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Fleagrass (*Adenosma buchneroides Bonati*) Acts as a Fungicide Against *Candida albicans* by Damaging Its Cell Wall

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Abstract

Fleagrass, a herb known for its pleasant aroma, is widely used as a mosquito repellent, antibacterial agent, and for treating colds, reducing swelling, and alleviating pain. The antifungal effects of the essential oils of fleagrass and carvacrol against *Candida albicans* were investigated by evaluating the growth and the mycelial and biofilm development of *C. albicans*. Transmission electron microscopy was used to evaluate the integrity of the cell membrane and cell wall of *C. albicans*. Fleagrass exhibited high fungicidal activity against *C. albicans* at concentrations of 0.5% v/v (via the Ras1/cAMP/PKA pathway). Furthermore, transmission electron microscopy revealed damage to the cell wall and membrane after treatment with the essential oil, which was further confirmed by the increased levels of β-1,3-glucan and chitin in the cell wall. This study showed that fleagrass exerts good fungicidal and hyphal growth inhibition activity against *C. albicans* by disrupting its cell wall, and thus, fleagrass may be a potential antifungal drug.

Keywords *Candida albicans* · Fleagrass · Carvacrol · Antifungal drug

Introduction

Candida albicans (*C. albicans*) is the most common pathogenic fungi in humans, causing a broad spectrum of diseases including skin, mucosal and systemic infections (Choi & Lee, 2015; Gow & Hube, 2012; Tran et al., 2020). Its ability to invade deep tissues for systemic infection is invariably associated with adhesion and hyphal and biofilm formation (Shahina et al., 2022). The mycelial form of *C. albicans* is more invasive than the yeast form and, therefore, plays an important role in its pathogenicity. In contrast, the yeast form is mainly involved in strain transmission (Choi & Lee, 2015). It has been found that the cell wall plays an important role in the virulence and mycelial morphogenesis of *C. albicans* (Arita et al., 2022). Biofilms, on the other hand, can form after the adhesion of *C. albicans* to host tissue or medical indwelling devices (Shahina et al., 2022). Once formed, the biofilm acts as a reservoir for pathogenic cells, which are highly resistant to drugs and the host immune system, and with the potential to spread disseminated bloodstream infections that can lead to invasive systemic infections of tissues and organs (Gulati & Nobile, 2016).

Despite the severe clinical distress and economic losses caused by *C. albicans*, there are only a few antifungal drugs available for clinical treatment, most of which are no longer effective against *C. albicans*. Given this challenging situation, there is an urgent need to develop new, highly efficient, non-toxic antifungal drugs as alternatives to conventional drugs. Plant-based essential oils (EOs) and their components (EOCs) are becoming increasingly popular due to their potent antimicrobial and antibiofilm activities (Shahina et al., 2022). Previous studies demonstrated that essential oils can be employed as natural preservatives, flavorings, antioxidants, antibacterial, and antifungal agents in various food products (Cebi et al., 2020).

Fleagrass (*Adenosma buchneroides Bonati*) was discovered during a survey conducted in Mengla County, Xishuangbanna, China, in the 1980s (Ma et al., 2019). Fleagrass, belonging to the genus *Adenosum*, is a well-recognized

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aromatic medicinal plant long favored by the Aini people in Southwest China as an insect repellent (Huang et al., 2023). Fleagrass has been shown to exhibit several pharmacological effects, including anti-rheumatic, anti-inflammatory, blood circulation-promoting, and analgesic properties (Gou et al., 2018). Furthermore, fleagrass exhibited strong mosquito-repellent activity and demonstrated positive insecticidal effects against *Callosobruchus maculatus* (Huang et al., 2023). The main constituents of fleagrass are γ-terpinene (34.86% v/v), carvacrol (22.2% v/v), and p-cymene (12.1% v/v) (Ma et al., 2019). According to the theory of "aromatic substances repel microorganisms" in Chinese medicine, the volatile oil of fleagrass should have the function of resisting various pathogens. Therefore, we hypothesized that fleagrass essential oil might exhibit antifungal properties.

Carvacrol is one of the components of fleagrass, which has been proven to have pharmacological effects such as antioxidant, anti-inflammatory, anti-pyretic, and anti-parasitic properties. Studies have shown that carvacrol and its essential oils exhibit antimicrobial activity against yeast/fungi and Gram-positive and Gram-negative bacteria (Imran et al., 2022; Nostro & Papalia, 2012; Wijesundara et al., 2021). Therefore, the aim of the present study was to investigate the antifungal activity of fleagrass and its specific mechanism of action, as well as to compare this activity with that of carvacrol.

Materials and Methods

Compounds, Strains and Culture Conditions

Fleagrass (Fle) was obtained from the Kunming Institute of Botany, Chinese Academy of Sciences, China. Carvacrol (Car) was purchased from Sichuan Vicky Biotechnology Co., Ltd, with batch number 22021006. Fluconazole (FLC) was purchased from Nanchang Hongyi Pharmaceutical Co., Ltd, with batch number 210125, and the FLC-resistant *C. albicans* strain SC5314-FR was obtained from our laboratory at the College of Chinese Materia Medica, Yunnan University of Chinese Medicine, China. The other *C. albicans* strains were kindly donated by Prof. Yuye Li from the First Affiliated Hospital of Kunming Medical University, China. The strains were stored in 30% glycerol at – 80 °C. Prior to the experiment, the strains were thawed and cultured in yeast extract-peptone-glucose-agar (YPDA) medium for at least 24 h.

