Journal of Ethnopharmacology 261 (2020) 113193

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Boesenbergia rotunda extract inhibits *Candida albicans* biofilm formation by pinostrobin and pinocembrin

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ARTICLE INFO

Keywords:
Boesenbergia rotunda
Candida albicans
Biofilm formation
Antifungal
Flavonoids
Gene expression

ABSTRACT

Ethnopharmacological relevance: *Boesenbergia rotunda* (L.) Mansf. (Zingiberaceae) is an indigenous plant of Southeast Asia. Based on ethnopharmacological use, the rhizome is recommended in the treatment of stomachache, leukoplakia, abscesses, and leukorrhea in Thailand primary health care system. *Candida albicans* often causes leukorrhea, and infection of many mucosal sites. Its infection leads to serious illness.

Aim of the study: This study aimed to investigate the effects of the ethanolic extract of the *B. rotunda* rhizome on *C. albicans* ATCC10231 in the stages of planktonic and biofilm formation and to explore the underlying mechanisms.

Materials and methods: The chemical composition of the extract was determined using ultra-performance liquid chromatography (UPLC). The planktonic growth of *C. albicans* was evaluated by the microdilution method, following EUCAST guidelines. For each stage of biofilm formation, the biofilm was assessed by the MTT assay. The biofilm structure was examined under a light microscope. The degree of cell surface hydrophobicity was measured. The mRNA levels of *ALS1*, *ALS3*, and *ACT1* were determined by RT-qPCR.

Results: The extract of *B. rotunda* consisted of 25% (w/w) pinostrobin and 12% (w/w) pinocembrin. All stages of *C. albicans* biofilm formation were significantly inhibited by the extract, whereas the planktonic growth did not change. Biofilm development greatly decreased due to the extract in a concentration-dependent manner, with an IC_{50} value of 17.7 μg/mL. Pinostrobin and pinocembrin demonstrated inhibitory effects during this stage. These results were in accordance with the microscopic evaluation. The filamentous form decreased with pinocembrin rather than pinostrobin. Moreover, the cell surface hydrophobicity was significantly decreased by 6.25 and 12.5 μg/mL of the extract and 100 μM of pinocembrin. The *ALS3* mRNA level was noticeably decreased by 12.5 μg/mL of the extract, 100 μM of pinostrobin, and 100 μM of pinocembrin. The *ACT1* mRNA level decreased significantly with pinocembrin. However, the *ALS1* mRNA level was not altered following all treatments.

Conclusion: The ethanolic extract of *B. rotunda* could inhibit biofilm formation of *C. albicans*, especially during the biofilm development stage, by means of reducing the cell surface hydrophobicity and suppressing the *ALS3* mRNA expression. Pinocembrin had a stronger effect on *ALS3* mRNA expression than pinostrobin. Only pinocembrin significantly decreased the *ACT1* mRNA level.

1. Introduction

Candida albicans, an opportunistic fungal pathogen, is the predominant species causing candidiasis, which results in morbidity and mortality in immunocompromised patients (Kaur et al., 2016; Melo et al., 2019). Its virulence factors include its ability for morphological transition and its capability of forming a biofilm (Mayer et al., 2013).

In the life-cycle of *C. albicans*, a transition between yeast, pseudohyphae, and true hyphae forms occurs. Filamentous forms, pseudohyphae and true hyphae, involve an invasion process. *C. albicans* grows as oval budding yeast cells in liquid medium and then adheres to surfaces such as medical devices and human tissues. Adhesion is the initial stage of biofilm formation. Then, cell proliferation and filamentation follow to form a complex network of polymorphic cells surrounded by an

