscopy

SEM analysis revealed that treatment of GI-FS1 with plumbagin resulted in distinct, concentration-dependent morphological alterations compared to the untreated control (Figure 8). Control spores and mycelia exhibited smooth surfaces, uniform morphology, and intact cellular architecture. In contrast, plumbagin-treated spores displayed progressive shrinkage, deformation, and surface irregularities, while the mycelia appeared thinner, distorted, and had increased surface roughness at the MIC; membrane rupture was observed at higher concentrations tested.

3.5 Penetration assay

To evaluate the effect of plumbagin on the penetration of GI-FS1 into plant tissue, an onion epidermal infection assay was performed (Figure 9). In the control, DMSO and carbendazim-treated groups, spores germinated and penetrated plant cells by 24 hours post-inoculation (hpi). In contrast, plumbagin-treated spores exhibited reduced germination at 5 $\mu\text{g/mL}$ or no penetration at 10 and 15 $\mu\text{g/mL}$. These results indicate that plumbagin effectively inhibits infection by blocking both conidial germination and penetration into host tissue, demonstrating its potential as a natural antifungal agent.

3.6 Cell viability assessment

The FDA/PI staining assay showed a clear impact of plumbagin on the viability of GI-FS1 spores (Figure 10A) and mycelia (Figure 10B). In the control and DMSO-treated groups, cells appeared healthy with

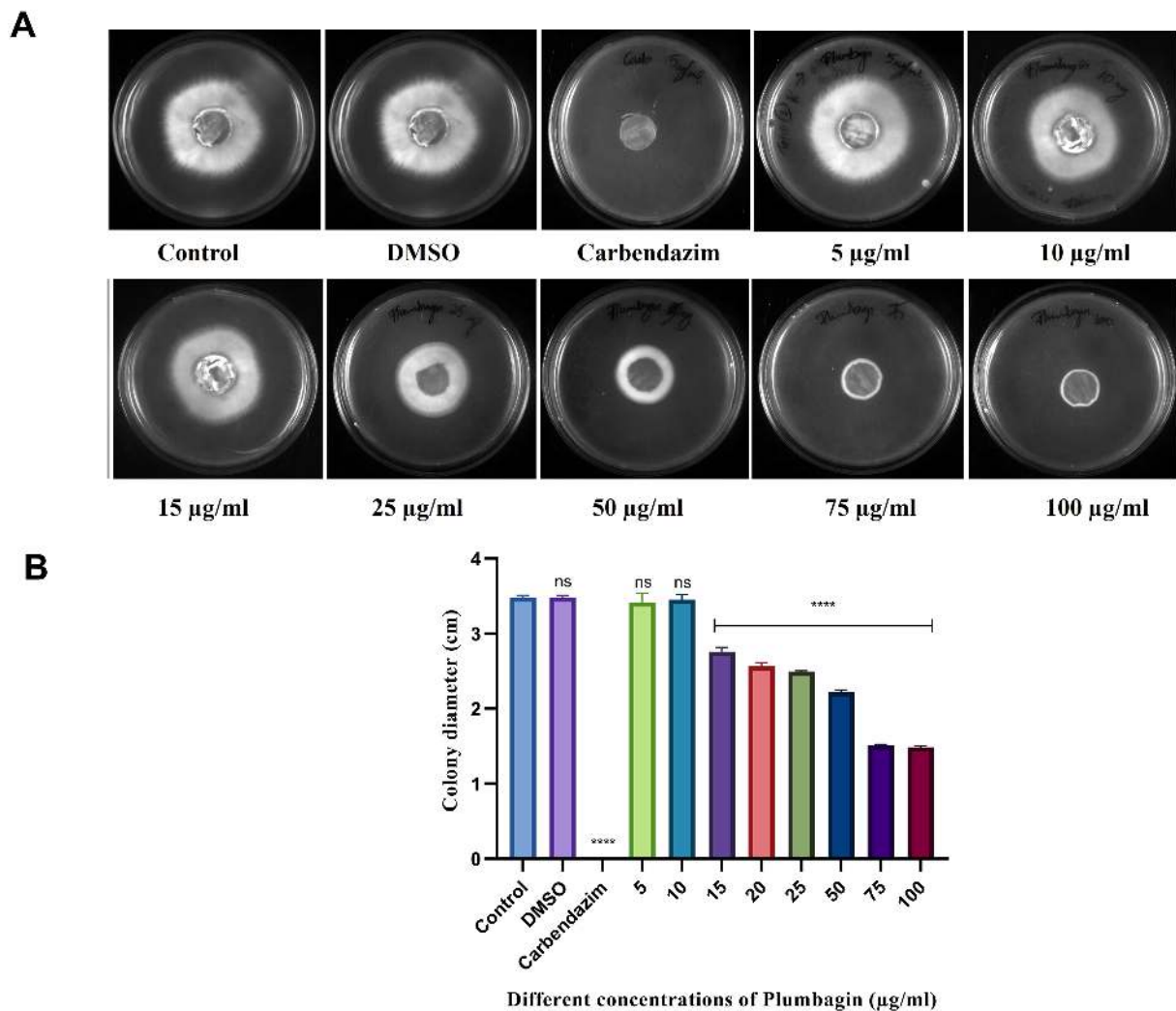


FIGURE 5

Radial growth assay demonstrating the inhibitory effect of Plumbagin on GI-FS1 mycelial growth. (A) Plumbagin showed a concentration-dependent reduction in radial growth, with marked inhibition from 25 µg/mL and almost complete suppression at 75–100 µg/mL. Carbendazim (positive control) effectively restricted mycelial expansion, while untreated control and DMSO (solvent control) had no inhibitory effect. (B) The bar graph depicts colony diameter (cm) corresponding to each treatment. Data are expressed as mean $\pm$ SD from three independent experiments consisting of three biological replicates ($P < 0.05$, ANOVA, Dunnett's multiple comparison test). '****' denotes highly significant differences ($P < 0.0001$); 'ns' indicates non-significant differences ($P > 0.05$) based on ANOVA followed by Dunnett's multiple comparison test.