Essential Oil Extraction

The air-dried ground Fle and Car materials were extracted using hydrodistillation for 6 h using the standard protocol for obtaining essential oils described in the Chinese pharmacopoeia (Chinese Pharmacopoeia Commission, 2020). The essential oil was then dried using anhydrous sodium sulphate, and stored at 4 °C until analysis. The essential oil was dissolved in hexane at a concentration of 20 µl/ml for gas chromatography (GC) analysis.

GC–Mass Spectrometry (MS) Identification

The GC/MS analyses were conducted using an Agilent 7890A gas chromatograph coupled with an Agilent model 5975C mass spectrometer (Agilent). The mass spectrometer operated under the following conditions: ionization voltage, 70 eV (EI); ion source temperature, 230 °C; quadrupole temperature, 150 °C; mass scan range, 30–500 amu.

GC column: HP-5 capillary column, 5% Phenyl Methyl Siloxan, 50 m length, 0.32 inner diameter, with a 0.52 µm film thickness (Agilent); carrier gas, helium (99.999%); flow rate, 1.5 ml/min; injector temperature, 250 °C; oven temperature program, initial temperature of 60 °C raised to 240 °C (hold 5 min) at 3 °C/min. 1 µl of essential oil solution was injected into GC for which the split injection mode (split ratio, 50:1).

Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) Assay

The susceptibility of different *C. albicans* strains to Fle was determined using the microdilution method (Manohar et al., 2001). Susceptibility was expressed as the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). Specifically, aliquots of 200 µl of *C. albicans* cell suspensions (1×10^5 cells/ml) with different Fle concentrations (0.03125%, 0.0625%, 0.0125%, 0.25%, 0.5%, and 1% v/v) were transferred into a series of 96-well plates. After a 24 h incubation, the MIC of Fle against *C. albicans* strain was examined. The MIC was defined as the lowest concentration required to arrest the growth of the fungi at the end of 24 h of incubation. Moreover, MFC was determined by passaging 100 µl aliquots of the medium drawn from 96-well culture plates with no visible growth to the naked eye after 24 h of incubation into Petri dishes. The growth of yeast colonies on Petri dishes was then scored. The lowest concentration of the antifungal agent at which negative growth or less than 3 colonies were recorded was considered the MFC. All experiments were repeated three times.

Antifungal Kinetics Assay

Candida albicans SC5314-FR was resuspended in centrifuge tube containing YPD medium to achieve a final concentration of 1×10^5 CFU/ml. Divide the cell suspension into seven groups, each with a total volume of 10 ml, and place them in centrifuge tubes accordingly. Different

concentrations of essential oils were added to six groups (treatment groups) based on the concentration indicated for the respective groups: Fle (0.0625%, 0.125%, and 0.25% v/v), Car (0.0175%, 0.035%, and 0.07% v/v). The remaining one served as the control group and contained fungi without any treatment. The samples were incubated at 37 °C in a constant temperature shaker, and the samples (200 µl) were collected from each group after 0 h, 4 h, 8 h, 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h. The collected samples were placed in 96-well culture plates. Each group had three replicate wells. The absorbance values were determined at 625 nm on a microplate reader, and the experiment was repeated three times. The concentration of Car used in the experiment was consistent with the Car content in fleagrass.

The Effect of Essential Oils on Mycelium Formation of *C. albicans*

The effect of different essential oils on the morphological transformation of *C. albicans* SC5314-FR was investigated using Spider medium and YPD + 10 fetal bovine serum (FBS) medium. Fle (0.125% v/v) and Car (0.035% v/v) were used as the concentrations for the essential oils, while the control group contained only fungi without any treatment. First, 3 ml of medium was added to the centrifuge tube, and then the fungal solution was added to achieve a fungal concentration of 1×10^5 CFU/ml in the medium. Finally, the essential oils were added to achieve concentrations of 0.125% v/v or 0.035% v/v of the essential oils. After mixing, the solution was dispensed into 24-well plates, with 1 ml per well, and each group had 3 replicate wells. The plate was incubated at 37 °C, and the morphological transformation of *C. albicans* was observed and recorded after 2 h, 4 h and 8 h of incubation. The experiment was repeated three times.

The Effect of Essential Oils on *C. albicans* Biofilm Formation

The XTT method was used to determine the Fle-mediated inhibition of *C. albicans* SC5314-FR biofilm (Pierce et al., 2008). *Candida albicans* was cultured overnight and then washed three times with phosphate-buffered saline (PBS). The *C. albicans* culture was resuspended in RPMI-1640 medium supplemented with 10% FBS, and the final concentration was adjusted to 1×10^6 CFU/ml. The prepared fungal suspension was added to 96-well plates with a volume of 100 µl per well, and each group had 3 wells. After incubation at 37 °C for 90 min, the top layer of the medium was discarded and the precipitate was washed with PBS three times. Thereafter, 100 µl of different concentrations of treatment-containing medium were added. The plates were further incubated at 37 °C for 24 h, after which the top layer of the medium was discarded, and the precipitate was washed three times with 200 µl PBS. Subsequently, 100 µl of 0.5 g/l XTT solution was added to each well, and the optical density (OD) value was measured at 490 nm after incubation at 37 °C for 2 h in the dark. The experimental group consisted of different concentrations of Fle (1%, 0.5%, 0.25%, 0.125%, 0.0625%, and 0.03125% v/v) and Car (0.28%, 0.14%, 0.007%, 0.035%, 0.0175%, and 0.0085% v/v), while the control group contained only fungi without any treatment. The experiment was repeated three times.