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https://doi.org/10.1016/j.jep.2020.113193
Received 13 May 2020; Received in revised form 9 July 2020; Accepted 14 July 2020
Available online 27 July 2020

exopolymeric matrix (Gulati and Nobile, 2016; Wall et al., 2019). The biofilm plays an important role in structural protection, leading to resistance of the biofilm to many antifungal agents, which is remarkably more than planktonic cells (Chandra et al., 2001). Moreover, the properties of the biofilm, such as its matrix components, efflux pumps, and metabolic plasticity, are associated with its resistance (Gulati and Nobile, 2016; Mayer et al., 2013). Recently, the agglutinin-like sequence (*ALS*) family, which encodes cell surface glycoproteins, has been identified as a specific gene for *C. albicans* biofilm formation (Hoyer et al., 2008). The expression of the *ALS1* gene was preferentially increased during biofilm development compared to that under planktonic conditions (Chandra et al., 2001; O'Connor et al., 2005). The biofilm mass and ability of the *C. albicans als3/als3* mutant to adhere to human epithelial decreased significantly when compared to the wild type (Murciano et al., 2012; Zhao et al., 2006). Moreover, it was demonstrated that the ALS3 protein adhered to endothelial and epithelial cells by binding to cadherin on the membrane of host cells, thereby inducing endocytosis (Phan et al., 2007). Thus, these genes might be novel targets for *C. albicans* biofilm eradication.

Boesenbergia rotunda (L.) Mansf. (synonyms; *Boesenbergia pandurata* (Roxb.) Schltr. and *Kaempferia pandurata* Roxb.), belonging to the Zingiberaceae family, is an indigenous plant of Southeast Asia (Smitinand, 2014). Its rhizome has been widely used throughout the region, and was selected to establish a monograph in the Standard of ASEAN Herbal Medicine (Ali et al., 2010; ASEAN Countries, 1993; Eng-Chong et al., 2012). The primary health care system in Thailand recommended it for the treatment of stomachache, leukoplakia, abscesses, and leukorrhea (Bunyapraphatsara et al., 1992). The ethno-pharmacological properties have led to scientific investigations for several infections including those caused by *C. albicans* (Chahyadi et al., 2014; Jitvaropas et al., 2012), commonly occurred in vagina, mouth, and other mucosal sites (Mayer et al., 2013). The *B. rotunda* rhizome extract has been reported for inhibitory effects on *C. albicans* as well as clinical isolates from AIDS patients (Phongpaichit et al., 2005). The metabolic activity of *C. albicans* biofilm was almost completely inhibited by the oils from the rhizome (Taweechaisupapong et al., 2010). Moreover, the rhizome extract decreased the adhesion of *C. albicans* on acrylic strips in a concentration-dependent manner (Sroisiri and Boonyanit, 2010), and preferably affected the infection of *C. albicans* and *Streptococcus mutans*, frequently found in dental cavity and related to dental plaque biofilm (Falsetta et al., 2014). With continual traditional uses and scientific evidences, the *B. rotunda* extract was used and developed by a specialized Thai traditional medicine hospital as an active ingredient in a modernized mouth care products (Wiwanitkit, 2020).

Flavonoids are a major chemical constituent of the rhizome (Chahyadi et al., 2014). Flavanones, a type of flavonoids, are predominantly found in the *B. rotunda* rhizome extract, and the three most abundant are pinostrobin, pinocembrin, and alpinetin (Tan et al., 2015). Pinostrobin and pinocembrin have exhibited effective antifungal activities (Ramirez et al., 2013; Wangkangwan et al., 2009). In addition, it was shown that bacterial biofilm formation for *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* decreased due to pinostrobin (Christena et al., 2015), and bacterial biofilm formation for *Enterococcus faecalis* and *S. mutans* decreased due to pinocembrin (Ong et al., 2017; Veloz et al., 2019). Consequently, it was hypothesized that the *B. rotunda* rhizome extract and major components would influence biofilm formation of *C. albicans*, but the mechanism of biofilm eradication remained unclear. This study aimed to investigate the effects of the ethanolic extract of the *B. rotunda* rhizome on *C. albicans* in the stages of planktonic and biofilm formation and to explore the underlying mechanisms.

2. Materials and methods

2.1. Plant material and preparation of the extract

The rhizomes of *B. rotunda* were collected from DMSC herbal garden, Nonthaburi, Thailand (N 13.8525, E 100.5314). The plant material was authenticated and kept as voucher specimen no. DMSC5205 at the Department of Medical Sciences Herbarium. Ten kilograms of the *B. rotunda* rhizomes were dried and ground. The powder was macerated 3 times with 95% (v/v) ethanol for 72 h. The supernatants were pooled, filtered, and evaporated under reduced pressure. The extract of *B. rotunda* was then stored at -20 °C until use.

