

In vitro and in vivo antifungal effects and mechanism of Daphnetin against *Candida albicans*

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Conflict of interest

The authors declare no conflict of interest.

Ethics approval

This study was performed in line with the principles of Lanzhou University on the Management of Laboratory Animals and were approved by the Lanzhou University Animal Ethics Committee.

**In vitro and in vivo antifungal effects and mechanism of Daphnetin
against *Candida albicans***

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Abstract

Pathogenic fungi have increasingly developed resistance to conventional antifungal drugs in recent years, and the incidence of fungal infections continues to rise globally; thus, they have become a global public health threat. *Candida* species, particularly *Candida albicans* (*C. albicans*), are the leading cause of invasive fungal infections (IFIs). In this study, we examined the activity of Daphnetin (DAP) against *C. albicans* in vitro and in vivo, as well as its antibiofilm effects, resistance profile, and antimicrobial mechanisms. DAP exhibited significant inhibition against both *C. albicans* and its biofilms, with a minimum inhibitory concentration (MIC) of 0.64 mg/mL and a minimum fungicidal concentration (MFC) of 1.28 mg/mL. Antifungal activity was concentration-dependent and associated with a low potential for the development of resistance. DAP exerted its effects through multiple pathways, including disrupting the integrity of the fungal cell membrane and cell wall, increasing membrane permeability, inhibiting biofilm formation, and

intracellular reactive oxygen species (ROS) production, and destroying mature biofilms. In addition, DAP and its thermo-responsive gel (DAP-TG) exhibited strong antifungal activity in mice infected with *C. albicans* in wounds, effectively promoting wound healing. Taken together, our results indicate that DAP exhibits marked in vitro and in vivo antifungal activity and antibiofilm effects in vitro, with minimal development of resistance. It represents a potent, safe, multi-target antifungal agent with promising potential as an ideal candidate for treating *C. albicans* infections.

Lay summary

C. albicans is a common pathogenic fungus. This study investigated the antifungal activity, antibiofilm activity, antifungal mechanism, and resistance of DAP against *C. albicans* in vitro for the first time. The antifungal activity and wound-healing effects of DAP and its thermosensitive gel were also evaluated in vivo. The results provide a new and promising strategy for combating *C. albicans* infections and overcoming fungal resistance.

Keywords Daphnetin, *Candida albicans*, antifungal activity, antifungal mechanism, drug resistance

Introduction

Fungal infections account for 6.5 million cases of illness each year and result in over 3.8 million deaths ^[1]. In recent years, invasive fungal infections have increasingly posed a significant threat to public health. They constitute a major component of antifungal resistance and have emerged as a new global crisis. Invasive candidiasis (including candidemia) is a predominant factor of morbidity and mortality in clinical settings ^[2].

Of these, *C. albicans* has become the primary causative agent of all healthcare-associated bloodstream infections. In individuals with profound immunosuppression, this opportunistic pathogen can disseminate hematogenously throughout the body and subsequently invade multiple visceral organs, resulting in life-threatening disseminated infection [2]. However, there are currently only three major classes of antifungal drugs, including azoles, echinocandins, and polyenes, that are approved for the management of IFIs. The efficacy of these drugs is significantly reduced because of the emergence of resistance within the pathogen population and the occurrence of adverse drug reactions [3]. Azole drugs (represented by fluconazole, FLC) are the most commonly used class of agents in clinical practice. Its mechanism of action involves inhibiting the activity of sterol 14 α -demethylase (CYP51), thereby blocking the biosynthesis of ergosterol [4]; however, with the widespread use of azole drugs, fungal resistance has significantly increased. Consequently, the growing clinical challenge of antifungal resistance underscores an urgent need for novel therapeutic agents [5, 6].

The isolation of novel antifungal compounds from natural plant extracts is a promising and effective strategy [7]. Terpenoids, alkaloids, flavonoids, and polyphenols exhibit good antifungal activity and may circumvent antifungal drug resistance [8]. In our previous study, we found that daphnetin (DAP), a natural coumarin derivative derived from plants of the *Daphne* genus, showed good biocompatibility, including low hemolytic and cytotoxic activity [9, 10]. It also exhibited activity against *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) in vitro. The antibacterial effect of DAP primarily occurred through the disruption of cell membrane and cell wall integrity, production of reactive oxygen species (ROS), and the inhibition of biofilm formation. Other studies have also shown that DAP exerts excellent antibacterial activity against bacteria, such as *Pseudomonas aeruginosa* [11] and *Helicobacter pylori* [12]. Nevertheless, the

antifungal activity and underlying mechanism of DAP against *C. albicans* remains unclear.

In this study, we investigated the antifungal activity, antibiofilm activity, antifungal mechanism, and resistance of DAP against *C. albicans* in vitro for the first time. The antifungal activity and wound-healing effects of DAP and its thermosensitive gel were also evaluated in vivo. The results provide a new and promising strategy for combating *C. albicans* infections and overcoming fungal resistance.

Materials and Methods

Strain, medicines, and reagents

The reference strain *C. albicans* ATCC 10231 was procured from the American Type Culture Collection. The fungi were cultivated in Yeast Extract Peptone Dextrose (YPD) medium (Qingdao Hope Biotechnology Co., Ltd.) at 37°C with agitation at 120 r·min⁻¹. Daphnetin (purity > 95%) was obtained from Shanghai Bide Pharmatech Co., Ltd (China). Yeast Extract Peptone Dextrose (YPD) liquid medium and Saccharose-Dextrose Agar (SDA) were purchased from Qingdao High-Tech Industrial Park Haibo Biotechnology Co., Ltd (China). Terbinafine hydrochloride (TBH), crystal violet (CV), glutaraldehyde fixative (2.5%), chloroform hydrate, and indocyanine green were procured from Shanghai McLean Biochemical Technology Co., Ltd (China). Poloxamer 407 and 188 were obtained from BASF (Ludwigshafen, Germany). Polyvinylpyrrolidone (PVP-K15) was procured from Dalian Meilun BioTechnology Co., Ltd (China). Phosphate-buffered saline (PBS, pH 7.4), Coomassie Brilliant Blue G-250, propidium iodide (PI) stain, ROS assay kit, and 4% paraformaldehyde were purchased from the Wuhan Saver Biotechnology Co., Ltd (China). The alkaline phosphatase (AKP) assay kit was obtained from Nanjing Jiancheng Biotechnology Co., Ltd (China).

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The antifungal activity of Daphnetin (DAP) against *C. albicans* was determined using the double dilution method based on the Clinical and Laboratory Standards Institute guidelines ^[13]. The mid-log phase *C. albicans* culture was diluted to 1×10^5 CFU/mL in YPD. Next, 100 μ L of the cell suspension was mixed with an equal volume (100 μ L) of DAP solution and added to 96-well microplates. The final concentration of DAP in the wells ranged from 0.08 to 2.56 mg/mL. The plates were incubated with continuous shaking at 120 rpm at 37°C for 24 h. Subsequently, the turbidity of the solutions was observed. The lowest concentration of samples that inhibited the growth of *C. albicans* was considered the MIC. The fungal suspension treated with TBH was used as a positive control, and that without DAP was used as a negative control. MFC was determined using the agar plate diffusion method ^[14]. After plating the broth from wells without visible *C. albicans* growth onto SDA plates, the MFC was considered the lowest concentration of the test drug that produced a $\geq 99.9\%$ decrease relative to the initial inoculum. The assay was performed in triplicate.

