Exploitation of the antifungal and antibiofilm activities of plumbagin against *Cryptococcus neoformans*

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**ABSTRACT**

*Cryptococcus neoformans* is an important opportunistic fungal pathogen that causes various infections. Here, the antifungal and antibiofilm activities of plumbagin against *C. neoformans* and the underlying mechanisms were evaluated. The minimum inhibitory concentration (MIC) of plumbagin against *C. neoformans* H99 was 8 \(\mu\)g ml\(^{-1}\). Plumbagin disrupted the cell membrane integrity and reduced the metabolic activities of *C. neoformans* H99. *C. neoformans* H99 biofilm cells were damaged by plumbagin at a concentration of 64 \(\mu\)g ml\(^{-1}\), whereas 48-h mature biofilms were dispersed at a plumbagin concentration of 128 \(\mu\)g ml\(^{-1}\). Whole-transcriptome analysis of plumbagin-treated *C. neoformans* H99 in the biofilm and planktonic states identified differentially expressed genes enriched in several important cellular processes (cell membrane, ribosome biogenesis, fatty acid synthesis, melanin and capsule production). Notably, plumbagin damaged biofilm cells by downregulating FAS1 and FAS2 expression. Thus, plumbagin can be exploited as an antifungal agent to combat *C. neoformans*-related infections.

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**Introduction**

*Cryptococcus neoformans* is a ubiquitous encapsulated fungus, and a major opportunistic human fungal pathogen. However, it can trigger serious infections preferentially in immune-compromised individuals and occasionally in those with weakened immunocompromised systems, being responsible for an estimated 220,000 cases of *C. neoformans* infections every year globally (Rajasingham et al. 2017). It can cause life-threatening meningitis and fungemia (Lin et al. 2015). Most human *C. neoformans* infections result from the inhalation of aerosolized cryptococcal yeast or basidiospores that are ubiquitous in various natural environments (Toberna et al. 2020). Such ubiquity of *C. neoformans* in the environment can be partly attributed to its ability to adapt to varying environmental conditions and to evade recognition and clearance by the immune system of humans. Recent studies have postulated that the capacity of *Cryptococcus* species to survive, proliferate, and thrive in diverse hostile niches exacerbates the virulence of the yeast in humans, leading to recurrent and difficult-to-treat infections (Brandao et al. 2015).

The biofilm formed by *C. neoformans* has been described as an important virulence trait that provides protection from microbial predators in natural habitats (Camacho and Casadevall 2018). The biofilm mode of growth affords greater resistance to antifungal agents and shields the pathogen from specific host immune system responses compared to their free-living counterparts (Kowalski et al. 2020). In particular, Ravi et al. (2009) reported that the biofilm formation by *C. neoformans* H99 on surfaces preferentially occurs under environmental conditions similar to those observed in its natural habitat outside of the human body. Moreover, previous studies have demonstrated that *C. neoformans* biofilms have been observed on artificial medical devices. These biofilms enable organisms to be less susceptible to conventional antifungal drugs, and to various innate antimicrobial molecules produced by the host’s immune system (Kernien et al. 2017). Importantly, as a result of biofilm fragmentation and detachment, these persistent populations within biofilms can serve as a potential reservoir for chronic and systemic infections in clinical settings. This highlights the importance of...
exploiting novel promising natural products and elucidating potential mechanisms of action against C. neoformans biofilms.

Recently, the development of high-throughput sequencing technology has made it possible to obtain a comprehensive interrogation of the whole transcriptome of pathogens related to their response to antimicrobials at an increasingly reasonable cost. For instance, comparative gene expression analysis of several fungal pathogens in response to antimicrobials has been reported using RNA-Seq (Cheng et al. 2020; Holland et al. 2014; Xiao et al. 2021). Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a plant-derived quinonoid constituent extracted mainly from the roots of Plumbago zeylanica (Joo et al. 2015). Plumbagin is of broad pharmaceutical interest because it is a potential therapeutic agent for the treatment of various cancers and chronic diseases (Rahman-Soad et al. 2021). Plumbagin exhibits various bioactivities, including anticancer, antioxidant, antimicrobial, anti-inflammatory, and antiparasitic activities (Dzoyem et al. 2007; Padhye et al. 2012; Sumsakul et al. 2014). Previous studies demonstrated that plumbagin exerts moderate antimicrobial activities against several pathogens including Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Candida albicans (Chen et al. 2020; de Paiva et al. 2003; Gupta et al. 2017). However, the antifungal action and the mechanism through which C. neoformans responds to plumbagin remain largely unexplored. The aim of this study was to investigate the antifungal and antibiofilm activities of plumbagin against C. neoformans H99 as well as its underlying mechanism of action, with the goal of contributing to the identification of potential targets for developing effective agents to combat C. neoformans biofilm-related infections.

Materials and methods

Reagents

Plumbagin (purity ≥ 98%) was obtained from Chengdu Pulis Biological Science and Technology Co., Ltd (Chengdu, China). Stock solutions of plumbagin were dissolved in 10% dimethyl sulfoxide (DMSO) and filter-sterilized. SYTO 9, propidium iodide (PI), FUN® 1 (FUN-1) and Calcofluor® White M2R stain (CWS) were obtained from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). All other chemicals and solvents were of analytical grade.

Strain and growth conditions

C. neoformans var. grubii, strain H99 (serotype A) was employed throughout this study. The yeast cells were cultured initially in 5 ml of yeast peptone dextrose (YPD) broth (1% yeast extract, 1% peptone and 2% glucose) at 30°C. After 24 h of incubation, the yeast cells were collected using centrifugation and washed twice with 10 mM phosphate-saline buffer (PBS, 137 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM disodium hydrogen phosphate and 1.47 mM potassium dihydrogen phosphate, pH 7.4). Then, the cells were resuspended in fresh YPD medium at a cell density of 1 × 10⁶ colony forming units (CFU) ml⁻¹ to acquire the standardized suspensions. To generate biofilms of C. neoformans, 1 ml of the standardized cell suspensions were transferred into individual wells of a 24-well microplate. Then, plumbagin was added into each well, at different concentrations when necessary, and incubated with the cells at 30°C.