2.2. Chemical analysis of the B. rotunda extract

Ultra-performance liquid chromatography (UPLC) (Acquity™ UPLC; Waters Corporation, MA, USA) was conducted to determine the chemical composition of the *B. rotunda* extract. The chromatographic condition consisted of an Acquity™ BEH C18 column (100 × 2.1 mm, 1.7 μm), a gradient of (A) water and (B) methanol–acetonitrile (1:1) as the mobile phase, and a photodiode array detector. The flow rate and column temperature were set at 0.5 mL/min and 35 °C, respectively. The *B. rotunda* extract, pinostrobin (99% purity; Sigma Chemical, MO, USA), and pinocembrin (95% purity; Sigma Chemical, MO, USA) were freshly prepared in methanol. The analysis was determined at a wavelength of 290 nm.

2.3. Microdilution assay: effect of the B. rotunda extract on C. albicans planktonic growth

C. albicans ATCC 10231 (American Type Culture Collection, MD, USA) was cultured on Sabouraud dextrose agar (Merck, Darmstadt, Germany) at 37 °C for 72 h. A colony was inoculated into Sabouraud dextrose broth (BD, MD, USA) and cultured overnight at 37 °C and 120 rpm. The yeast cells were centrifuged at 4,000 rpm for 10 min, and the pellet was then suspended in RPMI 1640 medium (Invitrogen Corporation, NY, USA) supplemented with 165 mM MOPS buffer (HiMedia Laboratories, Mumbai, India) and 2% (w/v) glucose (Merck, Darmstadt, Germany). Following the EUCAST guideline (Rodriguez-Tudela et al., 2008), the microdilution method was employed to determine the susceptibility of *C. albicans* to planktonic growth. The *B. rotunda* extract was freshly dissolved in DMSO at a concentration of 40 mg/mL and then serially double-diluted to the desired concentrations. In this study, 1×10^6 CFU/mL of *C. albicans* was treated with 3.125–200 μg/mL of the *B. rotunda* extract by the addition of an equal volume of the respective extracts and *C. albicans* suspension into 96-well round bottom microplates. The cells treated with 0.5% (v/v) DMSO and amphotericin B (WHO International Laboratory for Biological Standards, London, England) were the vehicle control and positive control, respectively. Samples under each condition were incubated at 37 °C for 24 h. Yeast growth was determined by comparing the absorbance of initial and final incubation at 600 nm. The minimum inhibitory concentration (MIC) was considered the concentration that inhibited growth of the yeast to 10% of the control.

2.4. Anti-biofilm formation assay

Biofilm formation was determined as described previously (Yan et al., 2019), with minor modifications. All stages of *C. albicans* biofilm formation: adhesion, biofilm development, and mature biofilm were examined with *B. rotunda* extract at concentrations of 3.125–200 μg/mL. For the adhesion stage, *C. albicans* was seeded at a density of 5×10^6 CFU/mL on 96-well flat bottom microplates, with or without the extract. Plates were incubated, undisturbed, at 37 °C for 90 min to allow attachment of the yeast cells on the polystyrene surface. For the development stage, *C. albicans* was allowed to adhere, and non-adherent cells

were removed by washing thrice. The adhered cells were cultured in the presence or absence of the extract at 37 °C for 24 h. For the mature biofilm stage, *C. albicans* adhered to microplates and grew for 24 h until the biofilm formed. Following washing thrice, the medium was replaced with the extract, and the cells were then incubated at 37 °C for 24 h. Each stage of biofilm formation was assessed according to the metabolic activity determined using the MTT assay (Grela et al., 2018), which *C. albicans* was washed thrice and 0.2 mg/mL of the MTT solution was added. The cells were incubated at 37 °C for 2 h. MTT was reduced in viable cells to obtain purple formazan crystals that were further dissolved with DMSO. Absorbance was measured at 570 nm. The RPMI 1640 medium supplemented with 0.5% (v/v) DMSO was the treatment condition used as the vehicle control. Amphotericin B was used as the positive control. Pinostrobin and pinocembrin were examined in the biofilm development stage as described above. Both compounds were used at concentrations of 6.25–200 μM.