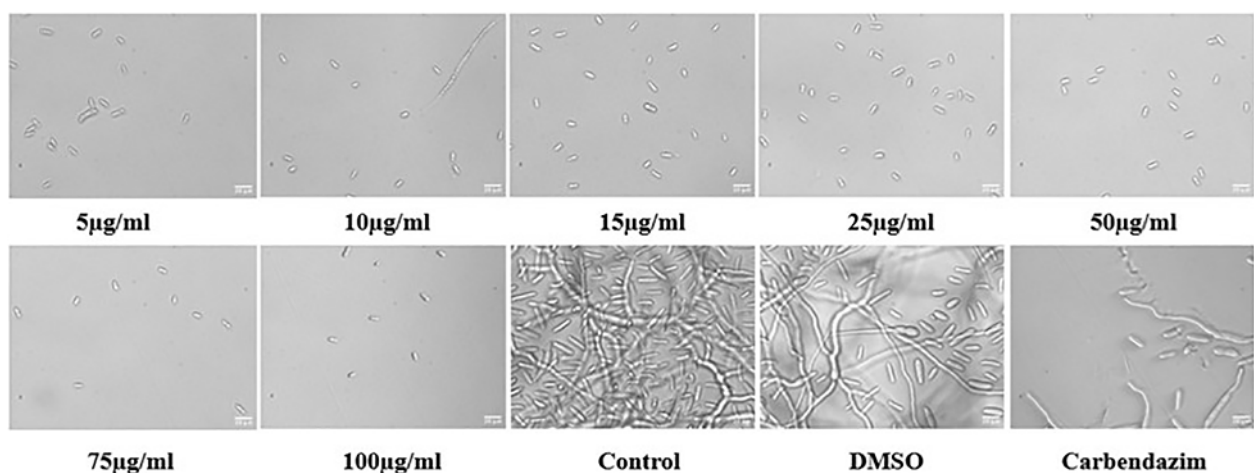


FIGURE 6

Inhibition of conidial germination of GI-FS1 by plumbagin in liquid media. Plumbagin significantly inhibited conidial germination at concentrations ≥ 15 µg/mL. Carbendazim, used as a positive control, caused distorted and lysed conidia, while DMSO and untreated controls exhibited normal germ tube formation and hyphal elongation. Images were captured after 24 hours of incubation at 28 °C.

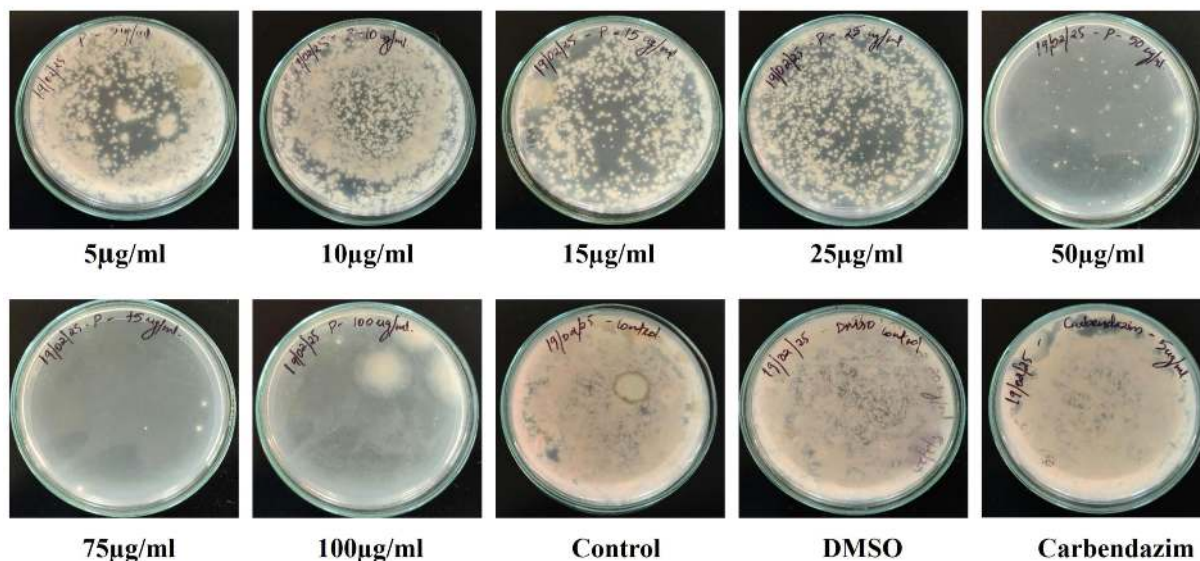
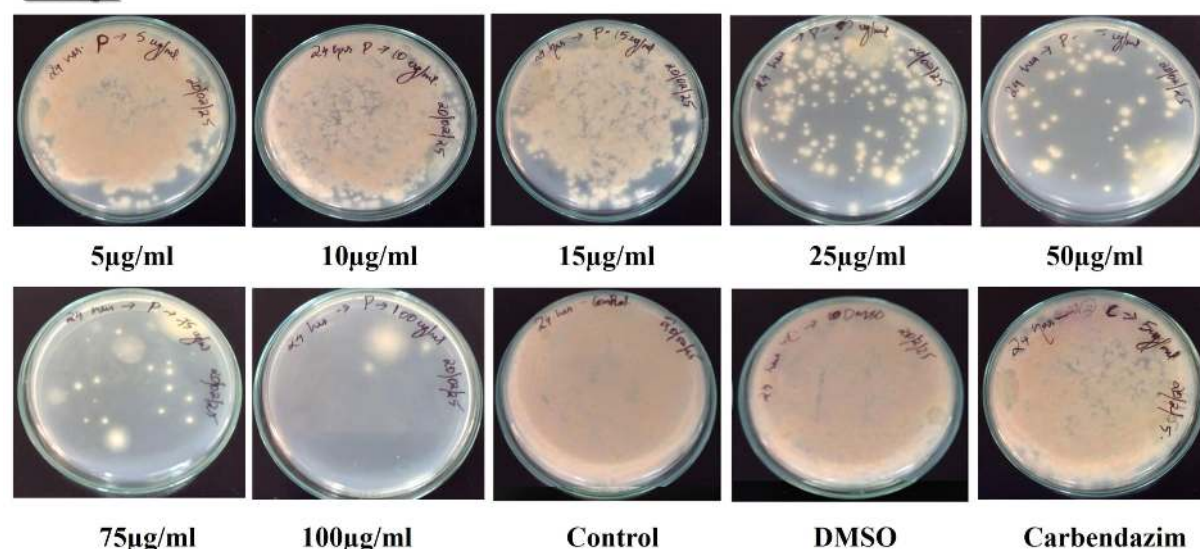
2 hpi**24 hpi**

FIGURE 7

Effect of plumbagin on GI-FS1 mycelial growth at 2 h and 24 h post-inoculation (hpi). Fungal cultures treated with plumbagin (5–100 µg/mL) and controls. Plumbagin caused dose-dependent inhibition, noticeable from 25 µg/mL and nearly complete at higher concentrations by 24 hpi. Carbendazim showed moderate inhibition, while DMSO and untreated controls displayed normal growth.

normal morphology and strong green fluorescence, indicating intact membranes and high viability. Carbendazim caused only moderate changes compared to the control. With plumbagin treatment, the effects became more evident as the concentration increased. At lower concentrations (5 and 10 µg/mL), most spores and hyphae still showed green fluorescence, suggesting that cell viability was largely maintained. However, at higher concentrations (15 and 20 µg/mL), a clear shift from green to red fluorescence was observed, along with visible structural damage. This indicates loss of membrane integrity and increased cell death. Quantitative analysis further supported these findings (Figure 10C). While spore viability showed a gradual decline, the effect was much stronger in mycelia. At 15–20 µg/mL,

mycelial cells showed almost complete loss of viability, confirming that plumbagin exerts a strong, concentration-dependent antifungal effect against GI-FS1 (Figure 10D).