Transmission Electron Microscopy of *C. albicans* Treated with Essential Oils

500 µl of *C. albicans* SC5314-FR (1×10^6 CFU/ml) was inoculated in the medium containing Fle (0.125% v/v), and the control group contained only fungi without any treatment. After incubation at 37 °C for 48 h, *C. albicans* cells were collected into 1.5 ml EP tubes containing fixing solution (3% glutaraldehyde:0.1 mol/l PBS = 1:5) using a cell scrape plate. The cells were then thoroughly mixed and left to stand at 4 °C for 5 min. Thereafter, the cells were centrifuged at 16,099×*g* for 10 min, and the supernatant was discarded. The pellet was fixed with 3% glutaraldehyde for 24 h, then with 1% osmium tetroxide for another 2 h. Dehydration was conducted using a graded acetone series. The tissue was then embedded, and the semithin sections were stained with methylene blue, while ultrathin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate. Finally, the sections were examined using a JEM-1400-FLASH transmission electron microscope.

β-1,3-Glucan Assay of the *C. albicans* Cell Wall

Four 50 ml centrifuge tubes were each filled with 40 ml of YPD medium, and different concentrations of essential oils (Fle: 0.03125% v/v and Car: 0.00875% v/v), FLC (0.03125% v/v) and 1×10^5 CFU/ml fungal suspension were added to the tubes. The control group contained only fungi without any treatment. The test tube was incubated at 37 °C on a constant temperature shaker at 150 rpm for 16–24 h. After incubation, the tubes were centrifuged at 2012×*g* for 5 min, and the supernatant was discarded, followed by washing the pellets 2–3 times with PBS. The pellets were then diluted with 5 ml of PBS. A fungal suspension with a final concentration of 1×10^7 CFU/ml was prepared, and the volume used for each group was 3 ml. Each group was treated with aniline blue staining solution at a final concentration of 0.1%. The groups were then incubated in a water bath at 80 °C for 30 min in the dark and cooled to room temperature before mixing. Thereafter, 200 µl of the solution from each group was added to a 96-well plate, with 3 wells per group. The fluorescence intensity of the solution was measured at an excitation wavelength of 398 nm and an emission

wavelength of 508 nm. The experiment was repeated three times.

Chitin Assay of the *C. albicans* Cell Wall

Fungal cells were prepared in tubes, and the pellets were obtained as described in the section 'β-1,3-glucan assay of the *C. albicans* cell wall.' The pellets were then diluted using an appropriate amount of PBS. A fungal suspension with a final concentration of 1×10^7 CFU/ml was prepared, and 3 ml of the suspension was used for each group. Thereafter, 1 ml of the fungal solution from each group was added into 5% CFW staining solution and mixed thoroughly. After incubation for 5 min at room temperature in the dark, the mixture was washed 3 times with PBS and resuspended in 1 ml of PBS. Subsequently, 200 µl of the solution of each group was added into three wells of a 96-well plate, and the fluorescence intensity was measured using an excitation wavelength of 355 nm and emission wavelength of 440 nm. The experimental groups consisted of FLC (0.03125% v/v), Fle (0.03125% v/v) and Car (0.00875% v/v), and the control group only contained fungi without any treatment. The experiment was repeated three times.

Quantitative Real-Time PCR Assays

Fungal cells were prepared in tubes as described in the section 'β-1,3-glucan assay of the *C. albicans* cell wall.' The collected fungal precipitate was ground into powder using liquid nitrogen and used for the RNA extraction, which was conducted using the Trizol method. The concentration and purity of the extracted RNA were determined using the Merinton SMA4000. The RNA was then reverse transcribed into cDNA following the instructions provided with the Promega A2081 Reverse Transcription Kit. The primer sequences are shown in Table 1. The primers were diluted to 10 µmol, and the samples of each group were loaded into 3 replicate wells of the 96-well plate. Water was used as a negative control. PCR amplification was performed using a three-step method. After amplification, the relative expression of each gene was quantified using the delta-delta Ct ($2^{-\Delta\Delta ct}$) method. The experimental groups were FLC (0.03125% v/v), Fle (0.03125% v/v) and Car (0.00875% v/v), and the control group contained only fungi without any treatment. The experiment was repeated three times.

Statistical Analysis

The experimental data were evaluated by calculating the mean ± standard deviation (SD) from at least three independent experiments. Analysis of variance (ANOVA) was used to assess the differences between the groups. Graphpad Prism8 was used for the statistical analysis. The results were considered statistically significant when the *P*-values were less than 0.05(*), 0.01(**), 0.001(***), or 0.0001(****), which served as the reference standard.