Determination of minimum inhibitory concentration (MIC)

The MIC of plumbagin against C. neoformans was determined according to the Clinical and Laboratory Standards Institute (CLSI) M27-A2 standard method, with minor modification. The standardized suspensions were diluted at a ratio of 1:100 in YPD medium. The resulting suspensions (1 × 10⁹ CFU ml⁻¹) were added into each well of a 96-well microplate, and then plumbagin was added into each well, yielding final concentrations ranging from 1 μg ml⁻¹ to 32 μg ml⁻¹. After 24 h of incubation at 30°C, the MICs were defined as the concentration that results in 90% growth inhibition compared to the negative control. Antifungal agents (amphotericin B, fluconazole; Sigma-Aldrich, Saint Louis, MO, USA) and 10% DMSO were used as the positive and negative control, respectively. Amphotericin B was dissolved in 10% DMSO to a final concentration of 0.1 mg ml⁻¹ and sterilized using filtration through a membrane filter with a pore size of 0.22 μm. The MICs were determined in triplicate.

Time killing kinetic assay

The time killing kinetic assay of plumbagin against C. neoformans H99 was carried out as previously described (Appiah et al. 2017). Overnight-grown cultures of C. neoformans H99 were prepared and diluted to a density of 1 × 10⁶ CFU ml⁻¹ in YPD medium. A 200 μl sub-sample of diluted culture was
transferred to each well of a 96-well plate, and plumbagin was added to each well to obtain a final concentration of 1 MIC, and further incubated at 30°C. Samples were taken at 0, 2, 4, 6, 8, 10, 12, and 24 h, and inoculated aseptically into YPD agar and incubated at 30°C for 24 h. The CFU number was determined.

Membrane integrity of fungal cells treated with plumbagin

The cell membrane integrity of *C. neoformans* H99 was assessed using confocal laser scanning microscopy (CLSM; Zeiss LSM 880 with Airyscan; Jena, Germany) as previously described with modifications (Qian, Yang, Wang, et al. 2020). The standardized suspensions were exposed to various concentrations of plumbagin (0, 1/4, 1/2, and 1 MIC) for 4 h, after which they were centrifuged at 600 × g for 10 min and resuspended in 10 mM PBS (pH 7.0). Then, a small sub-sample of the cell suspensions was transferred into fresh tubes. To visualize the cells, a mixture of fluorescent dyes (5 μM SYTO 9 and 5 μM PI) was transferred into each tube and thoroughly mixed. Following by co-incubation at 25°C for 15 min, the resulting samples were examined using a CLSM, where the fluorescence value of each cell suspension was measured at excitation/emission wavelengths of 488/520 nm (for SYTO 9) and 535/617 nm (for PI).

Metabolic activity of fungal cells exposed to plumbagin

The effect of plumbagin treatment on the metabolic activity of *C. neoformans* H99 was assessed using confocal laser scanning microscopy (CLSM) as previously described (Qian, Yang, Li, et al. 2020). The cultures of *C. neoformans* H99 cells (approximately 1 × 10⁶ CFU ml⁻¹) were co-incubated with plumbagin at different concentrations (0, 1/4, 1/2, and 1 MIC) at 30°C for 24 h. Then, the samples were washed thrice with 10 mM PBS and treated with the fluorescent dyes FUN-1 (5 μM) and CWS (5 μM). Following co-incubation for 20 min at room temperature, the cells were examined under a CLSM, at excitation/emission wavelengths of 470/590 nm and 488/617 nm for FUN-1 and CWS, respectively.

Adhesion assay

For the adhesion assay, the standardized suspensions were added to individual wells of a 24-well plate, each of which contained a sterilized glass coverslip, and then treated with different plumbagin concentrations (0, 1/4, 1/2, and 1 MIC) without shaking at 30°C for 4 h. Next, the cell supernatants were discarded from each well, and the coverslips were gently washed with 10 mM PBS to remove the non-adherent cells. After gentle washing, a mixture of the FUN-1 (5 μM) and CWS (5 μM) dyes was added, and the adhesion level of fungal cells on the coverslips was examined using a CLSM.

Biofilm inhibition assay

The inhibitory effect of plumbagin on *C. neoformans* H99 biofilm formation was assessed using field emission scanning electron microscopy (FESEM; Nano SEM-450, FEI, Hillsboro, OR, USA) as previously described, with a few modifications (Qu et al. 2016). Briefly, *C. neoformans* H99 cells (1 × 10⁶ CFU ml⁻¹) were exposed to plumbagin at various concentrations (0, 1/4, 1/2 and 1 MIC) at 30°C for 24 h. For the FESEM assay, after incubation, the biofilms on the glass coverslips were fixed in 2.5% glutaraldehyde (v v⁻¹) at −4°C for 2 h, and then washed thrice with 10 mM PBS. Subsequently, the fixed samples were dehydrated by rinsing with ascending grades of ethanol: 30%, 50%, 70%, 90%, and 100% ethanol for 10 min at each concentration. Finally, the morphology of the biofilm samples was observed under a FESEM. For the optical microscopy assay, the biofilms on the glass coverslips were washed twice with 10 mM PBS to remove the planktonic cells and then stained with 0.1% (w v⁻¹) crystal violet (CV) for 20 min. After staining, the samples were washed thrice with 10 mM PBS to wash off the unstained dye. Finally, the biofilms were examined using an optical microscope at 400 × magnification. For quantitative analysis of the biofilm biomass, overnight cultures were first diluted to an inoculum dose of approximately 1 × 10⁶ CFU ml⁻¹, and then exposed to various concentrations of plumbagin (0, 1/4, 1/2 and 1 MIC) in a 96-well plate for 24 h at 30°C. After incubation, the culture medium was removed. The 96-well plate was then rinsed twice with 10 mM PBS and stained with 0.1% CV (w v⁻¹) for 20 min at room temperature. Each well was washed twice with PBS, and then 200 μl of acetic acid (33%, v v⁻¹) was added to dissolve the CV bound to the fungal cells. Finally, the OD₅₇₀ was measured using a microplate reader.