Growth curves

The growth curve of DAP against *C. albicans* was determined based on a previous report ^[13]. The *C. albicans* culture in the mid-log phase was diluted to 1×10^5 CFU/mL. DAP was diluted with YPD to final concentrations of 0.32 mg/mL, 0.64 mg/mL, 1.28 mg/mL, and 2.56 mg/mL in 96-well plates containing the fungal suspension and incubated at 120 rpm at 37°C for 24 h. The fungal suspension without DAP was used

as the control. The 96-well microtiter plates were incubated at 120 rpm at 37°C for 24 h. Subsequently, the OD values at 600 nm for each well were measured at regular intervals using a microplate reader (Infinite M200, Tecan, Switzerland). The growth curve was plotted with time on the horizontal axis and OD₆₀₀ values on the vertical axis. The assay was performed in triplicate.

Leakage of cellular components assay

Cell membrane integrity was assessed by detecting the leakage of intracellular nucleic acids and proteins [14]. The *C. albicans* culture in mid-log phase was adjusted to 1×10^8 CFU/mL and centrifuged at 8000 rpm for 5 min. After rinsing twice with phosphate-buffered saline (PBS), the fungi were resuspended in PBS. The fungal suspension was mixed with an equal volume of DAP solution at final concentrations of 0.64 mg/mL, 1.28 mg/mL, and 2.56 mg/mL and incubated at 120 rpm and 37°C for 0, 0.5, 1, 2, 4, 6, and 12 h. Next, the inocula were removed and centrifuged at 8000 rpm for 5 min to collect the supernatant, which was filtered through a 0.22 µm filter membrane. The fungal suspension without DAP was used as a control. Subsequently, the OD values at 260 nm were measured using a spectrophotometer (nano-800, Shanghai Jinpeng Technology Co., Ltd., Shanghai, China). The assay was performed in triplicate.

The protein content in the filtrate was determined using Coomassie Brilliant Blue G-250 dye. Briefly, 50 µL of the filtrate was transferred to a sterile 96-well plate, 150 µL of Coomassie Brilliant Blue G-250 dye was added, and the plates were incubated for 3–5 minutes. Soluble protein leakage was measured using a microplate reader (Infinite M200, Tecan, Switzerland) at 595 nm, with PBS solution used as a control. The assay was performed in triplicate.

Cell wall permeability

As a periplasmic enzyme, AKP is detectable in the extracellular space only when the cell wall is compromised. Therefore, its extracellular levels directly reflect the state of cell wall integrity. The extracellular AKP content was determined by a previous method ^[15]. The fungi were resuspended in PBS and mixed with DAP solution at final concentrations of 0.64 mg/mL, 1.28 mg/mL, and 2.56 mg/mL and were incubated at 120 rpm and 37°C for 0, 0.5, 1, 2, 4, 6, and 6 h. Next, the inocula were removed and centrifuged at 8000 rpm for 5 min to collect the supernatant. The fungal suspension without DAP was used as a control. The content was determined using the AKPase assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The OD values at 520 nm were measured using a microplate reader (Infinite M200, Tecan, Switzerland). This assay was performed in triplicate.

Membrane permeability test

Fungal membrane integrity was assessed by propidium iodide (PI) staining based on a previous report ^[16]. The *C. albicans* culture in mid-log phase was adjusted to 1×10^8 CFU/mL, centrifuged at 8000 rpm for 10 min, and resuspended in PBS. The fungal suspension was mixed with DAP solution at final concentrations of 0.64, 1.28, and 2.56 mg/mL and incubated at 120 rpm and 37°C for 4 h. The suspension treated with TBH was used as the positive control, and that without DAP was used as the negative control. Fungal suspensions were subsequently stained with PI, Servicebio, Wuhan, China). After washing twice with PBS and staining with PI for 15 min in the dark, the cells were observed under a fluorescence microscope (BX53+DP74, Olympus, Japan).

Inhibition of biofilm formation and disruption of mature biofilm

The ability of DAP to inhibit biofilm formation by *C. albicans* was determined using the crystal violet (CV) staining method [18]. The mid-log phase *C. albicans* culture was adjusted to 1×10^5 CFU/mL and then treated with DAP at final concentrations of 0.32 mg/mL, 0.64 mg/mL, and 1.28 mg/mL, followed by incubation at 37°C with shaking at 120 rpm for 24 h. Next, the planktonic cells were removed, and the biofilms were gently washed with PBS. The biofilms were then fixed with 10% formaldehyde for 30 min and stained with 0.1% crystal violet (w/v) in water at room temperature for 15 min. After three additional washings in PBS, the biofilms were air-dried and solubilized with 30% (v/v) acetic acid. The fungal suspension treated with TBH was used as the positive control, and that without DAP was used as the negative control. Finally, the OD values at 590 nm were measured using a microplate reader (Infinite M200, Tecan, Switzerland). The assay was performed in triplicate.

The disruption assay for established biofilms also used the CV staining method described above. A mid-log-phase *C. albicans* culture was adjusted to 1×10^5 CFU/mL, added to a 96-well plate, and incubated at 120 rpm at 37°C for 24 h to allow biofilm formation. The culture medium was then gently removed, and the cells were washed twice with PBS. The subsequent steps were the same as in the biofilm inhibition assay.

Scanning electron microscopy (SEM) analysis

SEM samples were prepared based on the method described by Chan et al [19]. The mid-log phase *C. albicans* culture was adjusted to 1×10^8 CFU/mL. The fungal samples were collected after centrifugation at 8000 rpm for 5 min and were resuspended in PBS. The suspension was mixed with DAP solution at a final concentration of 2.56 mg/mL and incubated at 120 rpm at 37°C for 4 h, and the samples were washed twice

with PBS. The fungal suspension treated with TBH was used as a positive control, and the fungal suspension without DAP was used as a negative control.

The samples were fixed overnight at 4°C in 2.5% (w/v) glutaraldehyde and washed twice with PBS. Subsequently, samples were then subjected to graded ethanol dehydration (50%, 60%, 70%, 80%, 90%, and 100% v/v, 10 min per step), followed by centrifugation. Finally, the samples were dehydrated with tert-butanol for 2 h. After air-drying, samples were sputter-coated with a gold-palladium alloy and imaged using scanning electron microscopy (Aprio S, Thermo Fisher, USA) to observe morphological changes.

ROS measurements

ROS production in *C. albicans* cells was assessed by fluorescence assay following DAP treatment [16]. Specifically, the measurement was conducted using a ROS assay kit that detects reactive oxygen species based on the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA). The mid-log phase *C. albicans* culture was adjusted to 1×10^8 CFU/mL. The fungi were collected after centrifugation at 8000 rpm for 5 min and washed twice with PBS. The fungal suspension was first incubated with a fluorescent probe DCFH-DA at 37°C for 30 min in the dark, followed by washing with PBS to minimize extracellular fluorescence interference. Next, probed cells were treated at final drug concentrations of 0.64 mg/mL, 1.28 mg/mL, and 2.56 mg/mL for 0, 0.5, 1, 2, 4, and 6 h. A fungal suspension containing a ROS inducer was used as the positive control, and one without DAP was used as the negative control. After incubating for different time points, the ROS levels were quantified by measuring the fluorescence intensity (excitation wavelength = 488 nm, emission wavelength = 525 nm) with a microplate reader (Infinite M200, Tecan, Switzerland). The assay was performed in triplicate.

Serial passage assay for resistance development

The resistance of *C. albicans* to DAP was evaluated using a previously described method [20]. *C. albicans* cultures were serially passaged up to 15 times with sub-MIC levels of the antimicrobial agent, and the MIC was determined at each passage. MIC fold-change relative to the initial value was then calculated for each passage. TBH was used as a positive control.

Animal models of skin infections

The antifungal activity of DAP was assessed in vivo using a mouse skin wound model. Female Kunming mice (22–26 g) were obtained from the Medical Experimental Centre of Lanzhou University. All experimental procedures were performed in strict compliance with the regulations of Lanzhou University on the Management of Laboratory Animals and were approved by the Lanzhou University Animal Ethics Committee. A mouse skin infection model was established using relevant methods [21]. Mice were maintained under controlled environmental conditions: 25°C, 12-h light/dark cycle, and 40–60% relative humidity. After shaving the back of each mouse, the skin was rinsed with 70% ethanol. The mice were anesthetized via intraperitoneal injection of 10% chloroform hydrate. Subsequently, a 10-mm-diameter full-thickness excisional wound was induced on the dorsal skin of each mouse.