Table 1 Primers used in this study

Oligo name	Sequence (5′ to 3′)	Product length (bp)
RAS1-F	GTGGTGTTGGTAAATCCGCTT	178
RAS1-R	TCATGGCCAGATATTCTTCTTGTC	
CYR1-F	ACTTGGTGACTGCAGACTGG	110
CYR1-R	ACCCATACGAACCGACAACC	
EFG1-F	AATGTGGCCCAAATGACACG	131
EFG1-R	AGTTTCCAGGACGCCATCAA	
ECE1-F	GCCACTGGTGTTCAACAATCC	123
ECE1-R	AGTTTCCAGGACGCCATCAA	
ALS3-F	TGTTCCTGCCGGTTATCGTC	124
ALS3-R	GAAAGGTGCACGTTGCCAAT	
HWP1-F	CCGGAATCTAGTGCTGTCGT	185
HWP1-R	GCAGCACCGAAAGTCAATCTC	
ACT1-F	ACGGTGAAGAAGTTGCTGCT	180
ACT1-R	TGGATTGGGCTTCATCACCA	

Results

Chemical Analysis of the Essential Oils

The fleagrass essential oil obtained through hydrodistillation was analyzed using GC–MS. The GC–MS data were interpreted using the computer software AMDIS (Automated Mass Spectral Deconvolution and Identification System), developed by the National Institute of Standards and Technology (NIST). The chemical identities were further confirmed by comparing the MS spectra with those listed in the NIST20 mass-spectral libraries and comparing the retention indexes (RIs) with those reported in the literature (Adams, 2017). A total of 31 volatiles were identified, including five major constituents: carvacrol (27.94% v/v), γ-terpinen (27.78% v/v), p-cymene (12.41% v/v), carvacrol methyl ether (10.88% v/v), and β-bisabolene (5.26% v/v) (Table 2).

MIC and MFC Assays

The antifungal effect of fleagrass against different strains of *C. albicans* was studied, and the results are shown in Table 3. The results showed that fleagrass essential oil strongly inhibited SC5314-FR, ATCC-14053, ATCC-10231, and clinical FLC-resistant strains (CA23, CA318, CA550, and CA800). Additionally, its MIC and MFC activities were observed at concentrations below 0.5% v/v.

Table 2 Chemical components of fleagrass essential oil

No.	Compound	RI (Cal)	AI (Lit)	Percentage (%)
1	(3E)-Hexen-1-ol	852	844	0.07
2	α-Thujene	929	924	1.15
3	α-Pinene	937	932	0.28
4	β-Pinene	982	974	0.08
5	β-Myrcene	991	988	0.09
6	(Z)-3-Hexenyl acetate	1006	1004	0.05
7	α-Phellandrene	1009	1002	0.06
8	3-Carene	1015	1008	1.61
9	α-Terpinene	1020	1014	1.89
10	p-Cymene	1028	1020	12.41
11	Limonene	1032	1024	1.91
12	β-Ocimene	1048	1032	0.08
13	γ-Terpinene	1063	1054	27.78
14	Terpinolene	1093	1086	0.14
15	Linalool	1101	1095	0.06
16	Borneol	1173	1165	0.46
17	Terpinen-4-ol	1184	1174	0.18
18	α-Terpineol	1196	1186	0.24
19	Carvacrol methyl ether	1248	1241	10.88
20	Trans-ascaridol glycol	1276	1266	0.15
21	Thymol	1287	1289	0.12
22	carvacrol	1305	1298	27.94
23	Cis-3-hexenyl cis-3-hexenoate	1387	1383	0.08
24	Trans-β-caryophyllene	1433	1417	0.19
25	Cis-β-Farnesene	1460	1440	0.03
26	Humulene	1467	1452	0.90
27	β-Bisabolene	1516	1505	5.26
28	β-Sesquiphellandrene	1532	1521	0.14
29	Caryophyllene oxide	1599	1582	0.16
30	Humulene epoxide I	1615	1604	0.09
31	Humulene epoxide II	1626	1606	0.72
Total				95.17

All compounds were identified by matching their mass spectra with the NIST20 library and retention indices on the HP-5 column

Table 3 The antifungal activity of fleagrass against *Candida albicans*

Yeast strain	MIC % (v/v)	MFC % (v/v)
SC5314-FR	0.125	0.25
ATCC-10231	0.25	0.5
ATCC-14053	0.125	0.25
CA23	0.25	0.5
CA318	0.5	0.5
CA550	0.25	0.5
CA800	0.5	0.5

Results were from three independent experiments performed in triplicate (n = 3). MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration

Time-Kill Curve

The time-kill curve confirmed the significant inhibitory effect of fleagrass and carvacrol. Low concentrations of fleagrass consistently yielded better results than those of carvacrol from 4 to 36 h. However, both fleagrass and carvacrol exhibited significant inhibitory effects on the growth of *C. albicans* at medium and high concentrations (Fig. 1).

Effect of Essential Oils on Mycelium Formation of *C. albicans*

The mycelial formation of *C. albicans* SC5314-FR was observed by an inverted microscope, and the results are shown in Fig. 2. In the control group, long and interlocking hyphae were observed at 2 h, while dense, complex and intertwined hyphae were observed at 8 h in the mycelium-inducing liquid medium containing YPD + 10% FBS and Spider medium. However, the formation of hyphae in drug-resistant *C. albicans* was inhibited in liquid media containing fleagrass and carvacrol compared to the control.

Effect of Essential Oils on *C. albicans* Biofilm Formation

The inhibitory effect of fleagrass on *C. albicans* biofilm is shown in Fig. 3. Fleagrass and carvacrol strongly inhibited *C. albicans* biofilm formation, and the inhibition intensity was correlated with the concentration of essential oil. The inhibition increased with increasing concentration of essential oil. Fleagrass concentration of 0.03125–0.0625% v/v (0.0085–0.0175% v/v) inhibited biofilm formation to a greater extent than carvacrol, indicating that fleagrass exhibited better inhibitory activity than carvacrol under the same concentration. Furthermore, fleagrass also demonstrated superior inhibition of biofilm formation compared to carvacrol at higher concentrations.