2.5. Biofilm analysis by optical microscopy

The biofilm of *C. albicans* was observed under an Eclipse TS100 inverted microscope (Nikon Instruments Inc., NY, USA). Photographs were taken at 200× magnification.

2.6. Cell surface hydrophobicity assay

Cell surface hydrophobicity of *C. albicans* was assessed using the microbial adhesion to hydrocarbons assay (Rosenberg, 1984). At a density of 5×10^6 CFU/mL, *C. albicans* was treated with 3.125, 6.25, and 12.5 μg/mL of the *B. rotunda* extract, 100 μM pinostrobin, or 100 μM pinocembrin. These cultures were incubated at 37 °C for 24 h in order to form biofilms. The biofilm was washed and collected in PBS. For the planktonic specimen, *C. albicans* was cultured without treatment and collected at the same time point. The sample was centrifuged at 4,000 rpm for 10 min, and the pellet was suspended with PBS. The 800 μL of the cell suspension was pipetted into a glass tube and overlaid with 200 μL of xylene (Daejung, Gyeonggi-do, Korea). The mixture was vortexed for 2 min and set aside for 10 min. The cell suspension, lower phase, was taken to measure absorbance at 600 nm. The percentage of cell surface hydrophobicity was calculated by comparison of the absorbance between before and after mixing with xylene. The PBS without *C. albicans* was used as the vehicle control.

2.7. Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

The Presto Mini RNA Yeast Kit (Geneaid Biotech, New Taipei, Taiwan) was used to isolate RNA from the *C. albicans* biofilm. The biofilm was washed and collected in PBS. The cell suspension was centrifuged at 8,000 rpm for 10 min. The cells were suspended in sorbitol buffer supplemented with 200 U lyticase (Sigma Chemical, MO, USA) to form a spheroplast. Total RNA was purified using a spin column, following the manufacturer's instruction. The DNA contaminants were digested with 10 U of DNase I solution at room temperature for 30 min. Then, 50 ng of total RNA was reverse-transcribed and amplified using the Omniscript® Reverse Transcription Kit and the QuantiNova SYBR® Green PCR kit, respectively (Qiagen, Hilden, Germany). Primers were designed using the NCBI primer designing tool to recognize *ALS1*, *ALS3*, and *ACT1* genes, as illustrated in Table 1. The cycling protocol of cDNA amplification was designed for 40 cycles, consisted of denaturing at 95 °C for 5 s and annealing and extending the temperature to 60 °C for 10 s. This was performed using qTOWER^{3}G (Analytik Jena AG, Thuringia, Germany). The relative quantification of gene expression was analyzed using the $2^{-\Delta Ct}$ method, where C_t is the PCR cycle number where the reaction curve intersects the threshold line (Livak and Schmittgen, 2001).

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 5. Each experiment was conducted in triplicate, and the data was expressed as the mean ± SEM. The statistical difference was analyzed by either the *t*-test or one-way analysis of variance with Dunnett's multiple comparison post-test. In all cases, a *p*-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Chemical analysis of the B. rotunda extract

The ethanolic extract of the *B. rotunda* rhizome was yellowish brown in color and had a characteristic odor. UPLC chromatograms of the extract and two main constituents were presented in Fig. 1. Pinostrobin and pinocembrin appeared at retention times of 5.8 and 3.9 min, respectively. The amounts of pinostrobin and pinocembrin in the extract