The mice were randomly divided into six groups: a model group (Model, saline), a blank gel group (Thermosensitive gel, TG), a high-dose DAP thermosensitive gel group (DAP-TG-H), a low-dose DAP thermosensitive gel group (DAP-TG-L), a DAP aqueous solution group (DAP), and a positive control group (TBH thermosensitive gel, TBH-TG), with nine mice in each group. Data from all groups were

independently analyzed and statistically compared among the groups. The mid-log phase *C. albicans* culture was adjusted to 1×10^8 CFU/mL. Fifty microliters of the *C. albicans* suspension was applied to the wound site, which was then covered with a sterile dressing.

Drug-treated model mice and fungal burden measurement

A DAP thermosensitive gel (DAP-TG) was prepared following a previously reported method [10]. Briefly, 2.2 g of Poloxamer 407 (P407) and 0.1 g of Poloxamer 188 (P188) were added to cold water and stirred continuously. Next, 0.3 g of polyvinylpyrrolidone (PVP-K15) and the required amount of DAP were added to the mixture to yield DAP-TG (3 mg/mL). The TBH thermosensitive hydrogel (TBH-TG, 2.25 mg/mL) was also prepared by the same method, except that TBH replaced DAP.

Twenty-four hours after model induction, the wound was treated topically with saline, TG, DAP-TG-H (20 mg/kg), DAP-TG-L (10 mg/kg), DAP solution (20 mg/kg), and TBH-TG (12.5 mg/kg) continuously for 11 days. On days 1, 3, 5, 7, 9, and 11 after model induction, *C. albicans* was isolated from the mouse wound sites using sterile swabs and spread onto the surface of sterile SDA plates. The agar plates were incubated at 37°C for 24 hours, and fungal growth was observed.

Hematoxylin and eosin staining of wound tissue

Day 11 after treatment, skin tissue samples were collected from the wounds using sterile surgical scissors, fixed in 4% paraformaldehyde, and histological sections were prepared. The sections were sequentially dehydrated in 85% and 95% ethanol for 5 min each, stained in eosin solution for 5 min, and finally dehydrated and mounted. The specimens were examined under a fluorescence microscope (BX53+DP74,

Olympus, Japan) to observe the pathological changes in the skin wound tissue.

Wound healing

On days 1, 3, 5, 7, 9, and 11, photographs of the wounds of each mouse were taken to document wound healing. Wound area was calculated using ImageJ software, and the wound area % was determined as: $(\text{wound area at different times} / \text{initial wound area}) \times 100\%$.

Statistical analysis

Experimental data were analyzed statistically using Origin 2021 software, and all results are expressed as the mean $\pm$ SD. One-way analysis of variance was performed using SPSS 26.0 to assess statistical significance, and $P < 0.05$ was considered statistically significant.

Results

Minimum inhibitory concentration (MIC) and MFC

The in vitro antimicrobial activity of DAP against *C. albicans* was evaluated by determining its MIC and MFC using the microdilution and agar diffusion methods. The results indicated that the MIC of DAP and TBH against *C. albicans* were 0.64 and 0.016 mg/mL, respectively, whereas the MFC of DAP and TBH were 1.28 and 0.256 mg/mL. The MFC is the minimum drug concentration required to kill 99.9% of the fungus and serves as a supplementary reference indicator to the MIC. When the MFC: MIC ratio is ≤ 4 , the antimicrobial agent is considered to have bactericidal activity ^[22]. The MFC/MIC ratio for DAP was 2, which indicated good fungicidal activity against *C. albicans*.

Growth curve analyses

To further evaluate the antifungal activity of DAP, dynamic growth curves were plotted for *C. albicans* treated with various DAP concentrations (0.32 mg/mL, 0.64 mg/mL, 1.28 mg/mL, and 2.56 mg/mL) (Fig. 1). The OD600 value of the control group rapidly increased after 6 hours. In contrast, all drug-treated groups showed various inhibition effects, which became more pronounced as the concentration of DAP increased. At 0.32 mg/mL, DAP essentially inhibited the growth of *C. albicans* within 12 hours. After 12 hours, the inhibitory effect decreased, and *C. albicans* began to grow. At 0.64 mg/mL or higher, the OD600 values showed almost no change, indicating the complete inhibition of *C. albicans* growth, which also confirmed the accuracy of the MIC test results. At 24 hours, there was a significant difference in the antifungal activity of DAP at concentrations of 0.32 and 2.56 mg/mL. Taken together, DAP effectively inhibits the growth of *C. albicans* in a concentration-dependent manner.

Leakage of cellular components assay

The cell membrane is a key structure that is required for fungal survival. Under normal conditions, the cell membrane maintains its integrity and permeability, preventing the outflow of large molecules. When the cell membrane is damaged, large molecules, such as nucleic acids and proteins, leak into the culture medium [23, 24]. Therefore, the integrity of the cell membrane may be determined by measuring the amount of nucleic acid and protein that leaks into the culture medium.

Fig. 2A,B show the leakage of nucleic acids and proteins in *C. albicans* after 12 hours of treatment with various DAP concentrations (0.64, 1.28, and 2.56 mg/mL). In the blank control group (Control), the OD

values at 260 and 595 nm were unchanged with increasing incubation time, indicating that the cell membranes were not disrupted and that no nucleic acids or proteins leaked out. When *C. albicans* was cultured in DAP solutions at concentrations of 0.64, 1.28, and 1.28 mg/mL, the leakage of nucleic acids and proteins was significantly higher compared with the control group. The OD values of the culture medium at 260 and 595 nm increased with incubation time and exhibited some degree of concentration dependence. The higher the concentration of nucleic acids and proteins, the higher the OD values and the greater the leakage. The results also indicated that nucleic acid and protein leakage gradually increased as the duration of exposure increased. These results indicate that DAP disrupts the cell membranes of *C. albicans*, leading to increased membrane permeability and the leakage of intracellular nucleic acids and proteins into the culture medium. The higher the concentration of DAP and the longer the exposure time, the more severe the membrane disruption.

Permeability of the cell wall

AKP, a periplasmic enzyme, leaks into the extracellular milieu upon cell wall damage, leading to elevated extracellular AKP activity [25]. Therefore, the permeability of the cell wall may be assessed by detecting AKP leakage in the culture medium. Fig. 3 shows AKP leakage patterns after treating *C. albicans* with DAP concentrations of 0.64, 1.28, and 2.56 mg/mL for 12 h. As the incubation time increased, the OD values at 520 nm remained constant in the control group, and AKP activity in the supernatant remained very low, indicating that the cell walls remained intact with no AKP leakage. Compared with the control group, the OD values at 520 nm in the groups treated with DAP at concentrations of 0.64, 1.28, and 1.28 mg/mL were all higher, and the increase was concentration-dependent. The results indicate that DAP inhibits the growth

and reproduction of *C. albicans* by disrupting cell wall permeability.

Membrane permeability test

Pyridine iodide (PI) is an analog of ethidium bromide that selectively associates with DNA. After incorporation into double-stranded DNA, it emits red fluorescence, thus enabling the staining of DNA or cell nuclei. PI cannot penetrate intact cell membranes, but it can penetrate the disrupted cell membranes of cells in late apoptosis or dead cells [26, 27]. Therefore, PI staining may be used to assess cell membrane permeability. As shown in Fig. 4, intense red fluorescence was observed in the DAP group, whereas no red fluorescence was observed in the control group. At increased DAP concentrations, the fluorescence intensity of the fungi increased in a concentration-dependent manner. The results indicate that cell membrane permeability increases following DAP treatment.