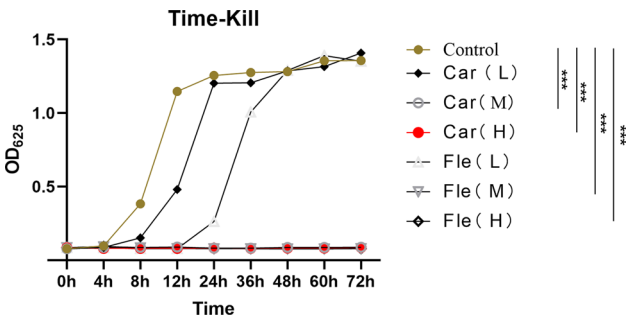

Fig. 1 Time-kill curve assay of the essential oils against *Candida albicans.* Car (L), carvacrol-low (0.0175% v/v); Car (M), carvacrol-medium (0.035% v/v); Car (H), carvacrol-high (0.07% v/v); Fle (L), fleagrass-low (0.0625% v/v); Fle (M), fleagrass-medium (0.125% v/v); Fle (H), fleagrass-high (0.25% v/v). ***$P < 0.001$ (n = 3)

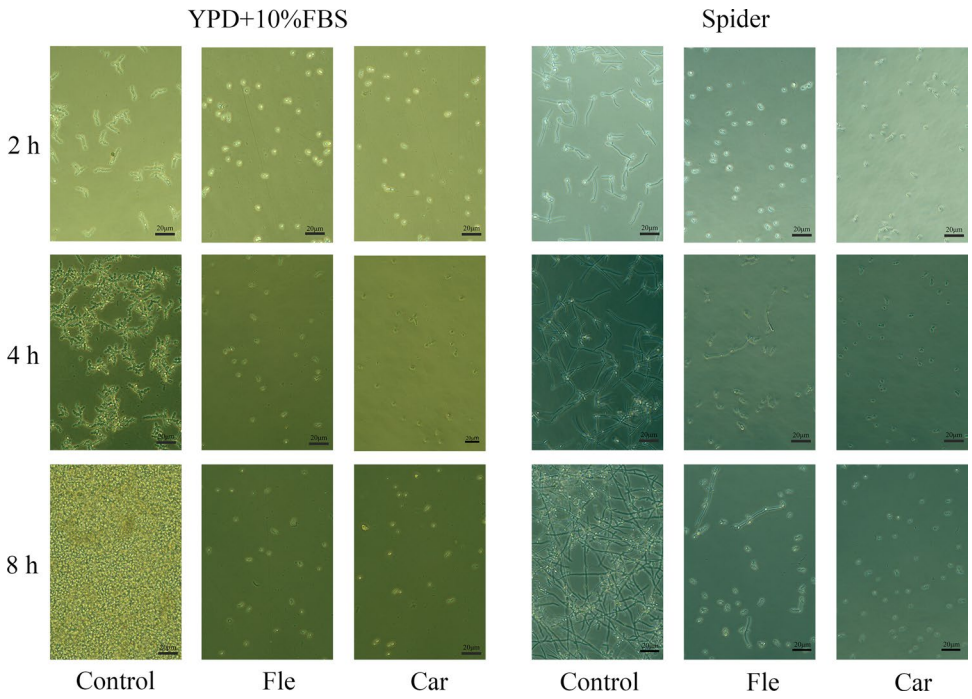

Fig. 2 Effect of essential oil on the hyphae formation of *Candida albicans* on yeast extract peptone dextrose (YPD) + 10% fetal bovine serum (FBS) medium and Spider medium (40×)

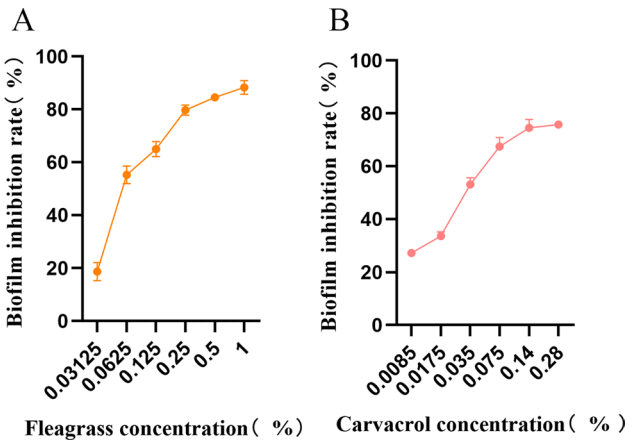

Fig. 3 Biofilm inhibition by different concentrations of essential oils. **A** Fleagrass (1%, 0.5%, 0.25%, 0.125%, 0.0625%, and 0.03125% v/v). **B** Carvacrol (0.28%, 0.14%, 0.007%, 0.035%, 0.0175%, and 0.0085% v/v)