3.7 Assessment of electrolyte leakage

Electrolyte leakage was measured to evaluate cell membrane integrity in GI-FS1 following plumbagin treatment (Figure 11). Increased conductivity indicates compromised membranes and loss of cellular contents. Conductivity increased dose-dependently with plumbagin concentrations from 5 µg/mL to 100 µg/mL. At concentrations ≥ 15 µg/mL, electrolyte leakage surpassed that

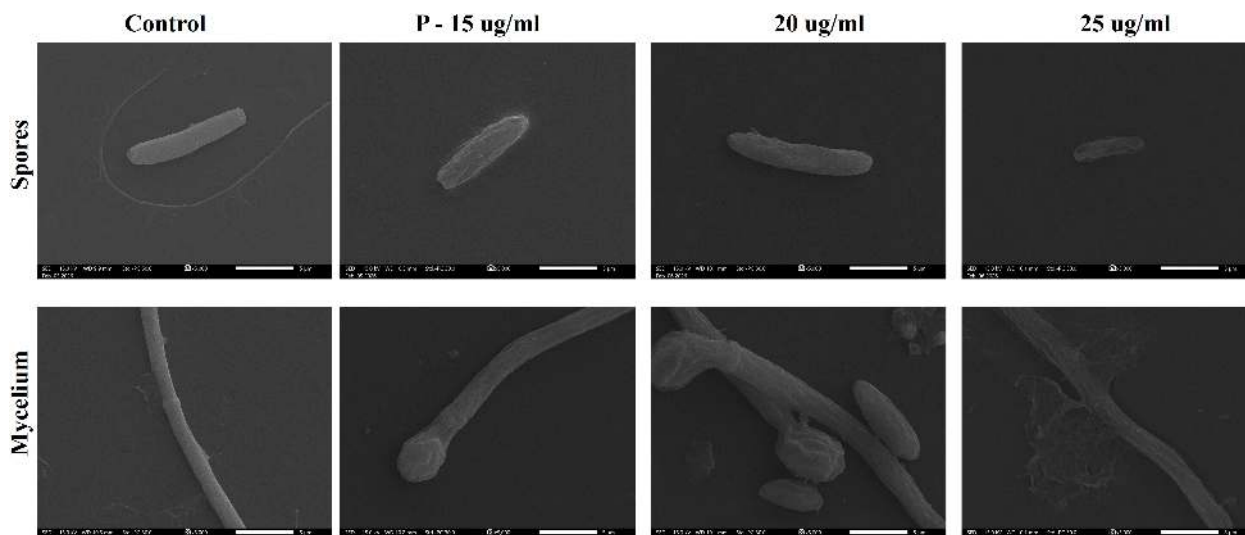


FIGURE 8

SEM images illustrating the concentration-dependent effect of plumbagin on the morphology of GI-FS1 spores and mycelia. Control spores and mycelia exhibit smooth surfaces and intact cellular architecture. In contrast, plumbagin-treated samples (15, 20, and 25 $\mu\text{g/mL}$) exhibit pronounced morphological alterations, including spore shrinkage, deformation, surface irregularities, and thinning and partial collapse of mycelia. White arrows indicate regions of pronounced morphological damage, such as surface roughening and structural distortion. These observations suggest loss of cellular integrity and support the antifungal activity of plumbagin against GI-FS1.

observed with carbendazim, reaching its highest value at 100 $\mu\text{g/mL}$. This indicates that plumbagin induces significant membrane damage, impairing fungal cell viability and function. Control and DMSO treatments showed minimal leakage, confirming that the observed effect is specific to plumbagin.

3.8 Reactive oxygen species detection

DCFH-DA staining demonstrated that plumbagin treatment leads to intracellular ROS accumulation in GI-FS1 in a concentration-dependent manner (Figure 12). As shown in (Figure 12A), control, DMSO, and carbendazim-treated samples exhibited minimal fluorescence, indicating low basal ROS levels. Similarly, treatment with 5 $\mu\text{g/mL}$ plumbagin resulted in only weak fluorescence. In contrast, higher concentrations (10–25 $\mu\text{g/mL}$) produced a marked increase in green fluorescence intensity, reflecting elevated ROS generation. Quantitative analysis

(Figure 12B) confirmed a significant, dose-dependent increase in ROS levels, with maximal induction observed at 25 $\mu\text{g/mL}$. These results suggest that Plumbagin's antifungal effect involves ROS-mediated oxidative stress leading to fungal cell damage.

3.9 DNA laddering assay

The DNA laddering assay was performed to evaluate genomic DNA integrity and to assess whether plumbagin induces apoptotic-like cell death in GI-FS1. Genomic DNA from untreated control cells showed intact, high-molecular-weight DNA with no signs of fragmentation (Figure 13, Lane L2). In contrast, DNA isolated from plumbagin-treated cells exhibited extensive smearing toward lower molecular weights, even though equal amounts of DNA were loaded (Figure 13, Lane L3). The absence of distinct oligonucleosomal ladder bands and the presence of diffuse smearing suggest non-specific DNA degradation rather than classical apoptotic DNA

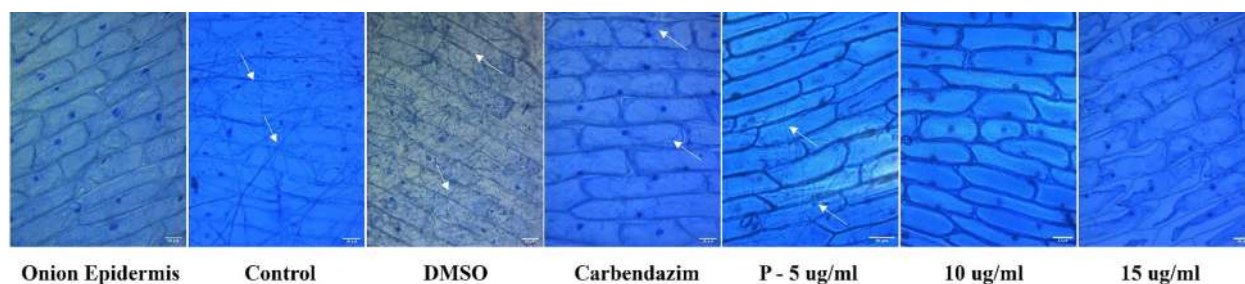


FIGURE 9

Light microscopy images (40X) showing GI-FS1 penetration in onion epidermis at 24 hpi. Scale bars = 20 μm . The experiment was repeated three times independently. Untreated control and DMSO-treated epidermis show extensive hyphal invasion (white arrows). Plumbagin (5 $\mu\text{g/mL}$) and carbendazim treatments showed reduced fungal penetration, whereas Plumbagin (10 and 15 $\mu\text{g/mL}$) showed no penetration, indicating strong antifungal activity.