Inhibit biofilm formation and disruption of mature biofilm

The inhibitory effects of various DAP concentrations on the formation of *C. albicans* biofilms are shown in Fig. 5A. As the concentration increased, the inhibition of *C. albicans* biofilms by DAP and TBH generally showed an upward trend. Furthermore, DAP concentrations of 0.32, 0.64, and 1.28 mg/mL significantly inhibited biofilm formation in a concentration-dependent manner. Inhibiting biofilm formation is an effective approach to combating drug resistance [28]. Our results indicate that DAP inhibits biofilm formation and thus serve as a candidate drug for overcoming resistance.

The ability of DAP solutions to disrupt established *C. albicans* biofilms is shown in Fig. 5B. Compared with the control group, treatment with various concentrations of DAP decreased the absorbance at 590 nm

from 2.72 to 0.78, 0.67, and 0.55, respectively. Cells treated with TBH decreased to 0.87, 0.76, and 0.50.

The results indicate that DAP significantly disrupts mature *C. albicans* biofilms in a concentration-dependent manner.

Scanning electron microscopy (SEM) analysis

The morphological changes in *C. albicans* before and after DAP treatment were observed using scanning electron microscopy (Fig. 6). The surface of the control group was uniform and preserved, and *C. albicans* exhibited typical oval and spherical shapes, indicating that the fungal cell membranes were not damaged. Compared with the control group, after treatment with DAP at a concentration of 2.56 mg/mL for 4 hours, the surface of *C. albicans* underwent significant changes, exhibiting obvious roughness, atrophy, wrinkling, and deformation. Following treatment with TBH, distinct pores and damage appeared on the cell surface. These results confirm that DAP disrupts the cell structure of *C. albicans* to exert its antifungal effect.

ROS measurements

Intracellular ROS concentration serves as a quantitative biomarker of oxidative stress severity [29, 30]. Therefore, intracellular ROS levels can be indirectly assessed by detecting intracellular fluorescence signals. Fig. 7 shows the changes in intracellular ROS levels of *C. albicans* cells within 6 hours following treatment with various concentrations of DAP. As the incubation time increased, the fluorescence intensity in the positive control group exhibited a rapid upward trend, indicating a rapid increase in ROS within the cells. In contrast, the fluorescence intensity in the negative control group remained at a consistently low level. Following treatment of *C. albicans* cells with DAP, the fluorescence intensity increased in line with rising

DAP concentrations and longer incubation times. These results indicate that DAP treatment results in the generation and accumulation of ROS within *C. albicans* cells, thereby exacerbating damage to the cell membrane. DAP exerts its antifungal activity by inducing the production and accumulation of ROS within *C. albicans*.

Serial passage assay for resistance development

To evaluate the development of resistance, serial passage assays were performed (Fig. 8). *C. albicans* was incubated at sub-MIC doses of DAP and TBH for 15 passages, respectively. The MIC fold change of TBH against *C. albicans* increased significantly with each passage. By the 15th passage, the MIC of TBH against *C. albicans* had increased 128-fold; however, the MIC fold-change for DAP against *C. albicans* remained consistently at 1, with no significant increase observed. The results indicate that DAP was unlikely to induce resistance in *C. albicans*.

Fungal burden measurement

Using a mouse model of skin wound infection with *C. albicans*, the effects of DAP were examined. As shown in Fig. 9, the number of colonies in the DAP-TG treatment group decreased in a time-dependent manner. Compared with the Model group, the *C. albicans* burden in the wound exudate from the backs of mice in the DAP-TG-H and DAP-TG-L groups was significantly decreased, and almost no colonies grew on agar plates on day 3 after modeling. The DAP-TG group exhibited reduced *C. albicans* wound infection compared with the DAP group. There was no significant difference in *C. albicans* growth on agar plates between the TG and Model groups. The results indicate that DAP and DAP-TG effectively inhibit *C.*

albicans infection in mouse wounds, with DAP-TG exhibiting stronger antifungal activity compared with free DAP. The results also indicate that loading DAP into a thermosensitive gel is beneficial for enhancing the antifungal activity of DAP.

H&E staining

Inflammatory cell infiltration correlates with the severity of *C. albicans* infection. H&E staining was used to examine inflammatory infiltration in wound tissues from the three groups of mice. As shown in Fig. 10, in the Model group, extensive inflammatory cell infiltration and edema were observed in the skin, and the skin tissue structure was severely disrupted. In contrast, DAP-TG treatment suppressed the inflammatory response. The number of inflammatory cells was markedly reduced, no edema was detected in the skin tissue, a large number of new hair follicles were present, and the skin structure remained intact, thus indicating a positive therapeutic effect. DAP-TG exhibited superior efficacy to free DAP, and the high-dose DAP-TG group showed better results compared with the low-dose DAP-TG group. The results indicate that DAP and its thermosensitive gel exert a positive therapeutic effect on inflammation in wounds with fungal infections.

Wound healing

The ability of DAP to promote wound healing was examined using a *C. albicans* infection model established in a mouse dorsal wound. As shown in Fig. 11A, wound morphology was documented via digital photography on post-treatment days 1, 3, 5, 7, 9, and 11 to quantitatively evaluate healing progression. The results indicated that wounds in all of the mice showed a tendency to heal, although the rate varied (Fig.

11B). Mice in the Model and TG groups showed the slowest wound healing rates. From 1 day after modeling until the end of the experiment, there was no significant difference in the wound area between the model and TG groups. Compared with the model group, the DAP group exhibited a significant difference in residual wound area from the 9th day after modeling until the end of the experiment. From the 7th day after injury until the end of the experiment, the residual wound area of the DAP-TG-L group showed a significant intergroup difference compared with the model group. Compared with the other groups, the DAP-TG-H group exhibited the fastest wound healing rate and the greatest antifungal activity. Specifically, it promoted wound healing from day 3 to day 11 relative to the Model group. Compared with the DAP group, the DAP-TG-H group showed significantly superior wound healing-promoting effects starting from day 3. Furthermore, on day 11, the DAP-TG-H group achieved superior therapeutic efficacy compared with the DAP-TG-L group. These results indicate that the antifungal effect of DAP-TG in vivo occurs in a concentration-dependent manner. Furthermore, although the drug loading of the DAP-TG-L group was half that of the free DAP group, the DAP-TG-L group still exhibited a superior wound healing effect, which was comparable to that of free DAP from day 3 until the end of the trial. In addition, the administration form of the thermosensitive hydrogel enhanced the antifungal and wound healing-promoting effects of DAP in vivo. Finally, the positive control TBH-TG group exhibited a relatively rapid wound healing rate. The results indicate that DAP and DAP-TG effectively promote the healing of *C. albicans*-infected wounds on the backs of mice, and DAP-TG achieved superior therapeutic efficacy compared with free DAP.

Discussion

Infections caused by pathogenic fungi have emerged as a global public health threat. Antifungal therapy

remains the standard approach to treating fungal infections in wounds; however, with the widespread use of antifungal drugs, drug resistance has posed a significant challenge to clinical treatment [31-33]. *Cryptococcus*, *Aspergillus*, *Candida*, and *Pneumocystis* collectively account for nearly 90% of life-threatening invasive fungal infections [34, 35]. Strains of the genera *Candida*, *Cryptococcus*, and *Aspergillus* exhibit a marked propensity for antifungal resistance development. Fungi can resist chemical agents in various ways, primarily through genetic mutations, overexpression of target enzymes, the action of efflux pumps, and biofilm formation. These mechanisms undermine the efficacy of virtually all known antifungal drugs. The widespread use of single-compound and single-target antifungal drugs can readily induce the development of multi-drug resistance in fungi, leading to an increase in cases of infections that are difficult to eradicate [36]. Biofilms secrete cellular products and autonomously produce extracellular polymers that protect bacteria within the biofilm, resulting in drug resistance at concentrations 10–1000-fold higher than that required to kill the same bacterial strains in a free state [37, 38]. *C. albicans* is the leading fungal etiologic agent in biofilm-related device infections, with medical implants serving as highly conducive substrates for its adherence and proliferation. Although free-floating *C. albicans* cells are not inherently resistant to commonly used antifungal drugs, their biofilms exhibit inherent multi-drug resistance. Natural products are an important source of new antifungal drugs [39]. We demonstrated for the first time the excellent antimicrobial activities of DAP, a natural coumarin derivative, against *C. albicans* and its biofilm. The mechanisms through which DAP combats *C. albicans* were also elucidated. Furthermore, we demonstrated for the first time that DAP does not readily induce resistance in *C. albicans*. Finally, we determined the effects of DAP and its thermosensitive gel on wound healing in mice. Taken together, the results provide insight for further studies and the application of DAP for the treatment of *C. albicans*-infected wounds.