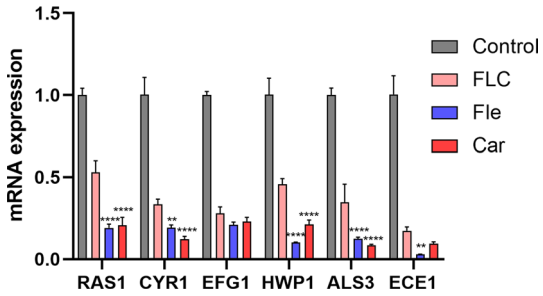

Fig. 4 The messenger RNA (mRNA) transcript levels of mycelium-associated genes (n = 3) under different treatments. $^{**}P < 0.01$, $^{****}P < 0.0001$ vs. FLC group. FLC, fluconazole (0.03125% v/v); Car, carvacrol (0.00875% v/v); Fle, fleagrass (0.03125% v/v)

Effect of Essential Oils on *C. albicans* Mycelium Associated Genes

qRT-PCR was utilized to determine the expression of key genes involved in the mycelium-associated pathway (Fig. 4). Compared to the control group, the fleagrass, carvacrol and FLC down-regulated the expression of mycelium-associated genes RAS1, CYR1, EFG1, HWP1, ALS3, and ECE1. The expression of RAS1, CYR1, HWP1, ALS3, and ECE1 showed a greater decline in the fleagrass and carvacrol groups compared to the FLC group ($^{**}P < 0.01$, $^{****}P < 0.0001$). The results also indicated that fleagrass and carvacrol exhibited the same mechanism of action against *C. albicans* by affecting the mycelium formation pathway.

The Effect of Essential Oils on C. albicans as Observed by Transmission *Electron* Microscopy

To investigate the morphological changes of *C. albicans* after exposure to essential oil, we used a transmission electron microscope to capture images of the fungal cells. The results are shown in Fig. 5. After treatment with essential oil for 48 h, the morphology of the fungi changed. The treated fungal cells had shrunken cell membranes, thinner and fractured cell walls, and unevenly distributed cytoplasmic

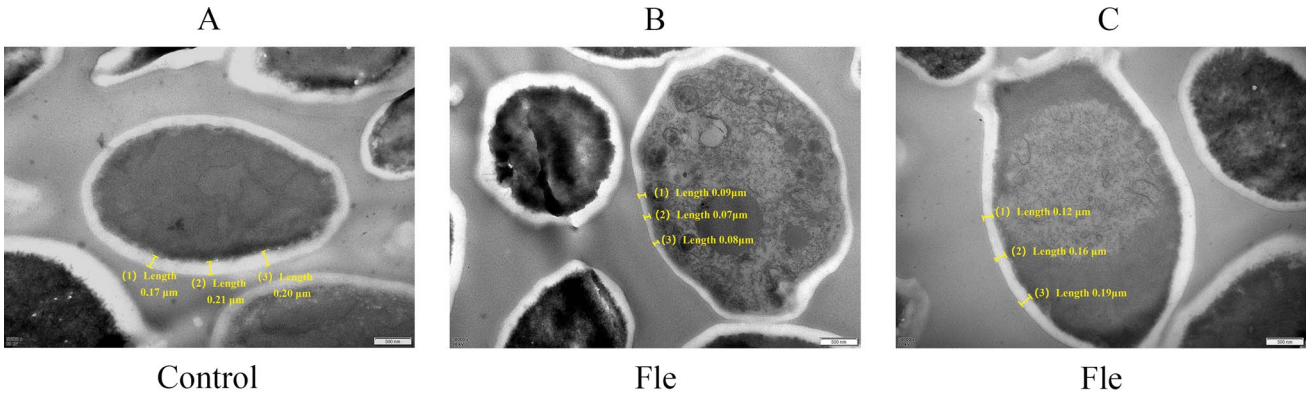

Fig. 5 Morphological changes of the fungi under transmission electron microscopy (6000×, n = 3). (A) Control group. (B, C) Fleagrass group (Fle, 0.125% v/v)

contents. However, the cell shape of the control group remained normal, exhibiting an oval shape, a smooth cell wall, and a uniform distribution of intracellular materials.

Effect of Essential Oils on β-1,3-Glucan of *C. albicans* Cell Wall

We analyzed the changes in fluorescence intensity among different groups by staining *C. albicans* with the aniline blue stain. As shown in Fig. 6, the fluorescence intensity of the strains in the FLC group exhibited noticeable changes compared to the control group ($^{*}P < 0.05$). However, fleagrass and carvacrol groups ($^{****}P < 0.0001$) exhibited higher efficacy than FLC, indicating that fleagrass and carvacrol could disrupt the cell wall structure, leading to glucan exposure. Furthermore, the effect of fleagrass was superior to that of carvacrol, with a notable difference ($^{\#}P < 0.05$).

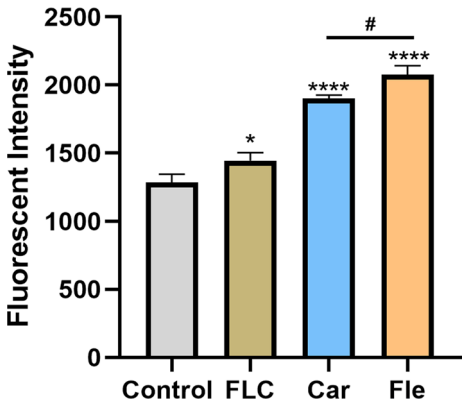

Fig. 6 Effect of essential oils and drugs on the β-1,3-glucan of *Candida albicans* cell wall (n = 3). $^{*}P < 0.05$, $^{****}P < 0.0001$, $^{\#}P < 0.05$. FLC, fluconazole (0.03125% v/v); Car, carvacrol (0.00875% v/v); Fle, fleagrass (0.03125% v/v)