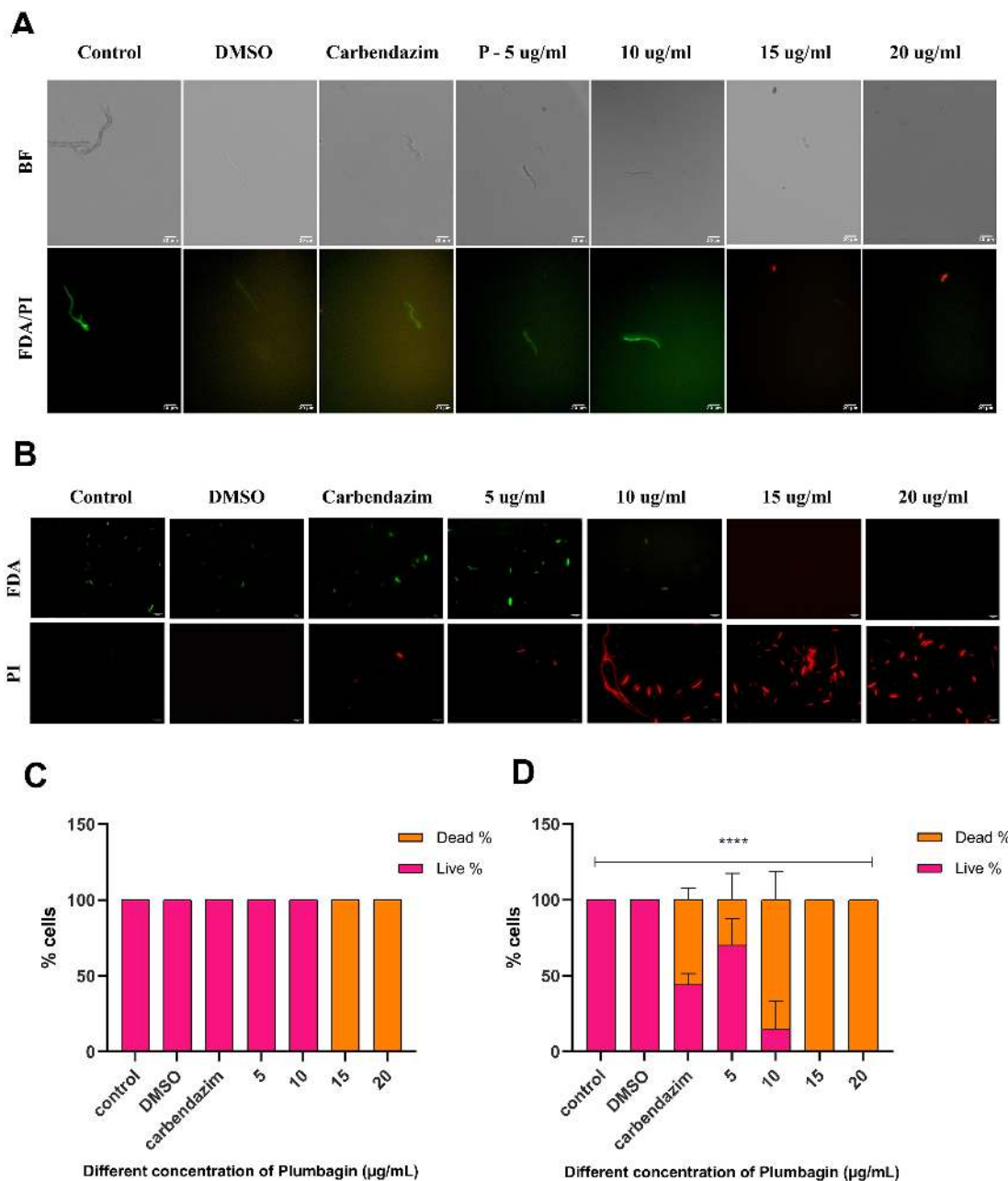


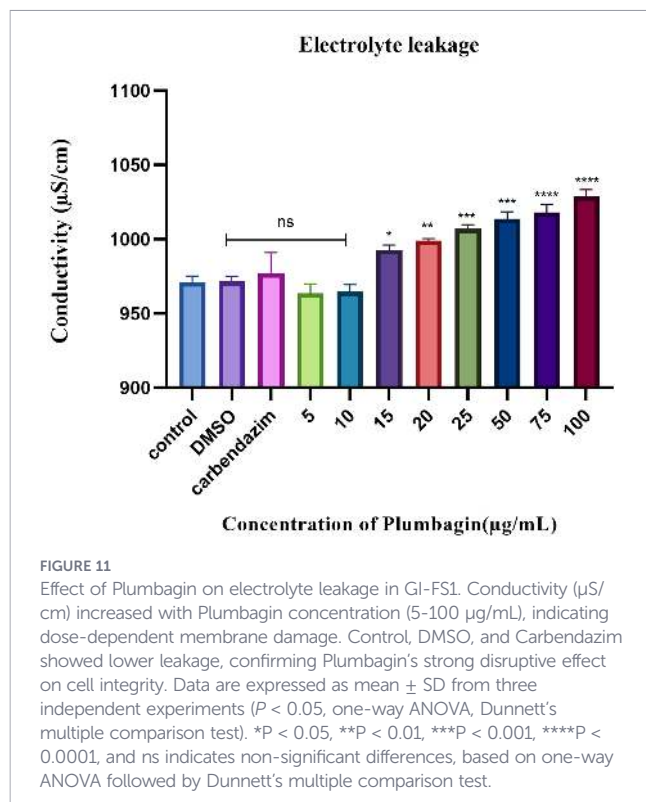
FIGURE 10

(A) FDA/PI staining of GI-FS1 spores after Plumbagin treatment (5–20 μg/mL). Scale bars = 20 μm. The experiment was repeated three times independently. Bright-field images show spore structure; fluorescence images indicate viability (green) and cell death (red). Higher Plumbagin concentrations (15–20 μg/mL) show increased red fluorescence, indicating loss of viability. Carbendazim shows partial effect, while DMSO and controls remain mostly viable. (B) FDA/PI staining of GI-FS1 mycelia after Plumbagin treatment (5–20 μg/mL). Green fluorescence (viable cells) decreases and red fluorescence (dead cells) increases with higher concentrations, indicating a dose-dependent loss of cell viability caused by Plumbagin. (C) Quantification of live and dead spore percentages based on fluorescence analysis. (D) Quantification of live and dead mycelial cells showing a significant, concentration-dependent increase in cell death following plumbagin treatment ($P < 0.05$, two-way ANOVA). **** denotes highly significant differences ($P < 0.0001$).

laddering. This pattern indicates that plumbagin-induced cell death may involve necrotic processes or late-stage apoptosis-like events rather than typical programmed DNA fragmentation. However, DNA fragmentation alone is insufficient to define the exact mode of cell death. Therefore, these results should be considered supportive evidence of plumbagin-induced genomic damage, and further molecular and biochemical analyses are necessary to clarify the underlying mechanism.