In the present study, DAP exhibited excellent antimicrobial activity against *C. albicans* with a MIC of 0.64 mg/mL and a MFC of 1.28 mg/mL. The growth kinetics curve for *C. albicans* indicated that the antifungal efficacy of DAP increased in a concentration-dependent manner. The antifungal curves indicated that at a concentration of 0.32 mg/mL, DAP could only inhibit fungal proliferation; however, at 0.64 and 2.56 mg/mL, DAP completely eradicated *C. albicans* cells within 12 hours of treatment. This trend was consistent with the results of the MIC and MFC assays. These results indicate that the inhibitory effect of DAP is primarily concentration-dependent and provides a basis for determining its optimal dosage. The MIC fold-change increase quantifies the extent of bacterial resistance evolution from single-mechanism fine-tuning (<8-fold) to multi-mechanism synergistic consolidation (≥ 64 -fold). The higher the MIC fold-change, the greater the loss of the therapeutic window and the higher the risk of cross-resistance [40]. After 15 consecutive passages, the MIC fold-change for DAP against *C. albicans* remained at 1, whereas that for TBH increased 128-fold, indicating that, compared with the positive control drug TBH, DAP was less likely to induce resistance. Following treatment with DAP, SEM analysis revealed that *C. albicans* cells displayed marked morphological changes and shrinkage. Leakage of intracellular nucleic acids and proteins, together with elevated extracellular AKP levels, indicated that the integrity of the cell membrane and cell wall structures was compromised. PI staining revealed increased fluorescence intensity, indicating that DAP increases the permeability of the cell membrane of *C. albicans*, thus indicating rapid fungicidal activity. Oxidative stress, which is characterized by the excess production of ROS, is considered a trigger of apoptosis, leading to DNA damage, lipid and protein peroxidation, telomere shortening, bacterial aging, and death, thereby inhibiting infection [41, 42]. Our results indicated that DAP leads to excessive accumulation of ROS within *C. albicans* cells, thereby causing cell death and exerting antifungal activity. Similarly, its

antifungal effect, which was mediated by the induction of excessive ROS production, also occurred in a concentration-dependent manner. Furthermore, CV staining revealed that DAP effectively inhibited the formation of *C. albicans* biofilms and disrupted the biofilms. The multifaceted fungicidal effects of DAP against *C. albicans*, as described above, conferred it with excellent anti-*C. albicans* activity. Its antifungal mechanism is very similar to that of DAP against *S. aureus* and *E. coli* reported in our previous studies ^[10], and may also be closely related to its rare induction of drug resistance in *C. albicans*.

Bacterial infections often cause pain and inflammation. Furthermore, the inflammatory response plays a significant role in impaired wound healing ^[43]. Two types of medication are generally used to treat bacterial infections, including antibiotics and anti-inflammatory drugs with analgesic properties ^[44]. With respect to patient compliance, pharmacoeconomics, and greater safety and a lower risk of side effects, the concurrent use of a single drug that combines antibacterial and anti-inflammatory effects has more advantages and has become a focus of current research ^[45, 46]. Notably, studies indicate that DAP exerts a variety of pharmacological effects, including anti-inflammatory ^[47, 48], antioxidant ^[49], anti-arthritis ^[50, 51], antibacterial ^[11, 12], and antitumor ^[52, 53] properties. DAP is currently administered as an anti-inflammatory and analgesic drug and is commonly prescribed for the treatment of rheumatoid arthritis ^[54]; however, its antifungal activity against *C. albicans* in animals, as well as its anti-inflammatory and wound-healing effects on wounds infected with *C. albicans*, have not been studied. Using a mouse skin wound model, we examined for the first time the in vivo efficacy of DAP and its thermosensitive gel, which exhibits antifungal and anti-inflammatory activities, in treating *C. albicans* infections. The results of fungal load analysis in mouse wounds, H&E staining, and skin wound healing indicated that DAP and its thermosensitive gel show excellent antifungal activity against *C. albicans* in vivo, while significantly reducing the fungal load at the

wound site, ameliorating the inflammatory response in wound tissue, and promoting the healing of infected wounds. On the one hand, DAP effectively inhibits further infection and deterioration of skin wounds in mice by killing *C. albicans*. On the other hand, it reduces the occurrence of inflammatory responses through its anti-inflammatory effects, which further promotes wound healing. Nevertheless, this preliminary conclusion requires detailed and systematic confirmation studies.

In our previous studies, we demonstrated that DAP thermosensitive gel could enhance antibacterial efficacy against *S. aureus* and *E. coli* in vitro compared with free DAP because of its favorable temperature responsiveness, drug-loading capacity, and excellent sustained-release properties. Based on these results, we evaluated the antifungal effects of DAP and its thermosensitive gel against *C. albicans* in vivo. Consistent with our previous in vitro results, the DAP thermosensitive gel exhibited superior anti-*C. albicans* activity compared with free DAP in vivo, with antifungal effects intensifying proportionally to administered dose. The results indicated that the antifungal effects of DAP could be enhanced effectively by modifying the dosage form.

In summary, DAP exhibits multiple antifungal activities and effectively inhibits biofilm formation and disrupts mature biofilms, while circumventing resistance in *C. albicans*. Furthermore, DAP shows excellent anti-*C. albicans* activity in vitro and in vivo. DAP effectively alleviates inflammatory responses in wound tissue and promotes the healing of infected wounds in vivo; thus, it is a promising candidate for combating *C. albicans* and may be used for the treatment of *C. albicans*-infected wounds.

Conclusions

In this study, we examined the antifungal and antibiofilm activities of DAP against *C. albicans* as well as

its mechanism of action and drug resistance. We further demonstrated that DAP holds therapeutic promise against cutaneous *C. albicans* infections. The results indicate that DAP has excellent antibacterial activity against *C. albicans* and its biofilms and is unlikely to induce drug resistance. The fungicidal mechanism of DAP may involve multiple pathways, including disrupting the integrity of the *C. albicans* cell membrane and cell wall, increasing cell membrane permeability, inducing the production of ROS within the cell, inhibiting the formation of *C. albicans* biofilms, and disrupting mature biofilms, through which DAP exerts its antifungal effects. Furthermore, in a mouse model of fungal infection in skin wounds, DAP and its thermosensitive gel exhibited excellent antifungal efficacy and wound-healing properties. Nevertheless, the precise molecular targets of DAP have not yet been fully elucidated and require further study using modern methods, such as gene editing and structural biology. In summary, our results indicate that DAP contains superior antifungal characteristics, such as rapid action, multiple antifungal mechanisms, and a low tendency to induce drug resistance. DAP is a potent and safe multi-target antifungal agent against *C. albicans* and has potential clinical value. Furthermore, it may serve as an ideal candidate for treating *C. albicans* infections, either alone or in combination with existing antifungal drugs to enhance therapeutic outcomes.