Effect of Essential Oils on the Chitin of *C. albicans* Cell Wall

We analyzed the changes in fluorescence intensity of *C. albicans* stained with CFW fluorescent dye in different groups under the inverted fluorescence microscope. As shown in Fig. 7a, the strains in the fleagrass and carvacrol groups exhibited noticeable blue fluorescence; however, the fluorescence produced by the treated strains in the control and FLC groups was relatively weak. The fluorescence intensity of each group was measured, and the results are shown in Fig. 7b. The increase in the fluorescence intensity of the fleagrass was highly significant ($^{****}P < 0.0001$), while that of carvacrol was significant ($^{**}P < 0.01$) compared to the control group. However, the FLC group showed minimal changes in fluorescence intensity. The results demonstrated that both fleagrass and carvacrol could increase the level of chitin in *C. albicans* cell wall, with fleagrass having a more pronounced effect ($^{\#\#\#}P < 0.001$).

Discussion

Candida albicans is a prevalent constituent of the human microbiome inhabiting the oral, upper respiratory, gastrointestinal, and vaginal tracts (Berman & Sudbery, 2002), recognized for its propensity to incite diverse infections through the manifestation of distinct virulent attributes across varying morphological states (Thakre et al., 2018). There is an urgent need to explore new-generation antifungal drugs due to the low efficacy, high toxicity, and drug resistance of the currently available antifungals (Jia et al., 2018).

Fleagrass, a safe natural product, is extensively utilized in the daily life of the Aini people in southwest China. Local residents use fleagrass as decorations or perfume by putting it in ear-holes or on caps. They also make a potion for relief

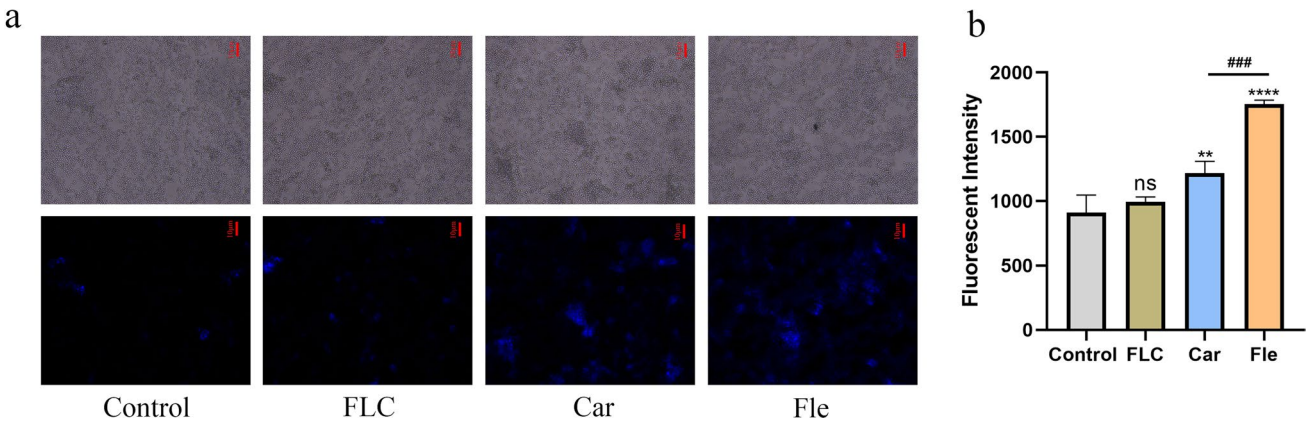

Fig. 7 **a** Inverted fluorescence microscopy showing chitin content of *Candida albicans* cell wall under different treatments (20×) (n = 3). The upper panel is the bright field. **b** Effect of essential oils and drugs on *Candida albicans* chitin (n = 3). $^{**}P < 0.01$, $^{****}P < 0.0001$, $^{###}P < 0.001$. FLC, fluconazole (0.03125% v/v); Car, carvacrol (0.00875% v/v); Fle, fleagrass (0.03125% v/v)

from headaches, influenza, or diarrhea by drinking it, and apply fresh crushed fleagrass to the skin to treat insect bites, as the sap helps in relieving itching and swelling (Gou et al., 2018). The present study found that fleagrass essential oil has superior fungicidal effect on *C. albicans*, which conform to the application scope of aromatic medicinal plant according to Chinese medicine theory.

In terms of composition, the present study identified that fleagrass essential oil contains mainly γ-terpinen (27.78% v/v), carvacrol (27.94% v/v), p-cymene (12.41% v/v), carvacrol methyl ether (10.88% v/v), and β-bisabolene (5.26% v/v). The results are consistent with previous studies (Ma et al., 2019). Interestingly, essential oils with both carvacrol and γ-terpinen as main components have been demonstrated superior antagonism against *C. albicans*, including trachyspermum ammi (Sharifzadeh et al., 2015), thymus pectinatus fisch (Vardar-Unlü et al., 2003), and satureja hortensis L. (Sharifzadeh et al., 2016). Therefore, it is expected that fleagrass essential oil exerts an antimicrobial effect on *C. albicans*. In this study, we demonstrated for the first time that fleagrass essential oil had strong inhibitory effects on different strains of *C. albicans* (SC5314-FR, ATCC-14053, ATCC-10231) and clinical FLC-resistant strains (CA23, CA318, CA550, and CA800), and 0.5 MIC fleagrass essential oil almost completely prevented the growth of *C. albicans*. This provides a theoretical basis and experimental foundation for future antimicrobial applications of fleagrass essential oil. Notably, the antifungal effect of carvacrol on *C. albicans* is well established (Acuna et al., 2023; Miranda-Cadena et al., 2021; Niu et al., 2020). Therefore, the present study further compared the antifungal effects of fleagrass essential oil and carvacrol on *C. albicans* in terms of mycelium formation, biofilm formation and molecular mechanisms to reveal the superiority of fleagrass essential oil.