3.10 Detection of apoptosis

The Acridine Orange/Ethidium Bromide (AO/EB) dual staining assay was employed to detect apoptotic-like features in GI-FS1 following treatment with plumbagin (Figure 14A). AO/EB staining revealed that Plumbagin induces apoptotic-like cell death in GI-FS1. Control cells showed mostly green fluorescence, indicating viability. In contrast, treated cells displayed increased orange and red fluorescence,



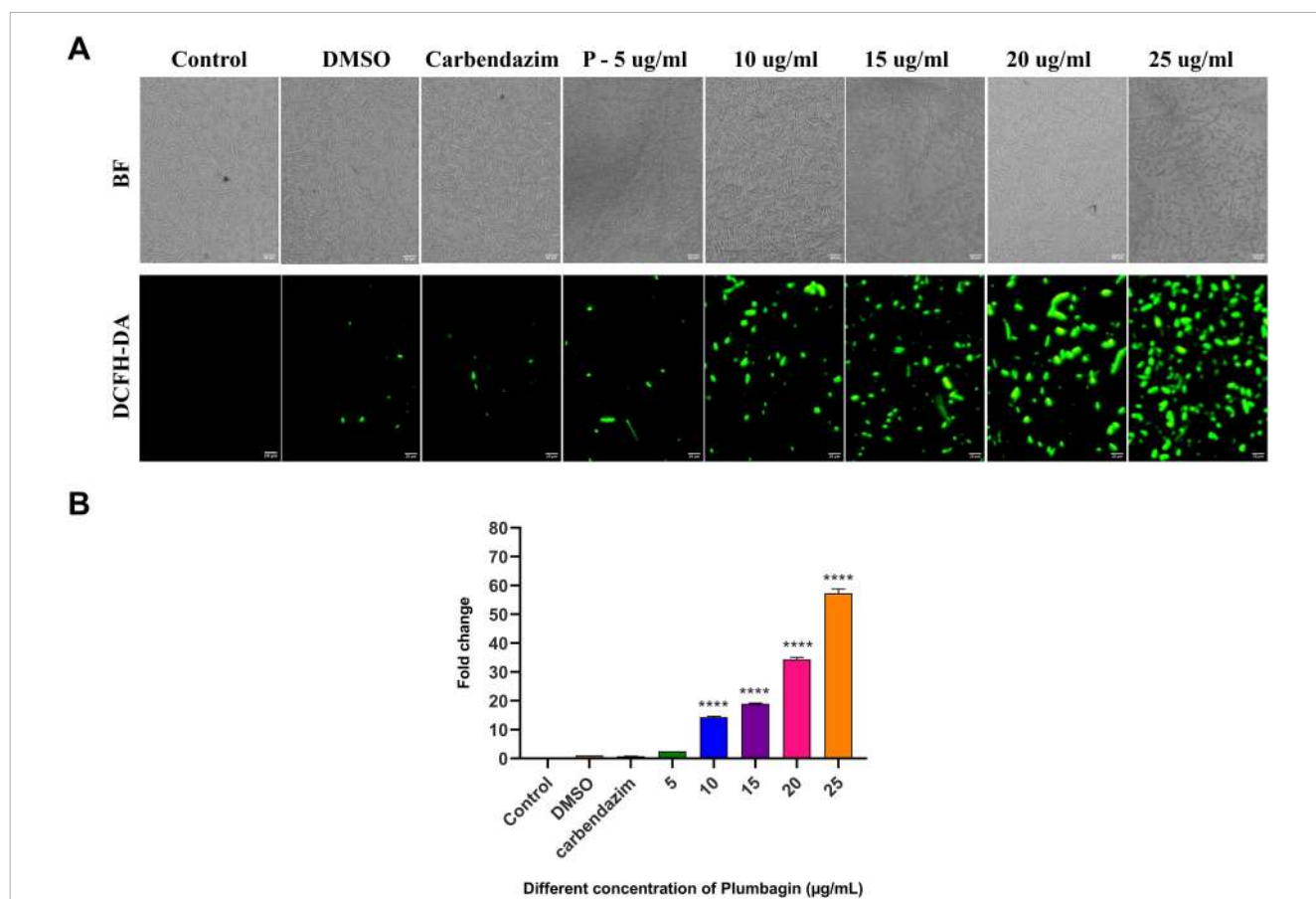
along with nuclear condensation and fragmentation—hallmarks of apoptosis. Plumbagin at a concentration of 15 µg/mL reduced the viability of cells by 30% compared to control (Figure 14B). These findings suggest that Plumbagin disrupts fungal viability by triggering programmed cell death mechanisms.

DAPI staining showed intact, well-defined nuclei in control cells, indicating healthy DNA and active growth (Figure 14C). In contrast, Plumbagin-treated cells displayed diffuse or condensed fluorescence with loss of clear nuclear structures, suggesting nuclear condensation or DNA fragmentation with an increase of 1.7 fold compared to control (Figure 14D). These changes are indicative of apoptosis- or necrosis-like cell death (Agus et al., 2018).

3.11 Molecular docking analysis

Molecular docking studies were performed to investigate the interactions between plumbagin and selected target enzymes from GI-FS1, namely Lanosterol 14 α -demethylase (CYP51), Cytochrome C, Topoisomerase I, and Topoisomerase II. The three-dimensional structures of all target proteins were generated by homology modelling using the SWISS-MODEL server, and docking simulations were carried out using AutoDock Vina.