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Figure 1. Growth curves of DAP against *C. albicans*. (n = 3, means ± SD, ***P < 0.001, ****P < 0.0001 vs control group).

Figure 2. Effect of DAP on cell membrane permeability, leakage of nucleic acids and from *C. albicans*. (A) Leakage of proteins. (B) (n = 3, means ± SD, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs control group).

Figure 3. The effect of DAP on the permeability of *C. albicans* cell walls. (n = 3, means ± SD, ****P < 0.0001 vs control group).

Figure 4. The effect of DAP on the cell membrane permeability of *C. albicans*. Scale bar = 10 μm.

Figure 5. The inhibitory effect of DAP on the formation of *C. albicans* biofilms. (A) The destructive effect of DAP on mature biofilm formed by *C. albicans*. (B) (n = 3, means ± SD, ***P < 0.001, ***P < 0.0001 vs control group).

Figure 6. The effects of DAP on the morphology of *C. albicans* were observed using a scanning electron microscope. Scale bar = 5 μm.

Figure 7. Intracellular ROS levels in *C. albicans* after 6 hours treatment with DAP at different concentrations. (n = 3, means ± SD, **P < 0.01, ****P < 0.0001 vs negative control group, ##P < 0.01, ###P < 0.001, ####P < 0.0001 vs positive control group).

Figure 8. Resistance development of *C. albicans* treated with sub-MIC DAP and TBH.

Figure 9. Fungal burden isolated from mouse wounds treated with different groups at different time points.

Figure 10. H&E stain of skin wounds in mice. Scale bar = 100 μm.

Figure 11. Image of a skin wound in mice at different time points following treatment with different group at different time points in a mouse. (A) Area of the skin wound in a mouse. (B) ($n = 3$, means $\pm$ SD, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ vs control group, $^{\#}P < 0.05$, vs DAP-TG-H group).

Figure 1.

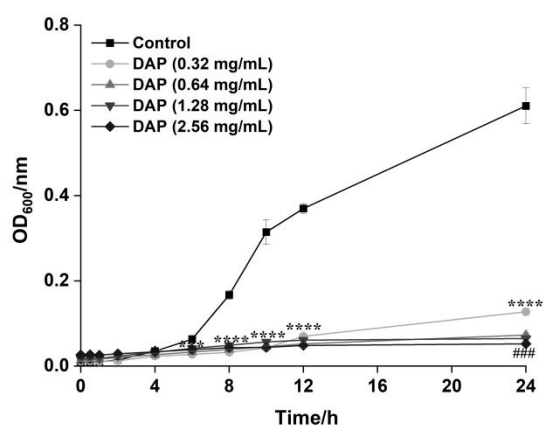


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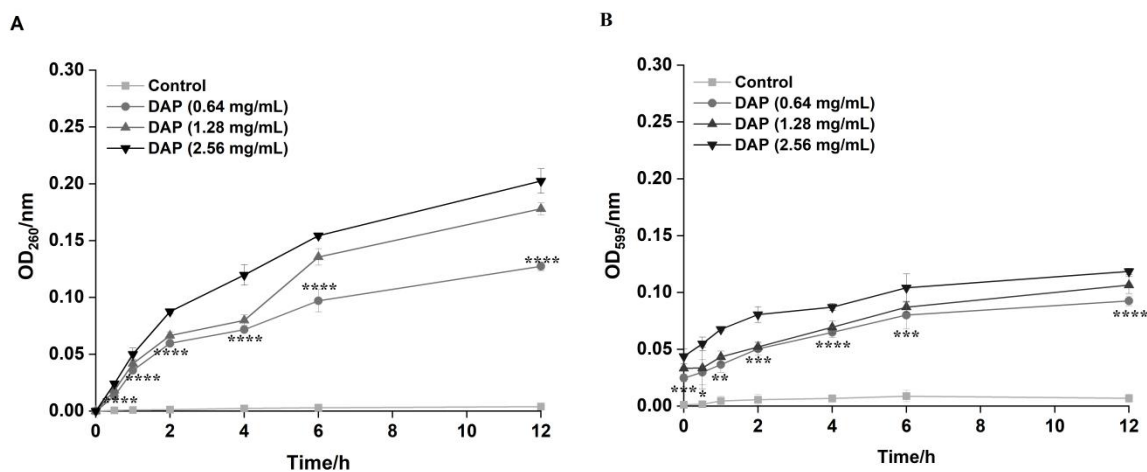


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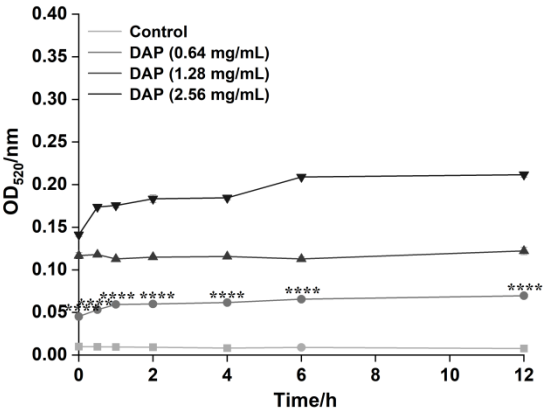


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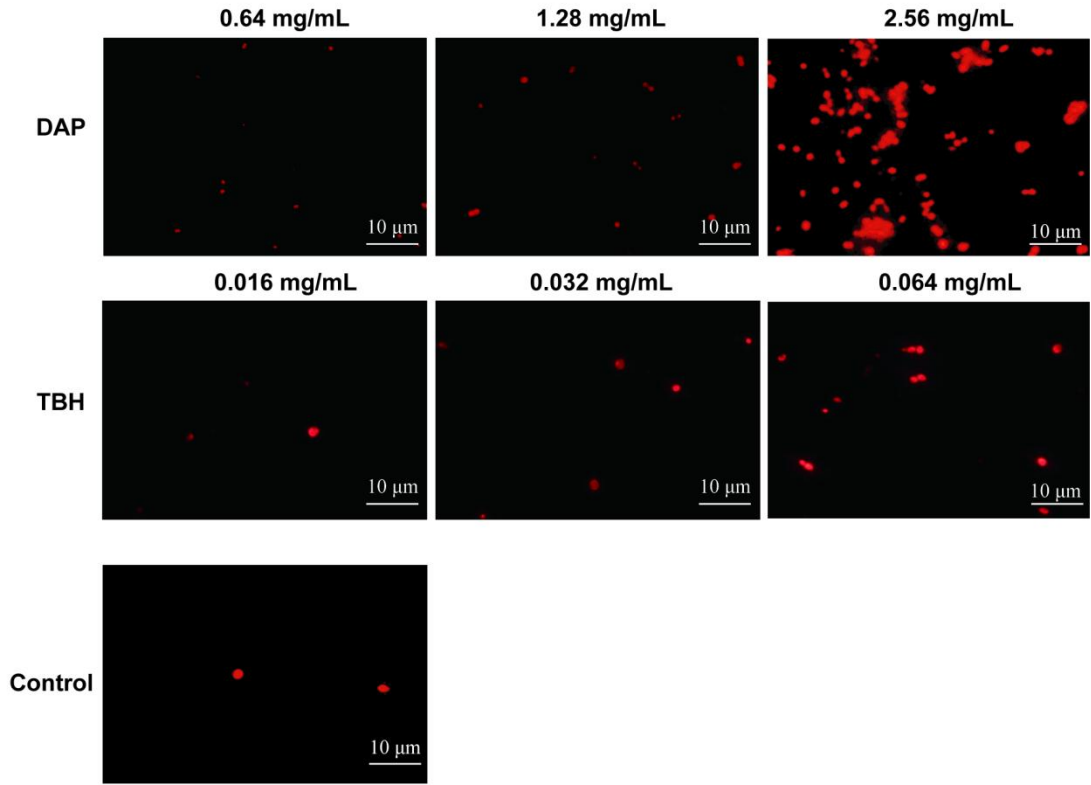