Biofilm dispersal assay

The biofilm-dispersal capability of plumbagin was evaluated quantitatively and qualitatively by
performing CV assays, optical microscopy, and FESEM analysis. The *C. neoformans* H99 cell suspensions (1 × 10⁶ CFU ml⁻¹) were dispensed into each well of a 24-well microtiter plate harboring a glass coverslip. After 48 h of incubation, the resulting biofilms were treated with various concentrations of plumbagin (0, 4, 8, and 16 MIC) for 6 h at 30°C. Subsequently, for qualitative analysis, the biofilm was observed using optical microscopy and FESEM. For quantitative analysis, the biofilm biomass was measured using the CV assay as described above.

**Assessment of cell damage within biofilms**

CLSM was employed to evaluate the damaging effect of plumbagin on biofilm cells according to a previously published method (Qian, Sun, et al. 2020). Briefly, *C. neoformans* H99 cells (approximately 1 × 10⁶ CFU ml⁻¹) were cultured for 48 h at 30°C on glass coverslips placed in each well of a 24-well plate, and the resulting biofilms were treated with various concentrations of plumbagin (0, 4, 8, or 16 MIC) for 6 h at 30°C. Then, the biofilm developed on the surface of each coverslip was washed twice with 10 mM PBS to remove planktonic cells and exposed to SYTO 9 and PI dyes. After incubation for 20 min, the stained viable and non-viable cells embedded in biofilms were observed using a CLSM.

**Capsule assay**

*C. neoformans* H99 cultures were established at an initial OD₆₀₀ of 0.5 (approximately 1 × 10⁶ CFU ml⁻¹), and treated with different concentrations of plumbagin (0, 1/2, 1, and 2 MIC). After incubation at 30°C for 24 h, samples were collected and washed thrice with 10 mM PBS to remove medium components. The precipitates were resuspended in liquid Dulbecco’s modified Eagle medium (DMEM; Thermo Fisher Scientific, Waltham, Massachusetts, USA) and cultured for 48 h at 30°C. Afterwards, an equal volume of culture and India ink (Phygene, Fuzhou, China) were mixed, and the capsule was visualized using optical microscopy at 1000 × magnification. To determine the capsule thickness, at least 50 cells were measured using ImageJ 1.39 software (Caroline et al. 2012). Capsule thickness was defined as the difference between the diameter of the total cell and that of the cell body.

**Melanin production assays**

Melanin production by *C. neoformans* H99 was analyzed according to previously reported methods with a few modifications (Oliveira et al. 2020). The melanin-inducing medium contained 15 mM glucose, 10 mM MgSO₄, 21.4 mM KH₂PO₄, 13 mM glycine, 3 μM thiamine, 1.5% (w v⁻¹) bacto agar and 1 mM Levodopa (L-DOPA) at pH 5.5. Plumbagin was added to the medium to obtain final concentrations of 0, 1/2, 1, and 2 MIC, mixed and poured on the plates. Then, 5 μl of *C. neoformans* H99 cells (approximately 1 × 10⁶ CFU ml⁻¹) was spotted on melanin-inducing medium. The agar plate was incubated at 30°C for 48 h, and melanin produced by fungal cells was assessed and photographed.

**RNA-sample preparation**

For the preparation of plumbagin-treated or untreated *C. neoformans* H99 biofilms, a starting inoculum of 1 × 10⁶ CFU ml⁻¹ was added into 90 mm round, glass Petri dishes containing 25 ml of YPD medium supplemented with or without 1/2 MIC plumbagin. The plates were incubated in an incubation chamber with shaking at 50 rpm at 30°C. Following 48 h of incubation, the plates were rinsed thrice with 10 mM PBS to remove unattached cells. Then, the biofilms formed on the bottom of the Petri dish were harvested by gentle scraping with a sterile scraper, suspended in 10 mM PBS and centrifuged at 10,000 rpm for 10 min. Finally, the cell pellets were washed twice with 10 mM PBS and frozen at −80°C.

The samples of plumbagin-treated and untreated *C. neoformans* H99 planktonic cells were prepared in flasks. Briefly, a suspension of 1.0 × 10⁶ CFU ml⁻¹ was added into the flask, and then incubated at 30°C for 22 h with shaking at 160 rpm. The resulting cultures were treated with or without plumbagin at MIC and further incubated for another 2 h with shaking at 160 rpm. After incubation, cells were collected using centrifugation at 1000 rpm for 5 min at 4°C and washed gently twice with 10 mM PBS. Subsequently, cells were frozen at −80°C and lyophilized for RNA sequencing (RNA-seq) analysis.

**RNA-seq**

cDNA library preparation and sequencing were conducted by Novogene Bioinformatics Technology Co., Ltd (Novogene, Tianjin, China). Total RNA samples for RNA-seq were extracted respectively from biofilm and planktonic cells in triplicate and prepared as
described above using RNAiso Plus (Takara, Dalian, China) according to the manufacturer’s instructions. The RNA concentration and quality were examined using a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) and Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany). cDNA libraries were constructed using the NEBNext RULtraTM RNA Library Prep Kit (NEB, Ipswich, MA, USA), according to the manufacturer’s recommendations, and quantified using the Agilent 2100 bioanalyzer. qRT-PCR analysis was performed to determine effective concentrations. Then, cDNA libraries were sequenced using a Novoseq sequencer (Illumina, San Diego, CA, USA) to obtain 150 bp paired-end reads.