Among the four target proteins, plumbagin exhibited the highest binding affinity towards CYP51 with a docking score of



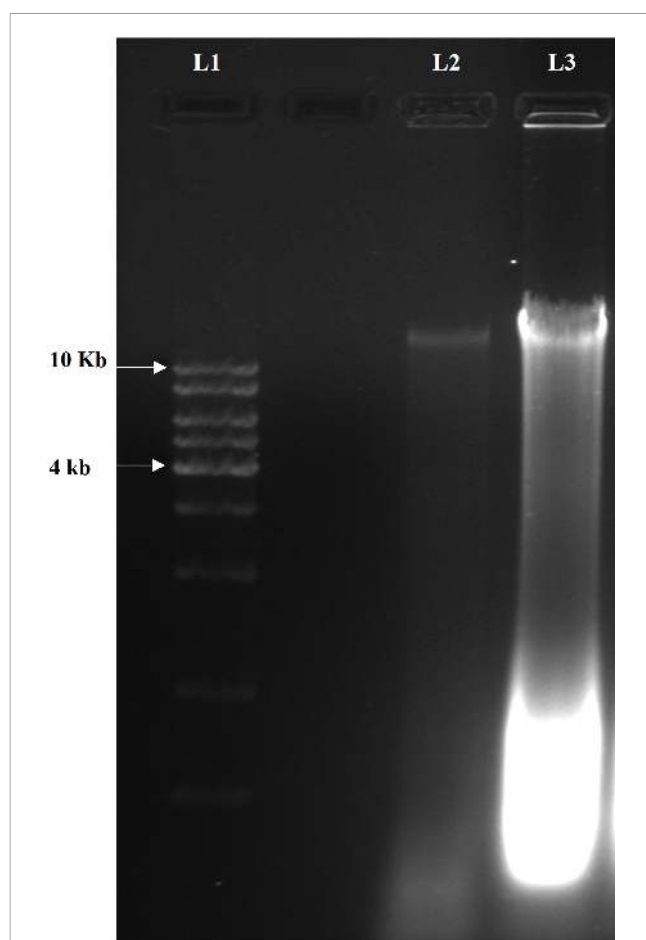


FIGURE 13
DNA laddering assay showing the effect of plumbagin on genomic DNA integrity in GI-FS1. Lane L1 contains the DNA molecular weight marker. Lane L2 shows intact high-molecular-weight genomic DNA isolated from untreated GI-FS1. Lane L3 shows genomic DNA isolated from plumbagin-treated GI-FS1, exhibiting pronounced DNA smearing indicative of treatment-induced loss of genomic integrity. Equal amounts of genomic DNA were loaded in all lanes.

-7.59 kcal/mol, indicating a strong interaction. The lowest binding affinity was observed with Cytochrome C, with a docking score of -4.7 kcal/mol. Docking conformations revealed that plumbagin binds stably within the predicted binding sites of each enzyme, as illustrated in the docking images. Interaction analysis showed that in the CYP51-plumbagin complex [Figure 15 (1)], the ligand formed interactions with key amino acid residues LYS (A:25) and GLU (A:21). In the Cytochrome C-plumbagin complex (Supplementary Figure 2), interacting residues included ALA (A:27), PHE (A:67), LYS (A:84), and LYS (A:68). For Topoisomerase I (Supplementary Figure 3), plumbagin interacted with the residue TRP (A:371), while in Topoisomerase II (Supplementary Figure 4), the interaction involved GLU (A:1186).

Overall, the docking results suggest that plumbagin exhibits variable binding affinities toward the selected GI-FS1 enzymes, with CYP51 showing the most favorable interaction. These findings indicate the potential of plumbagin to interact with key fungal enzymes and support its possible antifungal mechanism of action.

3.12 Leaf disc assay

The phytotoxic effect of plumbagin on ginger leaf tissues was evaluated by estimating total chlorophyll content following treatment with different concentrations of plumbagin (5–25 $\mu\text{g/mL}$). Total chlorophyll content serves as an indicator of photosynthetic integrity and leaf tissue health. The results demonstrated that control and solvent-treated (DMSO) leaf discs maintained comparable chlorophyll levels, indicating that the solvent did not exert any phytotoxic effect. Leaf discs treated with plumbagin at concentrations ranging from 5 to 20 $\mu\text{g/mL}$ showed only a marginal decline in total chlorophyll content compared with the control group (Figure 16). Statistical analysis revealed that these differences were not significant (ns), suggesting that plumbagin does not cause substantial damage to photosynthetic pigments within this concentration range. At the highest concentration tested (25 $\mu\text{g/mL}$), a slight reduction in chlorophyll content was observed; however, the reduction remained within a narrow range and did not indicate severe pigment degradation or tissue necrosis. The overall preservation of chlorophyll levels across treatments suggests that plumbagin exhibits minimal phytotoxicity toward host plant tissues at concentrations effective for antifungal activity. These findings indicate that plumbagin can potentially be utilized as a plant-protective antifungal compound with low risk of damaging host plant physiology, particularly photosynthetic function.

4 Discussion

Soft rot caused by *Fusarium* species remains a major constraint to ginger production worldwide, underscoring the need for alternatives to conventional fungicides that are both effective and environmentally compatible (Styawan et al., 2022; Yadav et al., 2023; Fanai et al., 2025). Within this context, the present study positions plumbagin, a naturally occurring naphthoquinone derived from *Plumbago rosea*, as the focal bioactive compound and demonstrates its strong antifungal activity against *Fusarium* sp. isolated from ginger. While accurate pathogen identification using cultural, morphological, and molecular approaches ensured experimental rigor (Zhang et al., 2010; Lee et al., 2012; Ivors, 2015; Raja et al., 2017), the central contribution of this work lies in elucidating how the chemical properties of plumbagin translate into a multi-target antifungal response.

Plumbagin markedly inhibited mycelial growth, spore germination, and overall fungal proliferation in a dose-dependent manner, with low MIC and MFC values (10 $\mu\text{g/mL}$ and 15 $\mu\text{g/mL}$, respectively). These findings align with earlier reports on the antifungal potential of plant-derived naphthoquinones (Padhye et al., 2012; Petrocelli et al., 2023) and reinforce their relevance as eco-friendly alternatives to synthetic fungicides (Nicolopoulou-Stamati et al., 2016; Bhandari et al., 2021; Mir et al., 2022; Deresa and Diriba, 2023). Importantly, the pronounced suppression of conidial germination, an essential step for host colonisation and