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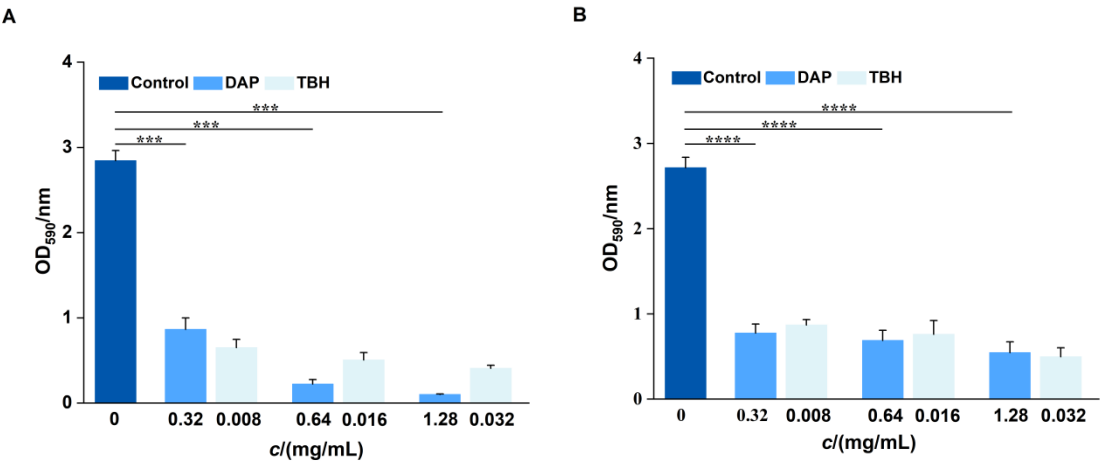


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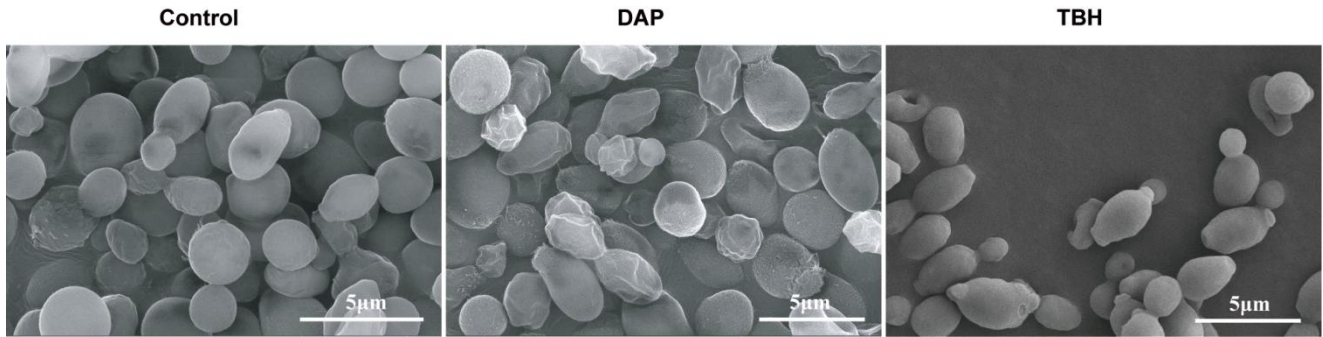


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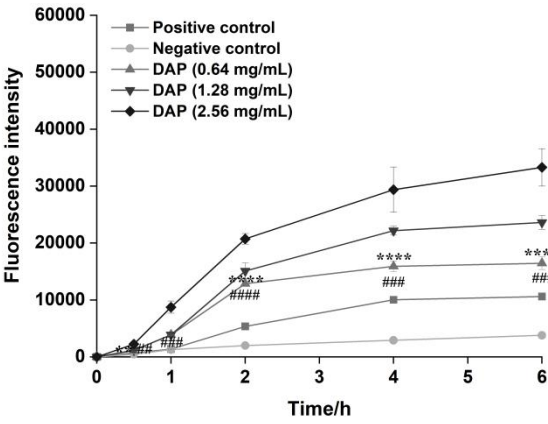


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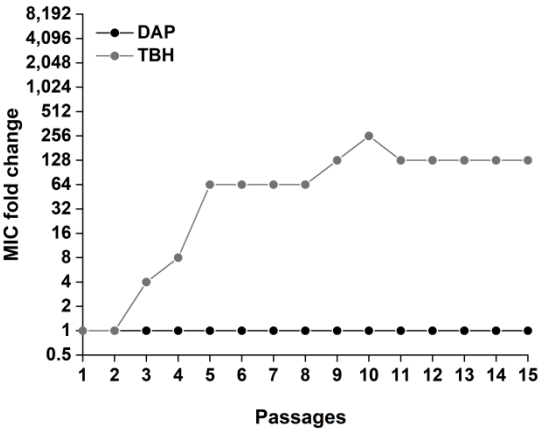


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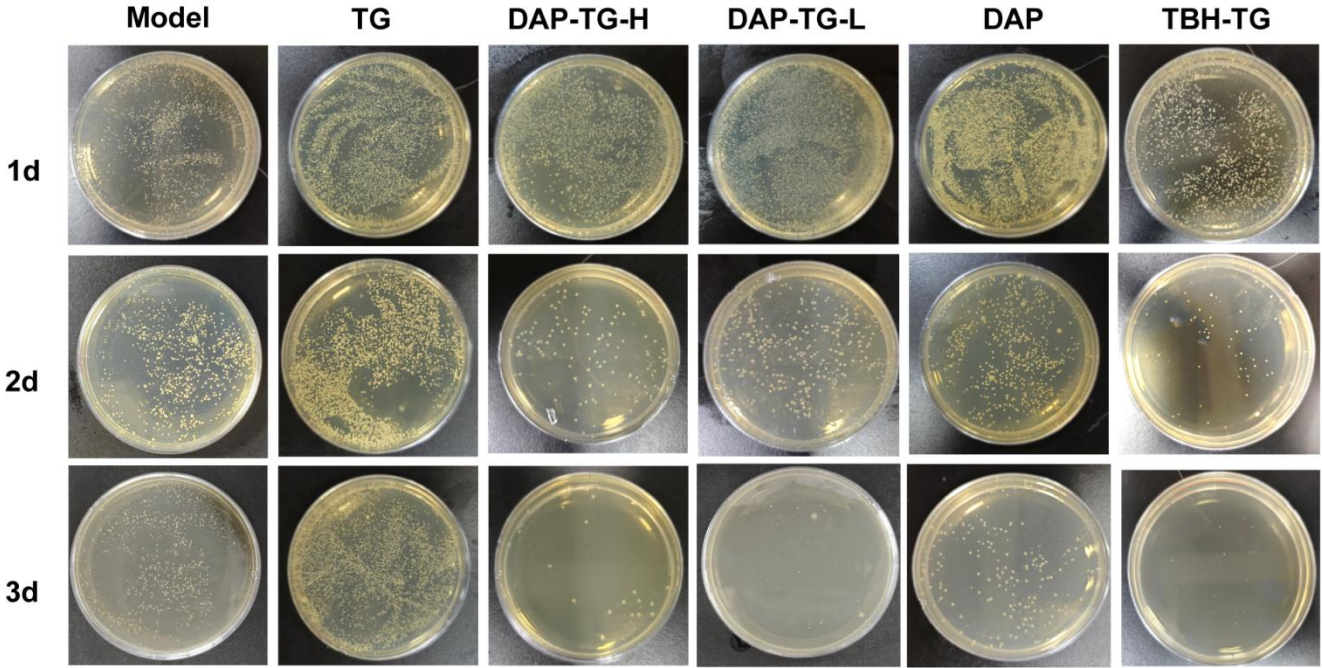