**Analysis of differentially expressed genes (DEGs)**

Filtering of the raw reads of four samples was carried out to produce high-quality, clean reads prior to assembly. All reads obtained were mapped to the C. neoformans reference genome (Assembly PRJNA411) using HISAT2 v2.0.5 (Kim et al. 2015). DEG analysis between the treated and untreated cell samples was performed using the Bioconductor software package DESeq2 in R 1.16.1 (Love et al. 2014). Relative gene expression was calculated using Fragments Per Kilobase of transcript sequence per Millions of base pairs sequenced (FPKM) and the log2FoldChange values were compared. P-values were adjusted to generate false discovery rates (padj), and a padj of < 0.005 or 0.001 was considered the significance threshold for the DEGs (Benjamini et al. 2001; Morrison et al. 2003).

**Functional enrichment analysis of the DEGs**

The DEGs were annotated and investigated using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database. GO enrichment was performed to explore the functions of the identified DEGs using the Bioconductor software clusterProfiler 3.4.4 in R package (Yu et al. 2012). Statistical enrichment of the DEGs was also implemented in the KEGG database for pathway enrichment (Kanehisa et al. 2019).

**Statistical analysis**

Experiments were independently conducted in triplicate. For multiple-group comparisons, statistical analysis was performed using SPSS software (SPSS 8.0 for Windows) (Qian, Yang, Li, et al. 2020). Analysis of variance was performed to detect any significant differences. A p value of ≤ 0.01 was considered statistically significant.

**Results**

**Plumbagin inhibited the growth of C. neoformans H99**

The MIC results showed that the MIC of plumbagin against C. neoformans H99 cultures was 8 μg ml⁻¹ (Supplementary material, Figure S1). Moreover, the 50% minimum biofilm inhibitory concentration (MBIC₅₀) of plumbagin against C. neoformans H99 was 3.8 μg ml⁻¹. The time kill kinetics profile of plumbagin against C. neoformans H99 at the MIC concentration showed a weak effect from 0 to 2 h, but the growth rate then decreased compared with that of the untreated control (Supplementary material, Figure S2). These values confirmed that plumbagin displayed potent growth inhibition against C. neoformans H99.

**Plumbagin treatment disrupted the cell membrane integrity of C. neoformans H99**

In principle, viable cells loaded with SYTO 9 should produce green fluorescence, whereas fungal cells with compromised membranes should present with red fluorescence after staining with PI. Figure 1A demonstrates changes in the cell membrane integrity of C. neoformans H99 treated with plumbagin at the final concentrations of 0, 1/4, 1/2, and 1 MIC. The untreated cells displayed bright green fluorescence, suggesting that the physical integrity of their cell membrane was intact. With the increase in the of plumbagin concentration, the green fluorescence diminished, and red fluorescence was distinctly enhanced. Moreover, strong red fluorescence emitted by membrane-compromised cells was observed in cultures treated with plumbagin at the MIC. Overall, our findings indicate that treating C. neoformans H99 with plumbagin disrupted the cell membrane integrity in a dose-dependent manner.

**C. neoformans H99 cells exhibited low level metabolic activity after plumbagin treatment**

As shown in Figure 1B, almost no red-to-yellow-green fluorescence conversion was observed in the untreated cells of C. neoformans H99, and thus, a bright blue fluorescent signal was observed around cells. In the presence of plumbagin at 1/4 or 1/2 MIC, the area...
showing red and blue fluorescence clearly decreased in the *C. neoformans* H99 cultures, when compared to that of the control group. By contrast, following exposure to plumbagin at the MIC, weaker red fluorescence and blue fluorescence signals were observed when compared with those of the untreated control group. Thus, higher plumbagin concentrations increased the red to yellow-green fluorescence conversion and reduced the blue fluorescence, revealing that *C. neoformans* H99 cells displayed low-level metabolic activity when exposed to plumbagin and eventually died.

**Plumbagin reduced the adhesion of *C. neoformans* H99 cells**

The images obtained using CLSM after 4 h of co-incubation with plumbagin are presented in Figure 1C and D. Following exposure to a gradually increasing plumbagin concentration, the fungal cells exhibited a gradual decrease in the number of adherent cells when compared with that of the untreated control group. In addition, a decrease in the cell metabolic activity of fungal cells was observed in the plumbagin-treated group at 1/2 MIC or higher, in spite of the occurrence of cell-matrix adhesion. Notably, the MIC of plumbagin could effectively inhibit the attachment of *C. neoformans* H99 to the glass surface.

**Plumbagin inhibited *C. neoformans* H99 biofilm-formation**

As shown in Figure 2A and B, a couple of purple patches were observed in the optical microscopy images of untreated biofilms, indicating that in the untreated groups, *C. neoformans* cells formed dense biofilms on the glass coverslips. The cell densities slightly decreased in the *C. neoformans* H99 biofilm exposed to plumbagin at 1/4 MIC. In contrast, in the presence of plumbagin at the MIC, the structure of the biofilms was dramatically affected, and only a few scattered cell aggregates of *C. neoformans* H99 were observed in monolayers. In addition, the effect of plumbagin on biofilm formation was quantitatively...
evaluated using the CV assay. Figure 2C displays that plumbagin treatment inhibited C. neoformans H99 biofilm formation in a dose-dependent manner. For the biofilm biomass, significant differences were observed between the 1/2 or 1 MIC-treated and untreated biofilms (p < 0.01). Collectively, these results showed that plumbagin at 1/2 or 1 MIC could effectively inhibit C. neoformans H99 biofilms formation.

Plumbagin efficiently dispersed mature C. neoformans H99 biofilms

As displayed in Figure 3A and B, the mature biofilms formed in the absence of plumbagin were dense and contained holes of different sizes and channels. In contrast, a significant decrease in the biofilm biomass occurred in the group treated with plumbagin at 8 MIC compared with that of the untreated group, suggesting that the number of planktonic cells and the amount of the biofilm matrix released from the biofilm increased in a dose-dependent manner. Moreover, treating C. neoformans H99 biofilms with plumbagin at 16 MIC resulted in a striking, almost complete disappearance of the biofilm architecture.