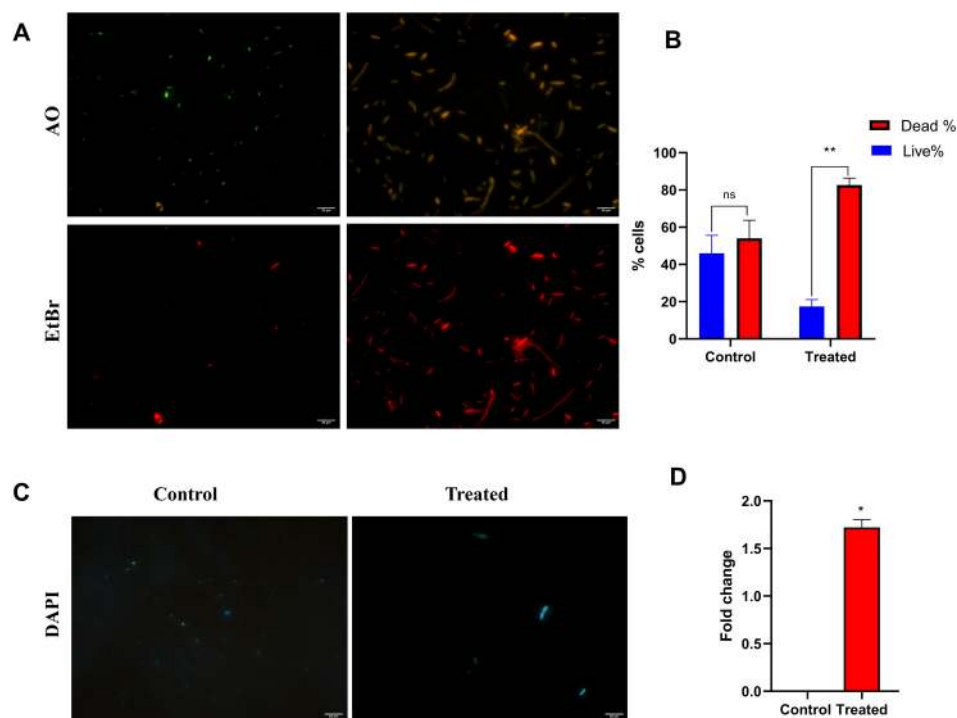


FIGURE 14

Plumbagin induces apoptosis-like changes in GI-FS1. (A) AO/EB staining shows predominantly green fluorescence (viable cells) in control, whereas treated cells exhibit increased orange-red fluorescence, indicating membrane damage and cell death. (B) Quantification of AO/EB staining reveals a significant increase in dead cells following plumbagin treatment. (C) DAPI staining shows uniform nuclear fluorescence in control cells, while treated cells display condensed and intensely stained nuclei, indicating chromatin condensation. (D) DAPI fluorescence intensity analysis shows a significant increase in treated samples compared to control ($P < 0.05$, Paired t-test). The symbols represent statistical significance levels: * $P < 0.05$, ** $P < 0.01$, and ns indicates non-significant differences ($P > 0.05$), based on a paired t-test.

disease establishment (Slawecki et al., 2002; Paola Díaz Dellavalle et al., 2010; Rongai et al., 2012), highlights plumbagin's capacity to interfere with early infection processes, a key requirement for effective biocontrol agents.

From a mechanistic perspective, the antifungal effects observed in this study can be integratively interpreted through the redox-active naphthoquinone scaffold of plumbagin. Naphthoquinones are well known to undergo redox cycling, leading to excessive intracellular ROS generation under aerobic conditions. Consistent with this chemical behavior, plumbagin treatment resulted in elevated ROS accumulation in GI-FS1, as detected by DCFH-DA staining. Such oxidative stress can disrupt redox homeostasis,

damage cellular macromolecules, and initiate downstream cell death pathways, as previously reported for redox-active antifungal compounds (Agus et al., 2018; Han et al., 2020).

Oxidative stress induced by plumbagin was accompanied by pronounced membrane damage, which is evidenced by increased electrolyte leakage and severe ultrastructural alterations observed through SEM analysis (Hu et al., 2024). These effects are mechanistically coherent with ROS-mediated lipid peroxidation and with the known ability of naphthoquinones to compromise membrane integrity by interfering with electron transport and sterol-associated processes. Cytological staining assays and DNA integrity analyses further revealed nuclear condensation, membrane

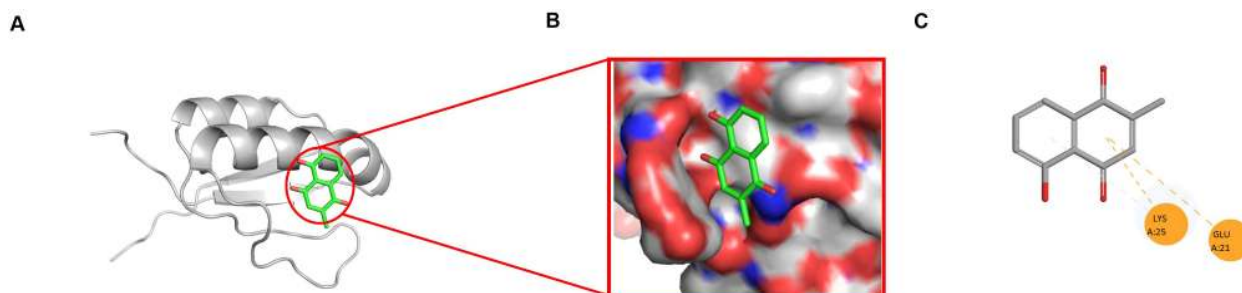
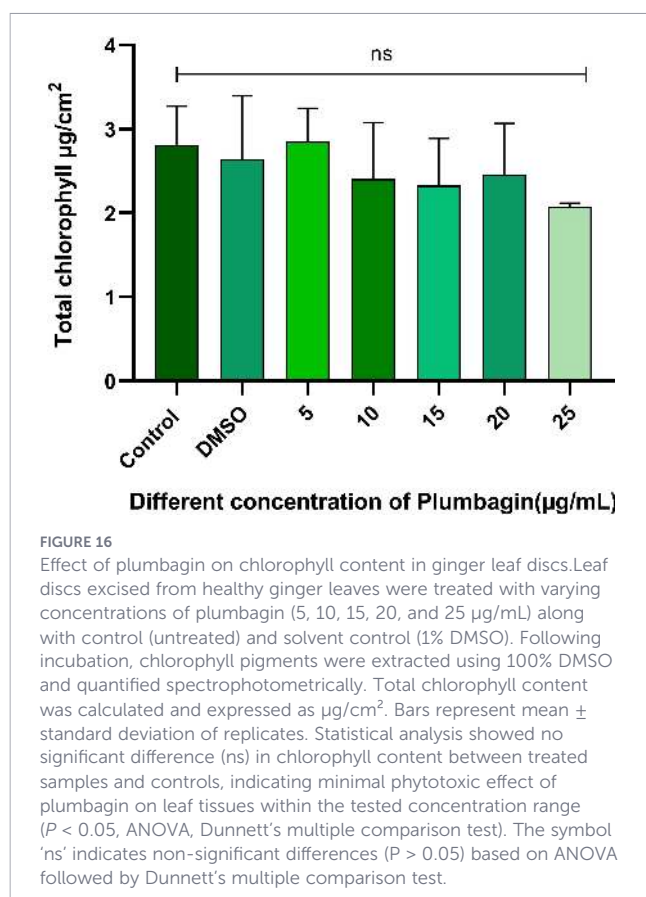


FIGURE 15

Molecular docking interaction of plumbagin with Lanosterol 14 α -demethylase (CYP51) of *Fusarium*. (A) Ribbon representation of the homology-modeled CYP51 protein showing plumbagin bound within the predicted active-site cavity (ligand shown in green). (B) Surface representation of CYP51 highlighting the ligand-binding pocket, illustrating stable accommodation of plumbagin within the active site. (C) Two-dimensional interaction map depicting the interaction of plumbagin with amino acid residues LYS (A:25) and GLU (A:21).



permeability changes, and extensive genomic DNA degradation. Rather than representing isolated phenomena, these observations collectively support a cascade model, wherein plumbagin-induced redox imbalance acts as a primary trigger, leading to membrane dysfunction, loss of cellular integrity, and ultimately apoptosis-like or necrosis-like fungal cell death (Agus et al., 2018; Hu et al., 2024). Such a chemically driven, multi-target mode of action is advantageous in antifungal applications, as it reduces the likelihood of resistance development compared with single-site fungicides such as carbendazim (Waterhouse et al., 2018; Koka et al., 2021).