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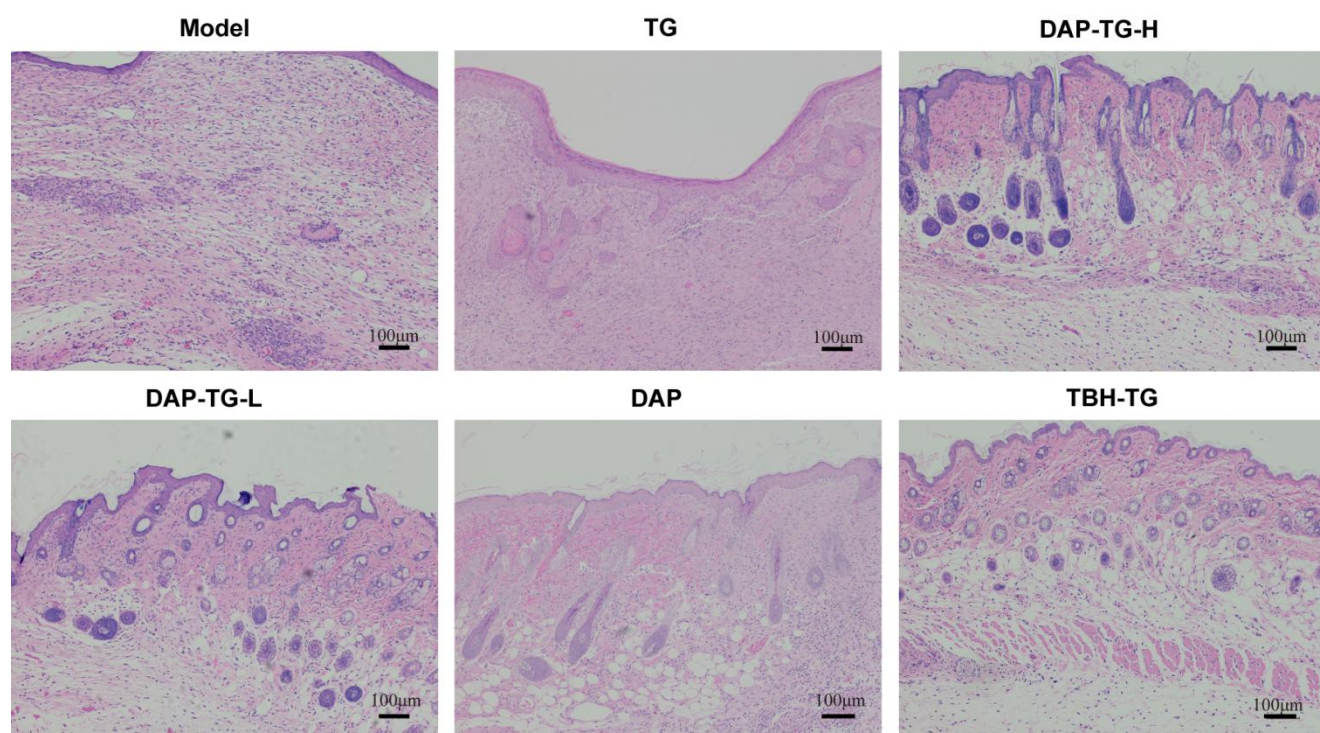
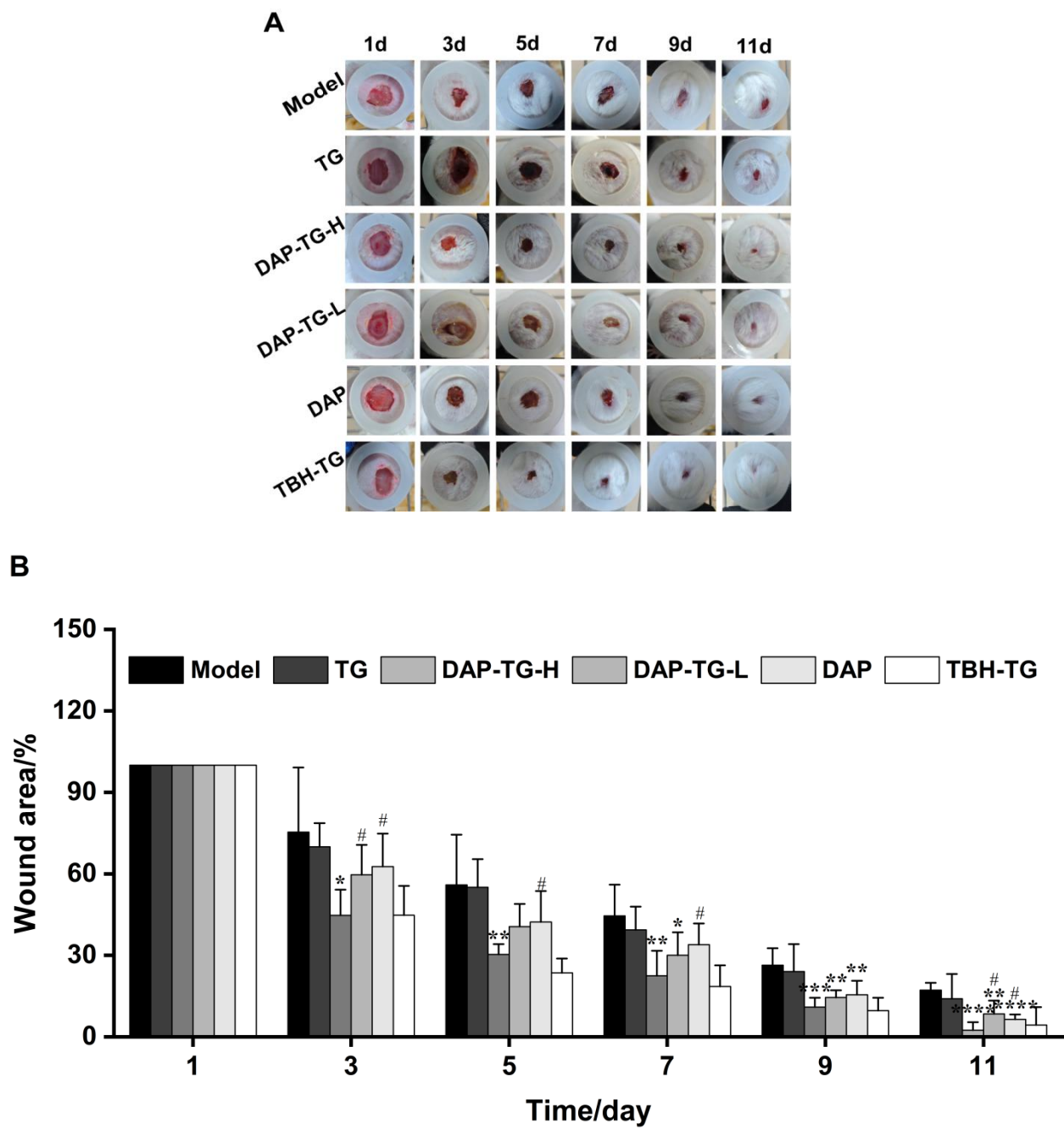


Figure 11.



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Author contribution

Yuqin Guo (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing), Xing Wang (Investigation, Visualization), Jia yi Gao (Formal analysis, Investigation), Junhong Fan (Methodology, Investigation), Shaohua Li (Methodology, Formal analysis), Xiaorui Shi (Formal analysis), Zhengfang Yu (Formal analysis), Zhiyan Zhou (Formal analysis), Huiju Liu (Formal analysis) and Lan Yu (Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing –review & editing)

Statements & Declarations

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Conflict of interest

The authors declare no conflict of interest.

Ethics approval

This study was performed in line with the principles of Lanzhou University on the Management of Laboratory Animals and were approved by the Lanzhou University Animal Ethics Committee.

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