The eradication activity of plumbagin on mature C. neoformans H99 biofilms was examined by performing CV assays (Figure 3C). Upon exposure to plumbagin at 8 MIC, the biofilms of C. neoformans H99 showed a significantly lower biomass (p < 0.05). Furthermore, significantly lower biofilm biomasses (p < 0.01) were observed following plumbagin exposure at 16 MIC. In addition, the CFU count results displayed a remarkable decline in C. neoformans H99 survival in a concentration-dependent manner when biofilms were exposed to plumbagin. Particularly, as shown in Figure 3C, all mature biofilms were almost eradicated under the presence of plumbagin at 16 MIC (p < 0.01).

Plumbagin damaged C. neoformans H99 cells within biofilms

As demonstrated in Figure 4, the cells inside mature C. neoformans H99 biofilms were evenly distributed and assembled into multiple layers. In the presence of plumbagin at 4 MIC, non-viable cells exhibiting low yellow or red fluorescence intensity were observed within biofilms, which shifted deeper into the image stack. Similar to plumbagin at 4 MIC, yellow or red fluorescence intensity due to non-viable cells markedly increased in the presence of plumbagin at 8 MIC. Furthermore, almost all cells within C. neoformans H99 biofilms were damaged when the plumbagin concentration was increased to 16 MIC, demonstrating that the number of non-viable cells within biofilms increased in a dose-dependent manner.

Plumbagin reduced the capsule size of C. neoformans H99

The C. neoformans capsular polysaccharide is mainly composed of glucuronoxylomannan (GXM) (Piccioni et al. 2013). As shown in Figure 5A and B, thick capsules were observed in untreated cells. In contrast, the capsule thickness of 1 MIC (p < 0.01) or 2 MIC-treated (p < 0.001) groups decreased compared to that of untreated group, and a marked loss of capsule was observed in the 2 MIC-treated group.

Plumbagin exposure caused changes to fungal melanin

Melanin is important for maintaining the cell wall and antioxidant activity of C. neoformans (Camacho...
et al. 2019; Wang and Casadevall 1994). To understand the effect of plumbagin in the melanin yield of \textit{C. neoformans} H99, the growth of \textit{C. neoformans} H99 on melanin-inducing medium was evaluated (Figure 5C). In the absence of plumbagin, \textit{C. neoformans} H99 produced abundant melanin. In contrast, in the presence of plumbagin at 2 MIC, no visible melanin around the colonies was observed.

Transcriptional response profiles of \textit{C. neoformans} H99 biofilm and planktonic cells exposed to plumbagin

To assess the effect of \textit{C. neoformans} H99 in response to plumbagin exposure, the whole transcriptome of \textit{C. neoformans} H99 biofilm and planktonic cells were evaluated using RNA sequencing. Illumina whole-transcriptome sequencing produced approximately $4.5 \times 10^7$ and $4.3 \times 10^7$ reads per sample of biofilm and planktonic cells, which resulted in 97% and 98% overall alignment rates, respectively. Then, RNA-seq analysis in \textit{C. neoformans} H99 biofilm cells exposed to plumbagin produced 2063 DEGs. Among these genes, 1006 DEGs were upregulated, whereas 1057 were downregulated (Figure 6A). Similarly, \textit{C. neoformans} H99 planktonic cells exposed to plumbagin produced 2665 DEGs, of which 1310 were upregulated and 1355 downregulated (Figure 6B).

Functional annotation and pathway analysis of the DEGs

Out of the total 2063 DEGs of the \textit{C. neoformans} H99 biofilm, 1006 with increased gene expression patterns were enriched in 80 KEGG pathways, of which nine KEGG pathways were significant with \textit{padj} < 0.05 (Table 1). Among these KEGG pathways, the most prominent pathways with upregulated expression in \textit{C. neoformans} H99 exposed to plumbagin treatment were enriched in amino acid degradation, peroxisome, fatty acid degradation, N-glycan biosynthesis, biosynthesis of secondary metabolites, phenylalanine, tyrosine and tryptophan biosynthesis, beta-alanine metabolism, nicotinate and nicotinamide metabolism. None of the most significantly altered genes showing the largest transcriptional response to plumbagin was enriched in the statistically significant pathways. In contrast, 1057 downregulated DEGs were mainly enriched in ribosome and ribosome biogenesis. Out of the total 2665 DEGs between the plumbagin-treated and untreated planktonic cells with KEGG pathway annotation, 1310 upregulated-DEGs were enriched in 84 KEGG pathways, of which eight were significant with \textit{padj} < 0.05 (Table 2). The pathways that were significantly upregulated in the planktonic state included peroxisome transporters, fatty acid degradation, fatty acid metabolism, valine, leucine and isoleucine degradation, basal transcription factors, carbon metabolism and beta-alanine metabolism. Moreover, 1355 downregulated-DEGs were enriched in 86 KEGG pathways, of which five were significantly (\textit{p} < 0.05) downregulated (ribosome, aminoacyl-tRNA biosynthesis, oxidative phosphorylation, biosynthesis of amino acids, amino sugars and nucleotide sugar metabolism). Among these, the ribosome pathway was the most significantly enriched KEGG pathway, indicating that ribosome formation is crucial for protein production, which necessitates cell growth and proliferation. The fungal ribosome content can be tightly coupled with the production of specific factors associated with energy production and protection from antimicrobials under stressful conditions. Therefore, the ribosome production of \textit{C.
neoformans H99 is viewed as a promising potential target for plumbagin.