Molecular docking analysis was employed to complement experimental findings and to generate hypotheses regarding potential intracellular targets of plumbagin. The strongest predicted interaction was observed with Lanosterol 14 α -demethylase (CYP51), a key enzyme in ergosterol biosynthesis, suggesting a possible contribution to membrane destabilization. The binding analysis of plumbagin with CYP51 (lanosterol 14 α -demethylase) revealed how plumbagin interacts with the enzyme's active site and demonstrated the stability of plumbagin within the binding pocket. The results indicate that plumbagin will be stabilized in the binding pocket by both specific electrostatic forces and the presence of hydrophobic aromatic types of interactions. One important characteristic of this binding is a pi-cation interaction between the aromatic naphthoquinone ring and Lys25, which likely helps orient the ligand within the substrate access channel and enhances binding specificity (Gallivan and Dougherty, 1999). Additionally, the overall

stability of the Plumbagin-CYP51 complex is enhanced through an additional pi-anion interaction with Glu21. Pi-anion interactions have not been given quite the same amount of attention in the literature compared to pi-cation interactions, but recent studies illustrate the importance of these interactions as they relate to molecular recognition and stabilization of a ligand (Simó Padial et al., 2017). The engagement of the electron-rich pi-system of the naphthoquinone system in plumbagin with the carboxylate group of Glu21 along with the Lys25 pi-cation interaction suggests that plumbagin occupies a decidedly different electrostatic environment. The combination of the two types of interaction is likely anchoring Plumbagin in a stable fashion to CYP51. Further validation through molecular dynamics simulations, binding free energy analysis, and enzymatic inhibition assays is required to confirm the stability and inhibitory potential of plumbagin under physiological conditions. Predicted interactions with Cytochrome C and DNA-associated enzymes may indicate secondary effects related to mitochondrial and DNA stress. However, these docking results remain preliminary and should be considered supportive of the biological findings rather than definitive evidence of target identification (Doyle et al., 2010; Waterhouse et al., 2018; Mir et al., 2022).

From an agronomic and biotechnological standpoint, the antifungal efficacy of plumbagin, combined with its natural origin, biodegradability, and reduced environmental persistence, aligns with global efforts to develop sustainable disease-management strategies (Parveen and Sharma, 2014; Suteu et al., 2020; Mir et al., 2022; Deresa and Diriba, 2023). The ability of plumbagin to disrupt fungal physiology at multiple levels reflects broader paradigms in plant-pathogen interactions, where specialized plant metabolites function as chemically sophisticated defense agents rather than single-target inhibitors (Arie, 2019; Suteu et al., 2020; Garagounis et al., 2021). Preliminary phytotoxicity evaluation using ginger leaf disc assays showed negligible chlorophyll loss at antifungal concentrations, suggesting plant compatibility. However, comprehensive phytotoxicity, formulation stability, and environmental persistence studies are required to validate plumbagin for field application.

The interpretation of plumbagin's antifungal mechanism is primarily based on *in vitro* experiments and morphological or cytological observations, and direct functional validation of specific molecular targets is lacking. Although ROS accumulation, membrane damage, and apoptosis-like cell death were consistently observed, the direct causal relationship between these effects and the intrinsic redox activity of plumbagin has not been experimentally confirmed. Furthermore, while molecular docking analyses suggest potential interactions with key fungal proteins, these predictions remain hypothesis-generating in the absence of biochemical or genetic validation. Addressing these limitations through targeted molecular, genetic, and omics-based approaches will be essential to refine and confirm the proposed mechanism of action (Windram et al., 2012; Ngo et al., 2019; Han et al., 2020).

This study highlights plumbagin as a chemically driven, multi-target antifungal compound active against *Fusarium* sp. GI-FS1. Through its redox-active naphthoquinone structure, plumbagin induces oxidative stress, disrupts membrane integrity, compromises nuclear stability, and interferes with essential fungal processes, resulting in effective growth inhibition and cell death. Collectively,

these findings support a mechanistic cascade in *Fusarium* sp., whereby plumbagin-induced ROS generation triggers membrane disruption, nuclear damage, and apoptosis-like cell death. By integrating experimental observations with cautious computational insights, this work advances the understanding of plumbagin's antifungal mode of action and supports its further development as a sustainable biocontrol candidate within integrated disease-management frameworks. Phytotoxicity assessment indicated minimal plant tissue damage at concentrations effective against the pathogen, suggesting potential host compatibility. Further studies focusing on formulation development, compound stability under field conditions, and delivery strategies will be important to facilitate the translation of laboratory findings into practical agricultural applications. These aspects will help optimize the efficacy, safety, and field-level performance of plumbagin-based disease management strategies.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repository and accession number(s) can be found in the article/[Supplementary Material](#).

Author contributions

SuS: Software, Investigation, Writing – review & editing, Formal analysis, Visualization, Writing – original draft, Data curation, Validation, Supervision, Methodology, Project administration, Conceptualization. BT: Visualization, Formal analysis, Validation, Methodology, Data curation, Writing – review & editing, Investigation, Writing – original draft. AS: Formal analysis, Writing – original draft, Visualization, Methodology, Software, Writing – review & editing, Data curation, Investigation, Validation. KY: Writing – original draft, Visualization, Data curation, Methodology, Validation. AsN: Visualization, Validation, Writing – original draft, Methodology, Data curation. NT: Methodology, Validation, Data curation, Visualization, Writing – original draft. SrS: Methodology, Writing – original draft, Data curation, Visualization, Validation. AR: Writing – original draft, Data curation, Methodology, Visualization, Validation. MA: Visualization, Writing – original draft, Validation, Methodology, Data curation. AkN: Methodology, Writing – original draft, Visualization, Data curation, Validation. PB: Writing – original draft, Methodology, Formal analysis, Data curation. AM: Data curation, Formal analysis, Writing – original draft. SP: Resources, Writing – original draft. BN: Project administration, Resources, Writing – original draft. CB: Methodology, Writing – review & editing, Project administration, Formal analysis, Validation, Data curation, Supervision, Conceptualization, Investigation, Visualization, Writing – original draft.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fagro.2026.1770272/full#supplementary-material>

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