The primary virulence factors of *C. albicans* include adhesion to host cells and tissues, formation of mycelia, and secretion of hydrolases (Wang et al., 2020). Mycelium formation stands as a critical virulence factor, facilitating the onset of life-threatening invasive infections and biofilm development (Thakre et al., 2018). The experimental results showed that low concentrations of fleagrass essential oil and carvacrol strongly inhibited the mycelium formation of *C. albicans* from 2 h onwards. Additionally, biofilm formation of *C. albicans* was disrupted upon exposure to different concentrations of fleagrass essential oils at 24 h after the treatment. The suppression of mycelium growth by lower concentrations of fleagrass essential oil likely facilitated the inhibition of biofilm formation, as morphogenesis is essential for initial adhesion (Wang et al., 2020). Therefore, fleagrass essential oil disrupts the adhesion of *C. albicans* and prevents the formation of an intact biofilm, which is one of the reasons for the antimicrobial activity of fleagrass essential oil. Notably, fleagrass essential oil exhibited a superior antifungal effect on *C. albicans* compared to carvacrol.

Mechanically, the Ras1/cAMP/PKA signaling pathway regulates the mycelial morphological transitions of *C. albicans* (Scorzoni et al., 2017). EFG1 plays a role in modulating the Ras1/cAMP/PKA signaling pathway, with its upregulation swiftly triggering the expression of genes specific to mycelium formation, including HWP1, ECE1, and ALS3 (Liu & Myers, 2017; Prasad et al., 2015; Redhu et al., 2016). To clarify the signaling pathway involved in the inhibitory effect of fleagrass essential oil on *C. albicans* mycelium, we examined the expression of mycelium-related genes using qRT-PCR. The results revealed that the expression levels of RAS1, CYR1, EFG1, HWP1, ALS3, and ECE1 were significantly down-regulated after the treatment of fleagrass essential oil. It is suggested that the fleagrass essential oil-mediated mycelium formation inhibition of *C.*

albicans is related to the Ras1/cAMP/PKA signaling pathway. Fleagrass essential oil impedes mycelium formation by downregulating the expression of genes associated with the mycelial pathway, thereby obstructing biofilm development, and curbing the pathogenicity of *C. albicans*. This further demonstrates the high potential of fleagrass essential oil as a clinical antifungal agent.

The cell wall and cell membrane are pivotal in maintaining cell viability, morphology, and virulence of *C. albicans* through various signaling pathways. Hence, even minor alterations in the structural integrity of the cell wall or membrane can result in growth inhibition of fungi (Shahina et al., 2022). In the present study, fleagrass essential oil-induced ultrastructural abnormalities in *C. albicans* were observed using electron microscopy. The TEM micrographs revealed that fleagrass essential oil disrupted the cell walls and membranes of *C. albicans*, resulting in the leakage of cytoplasmic contents.

In terms of composition, although chitin constituting less than 3% of the cell wall, it plays a very important role in the morphology and viability of *C. albicans*. It has been shown that cell wall damage often leads to an increase in chitin content and the presence of abundant mannoproteins on the outer surface of the cell wall, which covers the dextran and chitin layers, hinders the recognition by the body's immune system (Lin et al., 2016; Mora-Montes et al., 2011). Therefore, we determined the contents of β-1,3-glucan and chitin using aniline blue and CFW stains, respectively. Our findings reveal that fleagrass essential oil effectively compromises the cell wall of *C. albicans*, impairing its integrity and consequently precipitating cellular demise. It is presumed that disruption of the cell wall further impeded the mycelium and biofilm formation of *C. albicans*, thereby reducing the pathogenicity of *C. albicans*.

Notably, the present study confirmed that fleagrass essentiall oil exhibited more superior antifungal effects on *C. albicans* than carvacrol in terms of mycelium formation, biofilm formation and molecular mechanisms. As a natural product, fleagrass essentiall oil boasts a more agreeable fragrance, greater accessibility, broader applicability, and a less complex extraction method compared to carvacrol. Therefore, fleagrass essentiall oil may be a more suitable anti-*C. albicans* drug. Fleagrass essentiall oil also holds potential as a natural antimicrobial agent within the food industry for managing *C. albicans* contamination. Therefore, the essential oil or bioactive extracts of fleagrass can be fully utilized for product development. This could enhance the application of fleagrass in medicine and hopefully provide new therapeutic options for controlling fungal infections.

In conclusion, the essential oil derived from fleagrass demonstrates potential as an antifungal agent against *C. albicans*. Fleagrass essential oil inhibited the mycelium and biofilm formation of *C. albicans* by disrupting the cell wall integrity of the *C. albicans*, ultimately resulting in the death of the *C. albicans*. Therefore, fleagrass has a very important research value in future antifungal agent studies.

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Data Availability The data presented in this study are available on request from the corresponding author.

Declarations

Conflict of interest The authors declare no conflict of interest.

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