**Exposure to plumbagin altered the cell membrane-related gene expression of C. neoformans H99 biofilm and planktonic cells**

Figure 7 displays that 18 ergosterol (ERG)-associated genes were differentially expressed in our RNA-seq data. Among them, seven DEGs exhibited obvious increases in expression upon plumbagin treatment in the biofilm state, whereas three DEGs were significantly upregulated and eight DEGs were significantly downregulated in the planktonic state (Figure 7). Interestingly, the expression of ERG11 was significantly upregulated in the biofilm state, whereas in the planktonic state, ERG11 was downregulated, indicating that the different microbial growth stages complicate the effects of plumbagin on the expression of the same gene. Moreover, a total of 27 inositol-related DEGs were observed in our data. Among these genes, six and eight DEGs were significantly upregulated, and five and eight DEGs were significantly downregulated in both the biofilm and planktonic states, respectively. Notably, myo-inositol 1-phosphate synthase (INO1), the key enzyme in the synthesis of internal inositol, was 0.55-fold and 3.38-fold downregulated in the biofilm and planktonic states, respectively. Thus, INO1 represents a potential antimicrobial drug target.

**Ribosome biogenesis-related genes were downregulated in plumbagin-treated C. neoformans H99 biofilm and planktonic cells**

As shown in Figure 7, 19 DEGs involved in ribosome biogenesis were observed in plumbagin-treated C. neoformans H99 cells in the biofilm and planktonic state. Ribosomes are composed of structural components encoded by ribosomal protein (RP) genes and are the sophisticated molecular machines for protein synthesis as directed by the genetic information encoded by mRNAs. In this study, 12 and seven DEGs in the plumbagin-treated C. neoformans H99 cells were downregulated in the biofilm and planktonic states, respectively. Moreover, the downregulated expression levels of RPL27 (ribosomal protein L27), RPL17, RPL22, RPL2, RPL9B, RPL30 and UBI1 genes in the C. neoformans H99 biofilm state were similar to those in the corresponding planktonic state. In contrast, the expression of RPL39, NOP1, MRPS18 and NOG2 were downregulated in the biofilm state, but were unaltered in the planktonic state.

**Addition of plumbagin downregulated the expression of genes involved in fatty acid synthesis in C. neoformans H99 biofilm and planktonic cells**

As displayed in Figure 7, the levels of FAS1 and FAS2 were significantly downregulated in the biofilm state, whereas they were unaltered in the planktonic state. FAS1 and FAS2 in C. neoformans H99, which encode the two subunits of the essential fatty acid synthase, are essential for in vitro and in vivo growth in conditions with and without exogenous fatty acids (Chayakulkeeree et al. 2007). Notably, the levels of the secreted phospholipase B (PLB1) gene that necessitates initiation of pulmonary infection and dissemination by C. neoformans H99 from the lung through the lymphatics and blood vessels (Ganendren et al. 2006), was downregulated in the planktonic state, while it was unaltered in the biofilm state.

**Plumbagin treatment regulated the expression of genes involved in the virulence of C. neoformans H99 biofilm and planktonic cells**

As demonstrated in Figure 7, in the biofilm state, five capsule-related genes, PDR802, MBS1, YAP1, CLR3 and URA5, were downregulated by 1.56-, 0.90-, 0.79-,...
0.50- and 0.43-fold, respectively. Compared to the biofilm state, 21 capsule-associated DEGs were observed in the planktonic state, of which 12 were downregulated, and nine were upregulated. Moreover, Figure 7 shows that eight melanin-related DEGs were significantly upregulated in the biofilm state. In contrast, 14 melanin-related DEGs were detected in the planktonic state, among which only three DEGs were downregulated, and 12 were upregulated. Interestingly, the levels of MP98 and MP88 transcription in the plumbagin-treated biofilms were upregulated, while they were downregulated in the planktonic state. Furthermore, the levels of SNF102 were significantly upregulated in both the biofilm and planktonic states.

Discussion

*C. neoformans*, commonly occurring within specific host niches, is a potential human health hazard. During infection, the fungi frequently transition to a complex community architecture known as a biofilm, enabling them to proliferate as communities of surface-adherent cell aggregates (Mukherjee et al. 2005). The versatility shown by biofilm cells proved their high tolerance to antifungals and ability to withstand host defenses (Nett et al. 2010). Combating *C. neoformans*-associated infections caused by biofilms using conventional antifungals is challenging, highlighting the necessity to identify novel antifungal agents. In this context, natural products with remarkable characteristics such as remarkable chemical diversity, and less human toxicity, have attracted much attention from researchers (Patra et al. 2018). For instance, curcumin has been evaluated for its antifungal potential with a maximum tolerance dose of 12,000 mg d⁻¹ in Phase I clinical trials (Lao et al. 2006). The data generated in the present study demonstrated the robust
antibiofilm activities of plumbagin against *C. neoformans* H99.

The MIC of plumbagin against the planktonic cells of *C. neoformans* H99 culture was 8 \( \mu \text{g ml}^{-1} \). Nair et al. (2016) found that plumbagin can act as an antimicrobial agent against both *C. albicans* ATCC2091 and *S. aureus* strain SA113 (ATCC35556), in which the MIC of plumbagin against both these pathogens was 5 \( \mu \text{g ml}^{-1} \). Subramaniya et al. (2011) reported that plumbagin is non-toxic to normal cells.

Table 1. Significantly enriched KEGG pathways for genes with increased and decreased expression of *C. neoformans* biofilm cells treated with 1/2MIC plumbagin.

| Regulation | KEGG ID | Description | padj |
|------------|---------|-------------|------|
| Up         | cnb00280| Valine, leucine and isoleucine degradation | 2.24 \( \times 10^{-5} \) |
|            | cnb04146| Peroxisome | 2.05 \( \times 10^{-4} \) |
|            | cnb00071| Fatty acid degradation | 1.03 \( \times 10^{-3} \) |
|            | cnb00513| Various types of N-glycan biosynthesis | 2.76 \( \times 10^{-3} \) |
|            | cnb00510| N-glycan biosynthesis | 3.56 \( \times 10^{-3} \) |
|            | cnb11110| Biosynthesis of secondary metabolites | 1.12 \( \times 10^{-2} \) |
|            | cnb00400| Phenylalanine, tyrosine and tryptophan biosynthesis | 2.14 \( \times 10^{-2} \) |
|            | cnb00410| Beta-alanine metabolism | 2.14 \( \times 10^{-2} \) |
|            | cnb00760| Nicotinate and nicotinamide metabolism | 4.07 \( \times 10^{-2} \) |
| Down       | cnb03010| Ribosome | 5.98 \( \times 10^{-3} \) |
|            | cnb03008| Ribosome biogenesis in eukaryotes | 5.40 \( \times 10^{-3} \) |

Table 2. Significantly enriched KEGG pathways for genes with increased and decreased expression of *C. neoformans* planktonic cells exposed to MIC plumbagin.

| Regulation | KEGG ID | Description | padj |
|------------|---------|-------------|------|
| Up         | cnb04146| Peroxisome | 6.75 \( \times 10^{-10} \) |
|            | cnb02010| ABC transporters | 2.44 \( \times 10^{-3} \) |
|            | cnb00071| Fatty acid degradation | 5.79 \( \times 10^{-3} \) |
|            | cnb01212| Fatty acid metabolism | 5.79 \( \times 10^{-3} \) |
|            | cnb00280| Valine, leucine and isoleucine degradation | 5.79 \( \times 10^{-3} \) |
|            | cnb03022| Basal transcription factors | 1.01 \( \times 10^{-2} \) |
|            | cnb01200| Carbon metabolism | 2.03 \( \times 10^{-2} \) |
|            | cnb00410| Beta-alanine metabolism | 5.24 \( \times 10^{-2} \) |
| Down       | cnb03010| Ribosome | 1.70 \( \times 10^{-18} \) |
|            | cnb00970| Aminocyl-tRNA biosynthesis | 1.25 \( \times 10^{-4} \) |
|            | cnb00190| Oxidative phosphorylation | 2.13 \( \times 10^{-3} \) |
|            | cnb01230| Biosynthesis of amino acids | 3.07 \( \times 10^{-3} \) |
|            | cnb00520| Amino sugar and nucleotide sugar metabolism | 4.45 \( \times 10^{-2} \) |
such as peripheral blood mononuclear cells with an IC50 > 100 μM (20 μg ml−1). Similarly, Sumsakul et al. (2014) showed that plumbagin showed no toxicity to mice when administered at concentrations up to 25 mg kg−1 daily for four days, and that it was effective in controlling malaria caused by *Plasmodium falciparum*. The cell membrane integrity of *C. neoformans* H99 exposed to plumbagin was examined using CLSM in combination with PI and SYTO 9 staining. CLSM images demonstrated that plumbagin at the MIC compromised the cell membrane integrity of *C. neoformans* H99, as evidenced by the bright red fluorescence, revealing higher levels of internal PI staining for membrane-compromised cells. Similarly, Zhao et al. (2019) recently showed that exposing *Magnaporthe oryzae* mycelia to sanguinarine at 10 μg ml−1 eventually disrupts the cell membrane integrity. Moreover, similar phenomena were also observed in *C. neoformans* H99 cells according to FUN-1 and CWS analysis, in which plumbagin treatment enhanced the total numbers of metabolically inactive or dead fungal cells in a concentration-dependent manner. Furthermore, the efficacy of plumbagin against *C. neoformans* H99 cells within the biofilm
was evaluated. The current results show that *C. neoformans* H99 biofilm cells were killed by plumbagin at 16 MIC, as were planktonic cells. Similarly, Alalwan et al. (2017) reported that an 80% decrease in the metabolic activity of *C. albicans* SC5314 biofilms cells was observed when cells were treated with 200 μg ml⁻¹ curcumin.

Next, the current study evaluated the inhibition of *C. neoformans* H99 biofilms by plumbagin. The results demonstrated that plumbagin significantly inhibited the biofilm biomass at concentrations of 4 μg ml⁻¹. Specifically, the biomass was reduced by 66.7% ± 3.19%. In comparison, plumbagin at 128 μg ml⁻¹ demonstrated 85%, 66%, and 52% inhibition of biofilm formation in *E. coli, S. aureus* and *K. pneumoniae*, respectively (Adusei et al. 2019). Together, these observations imply that the action of plumbagin was more effective against biofilm formation by *C. neoformans* H99. Mature biofilms usually show resistance to treatment and require a higher dose of antimicrobials for biofilm eradication. Zuo et al. (2016) showed that two halogenated quinoline analogs at a concentration range of 6.25–62.5 μM eradicated mature *C. neoformans* biofilms. Together, these findings indicate that plumbagin may not only serve as a promising antimicrobial agent for killing *C. neoformans* H99 planktonic and biofilm cells, but also effectively inhibit/disperse biofilm formation by *C. neoformans* H99.

The *C. neoformans* capsule is a complex structure that is required for virulence. The current study which investigated the effect of plumbagin on the capsule production of *C. neoformans* H99 demonstrated that the addition of plumbagin at 16 μg ml⁻¹ inhibited capsular formation. Kong et al. (2017) found that the effects of hypoxia on capsule formation by different strains vary. In addition, melanin synthesis by *C. neoformans* has protective effects and induces host damage as a virulence factor. In the present study, almost invisible melanin production around the colony was observed at 16 μg ml⁻¹ of plumbagin. Similar results were found in a recent study, in which coumaric acid analogs were used as an inhibitor of fungal growth with remarkable inhibitory activity on melanin biosynthesis in *C. neoformans* (Oliveira et al. 2020).

To further investigate the action mechanism of plumbagin against *C. neoformans* H99, RNA-Seq was applied to further explore the transcriptomic profile of *C. neoformans* H99 in the biofilm and planktonic state after treatment with plumbagin. The RNA-Seq data showed that the gene expression of *C. neoformans* H99 biofilm and planktonic cells were extensively altered by plumbagin treatment. Altered genes were related to the cell membrane, ribosome biogenesis, and fatty acid synthesis, and certain genes encoded virulence factors. Members of the ERG gene family are involved in the ERG biosynthesis, of which lanosterol 14-demethylase (ERG11) plays a critical role in the resistance of planktonic cells toazole of fungal cells. In the present study, the expression of ERG11 significantly decreased in the *C. neoformans* H99 planktonic state. Similarly, it has been demonstrated that sterol 14α demethylase encoded by ERG11 is the primary target for the azole class of antifungals (Strzelczyk et al. 2013). These results suggest plumbagin might affect the permeability of cell membranes by downregulating the expression of ERG11 in the *C. neoformans* H99 planktonic state, which was consistent with the results of the membrane integrity assay. In contrast, in the current study, the expression of ERG11 was increased in the *C. neoformans* H99 biofilm state. Similarly, Song et al. (2009) reported that the expression of ERG11 in *C. glabrata* was unaffected during biofilm growth. Inositol is an essential nutrient with important structural and signaling roles, which is involved in fungal infection pathogenesis (Xue 2015). To investigate the effect of plumbagin on inositol uptake and metabolism genes in *C. neoformans* H99, the expression of inositol-related genes was compared in plumbagin-treated *C. neoformans* H99 biofilm and planktonic cells. An earlier study in *C. neoformans* indicated that inositol is required for fungal infection, and that inositol is important for disease development as a signaling molecule and/or as an essential nutrient (Porollo et al. 2014). Notably, INO1, the key enzyme in the synthesis of internal inositol, was downregulated in both the biofilm and planktonic states, indicating that plumbagin can serve as a potential antifungal drug target by inhibiting the inositol biosynthesis of *C. neoformans* H99.

Ribosomes provide the basis for protein production and, thus, drive cell growth. The current data showed that 19 genes involved in ribosome biogenesis were significantly downregulated after plumbagin treatment, both in the biofilm and planktonic states, indicating that the maintenance of ribosome synthesis was affected due to the slowing of active metabolism and growth. Ribosome formation is crucial to cell growth and proliferation. Ribosomes are assembled logistically by more than 200 ribosomal proteins and RNA factors in a highly coordinated manner (Ameismeier et al. 2020). The fungal ribosome content can be tightly coupled to the production of
specific factors associated with energy production and protection from antimicrobials under stressful conditions (Tavares et al. 2019). In agreement with these results, a downregulation of ribosomal proteins was observed in a C. gattii (another species causing cryptococcosis) fungal biofilm in the presence of fluconazole (Chong et al. 2012). Therefore, fungal ribosome production is viewed as a promising potential target for antimicrobial agents.

Fatty acids are major and essential constituents of all eukaryotic cells. They not only provide structural integrity and energy for various metabolic processes but also function as signal transduction mediators (Lim et al. 2017). Interestingly, the present study supports the fact that treating C. neoformans H99 with sub-MIC of plumbagin downregulated the expression levels of both FAS1 and FAS2 in the biofilm state. A previous study has shown that fluconazole potency against C. neoformans increased and fungicidal activity was exhibited when either FAS1 or FAS2 expression was suppressed (Chayakulkeeree et al. 2007). Similarly, in the current study it was observed that the PLB1 gene involved in fatty acid synthesis was downregulated in response to plumbagin treatment in the planktonic state. PLB1 encodes a membrane-associated phospholipase, and its in vivo role may involve ‘fatty acid remodeling’ of phospholipids (Tavares et al. 2019). These observations indicated that plumbagin may exhibit moderate antifungal activities against C. neoformans H99 by targeting fatty acid biosynthesis.

Primary virulence factors in Cryptococcus spp. include the capsule, melanin production and cell wall integrity. The Cryptococcus capsule modulates immune responses, enhances pathogenicity, and confers protection against oxidative stress (Zaragoza 2019). For C. neoformans, the polysaccharide capsule is essential for biofilm formation on medical devices (Martinez and Casadevall 2015). The expression of PDR802 in C. neoformans H99 was increased in the biofilm state. Reuwsaat et al. (2021) reported that the PDR802 mutant displayed an even more pronounced hyper-capsular phenotype, suggesting that plumbagin could decrease capsule synthesis by C. neoformans H99 by upregulating the expression of PDR802 in both the biofilm and planktonic states. In the planktonic state of C. neoformans H99, three genes, CAP10, CAP59, and CAP64, which are required for capsule formation, were downregulated (Imanishi et al. 2017). Interestingly, CAP59 was considered to be associated with the RPL27 gene, which encodes a ribosomal protein that is a component of secretory vesicles transporting capsular polysaccharides in C. neoformans (Chang and Kwon-Chung 1999; Rodrigues et al. 2007). Moreover, melanin is another important virulence factor that protects fungal cells from oxidative damage, antifungal assault and high temperature, and also modulates host immune responses (Garcia-Rubio et al. 2019). The current study indicates that plumbagin induced the expression of several genes involved in melanin production. Among these genes, GAT201 was upregulated by 0.47-fold in the biofilm state, which was consistent with the previous observation that a GAT201 overexpression strain showed decreased melanization (Liu et al. 2008).

In conclusion, the current results strongly suggest that plumbagin is effective in inhibiting both C. neoformans H99 planktonic cells and biofilm formation. Plumbagin influences the cell membrane integrity, metabolic activity, adhesion, capsule formation, and melanin production of C. neoformans H99 cells. In addition, plumbagin inhibited the biofilm and planktonic cells of C. neoformans H99 by regulating the expression of associated genes. DEGs identified through RNA-seq analysis were related to cell membrane integrity, ribosome biogenesis, fatty acid synthesis, and virulence factors. This study provides a general overview of gene regulation in C. neoformans H99 biofilm and planktonic cells after plumbagin treatment. Therefore, RNA-seq data provide valuable resource to elucidate the antifungal mechanisms and potential antifungal targets of plumbagin against C. neoformans H99.

**Competing interest**
The authors declare no competing interests.

**Data accession**
Our sequenced data has been deposited at GenBank under the accession GJKG00000000.

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