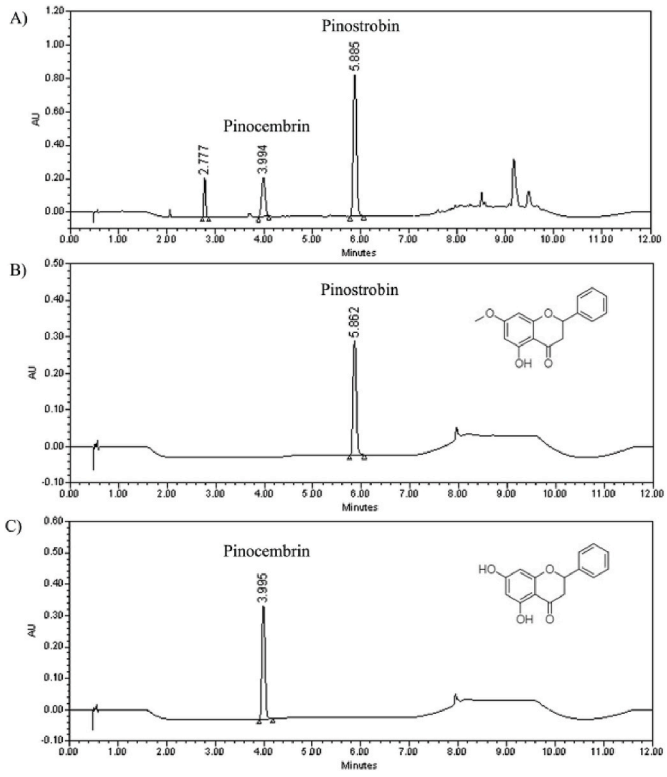

Fig. 1. Ultra-performance liquid chromatography (UPLC) chromatograms of the *B. rotunda* extract (A), pinostrobin (B), and pinocembrin (C).

Table 1
Primer sequences for RT-qPCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product size
ALS1	GTCAACAGCACAACCTCCGAT	TGACAATACCAGAAGAACTGGACA	187 bp
ALS3	AGGGACCAGGAACCCCAACT	TGTATCTCCCGGACTTGCACT	77 bp
ACT1	AGAGACTTGACCAACCATTTGTCC	CGAAATCCAAAGCAACGTAACACA	124 bp

were 25.2% and 11.6% (w/w), respectively.

3.2. Microdilution assay: effect of the B. rotunda extract on C. albicans planktonic growth

The planktonic growth of *C. albicans* was not altered when treated with the *B. rotunda* extract for 24 h (Fig. 2A). However, *C. albicans* was susceptible to amphotericin B, the positive control. *C. albicans* growth decreased significantly due to amphotericin B in a concentration-dependent manner, with an IC_{50} value of 0.3 μM and MIC of 1 μM.

3.3. Anti-biofilm formation assay

3.3.1. Effect of the B. rotunda extract on C. albicans biofilm formation

All stages of *C. albicans* biofilm formation were inhibited by the *B. rotunda* extract (Fig. 2B–D). Adhesion on the polystyrene surface decreased by approximately 20% with the extract at concentrations of 25–200 μg/mL. Biofilm development and the mature biofilm stage decreased significantly in a concentration-dependent manner. The inhibitory effect of the extract on biofilm development was shown with an IC_{50} value of 17.7 μg/mL. However, this result was less prominent in the mature biofilm. The maximum inhibitory effect reached 33.2% when it was treated with 200 μg/mL of the extract. For the positive control, *C. albicans* biofilm formation was dramatically inhibited by amphotericin B. The IC_{50} values for adhesion, biofilm development, and mature biofilm stages were less than 0.125, 0.4, and 1.4 μM, respectively.

The concentration response of the *B. rotunda* extract on biofilm development of *C. albicans* was also determined under light microscopy (Fig. 3). These observations were concordant with the results of the MTT assay. The number of *C. albicans* remaining on the surface in the presence of 200 μg/mL of the extract was similar to that observed in the presence of 1 μM amphotericin B.

3.3.2. Effect of pinostrobin and pinocembrin on the biofilm development of C. albicans

C. albicans during biofilm development stage was significantly inhibited by pinostrobin and pinocembrin in a concentration-dependent manner (Fig. 4). At a concentration of 200 μM, the biofilm decreased by 42% and 53% due to pinostrobin and pinocembrin, respectively. Structural determination under light microscopy showed that the decrease in the *C. albicans* biofilm correlated with the results of the MTT assay (Fig. 5). Moreover, the filamentous form of *C. albicans* decreased due to pinocembrin rather than pinostrobin.

3.4. Cell surface hydrophobicity assay

As seen in Fig. 6, the degree of hydrophobicity in the *C. albicans* biofilm was two-fold higher than that under the planktonic condition. In the biofilm stage, the hydrophobicity decreased significantly with the *B. rotunda* extract at concentrations of 6.25 μg/mL and 12.5 μg/mL. It also decreased with the 100 μM pinocembrin. However, the cell surface hydrophobicity of *C. albicans* was not altered by pinostrobin.

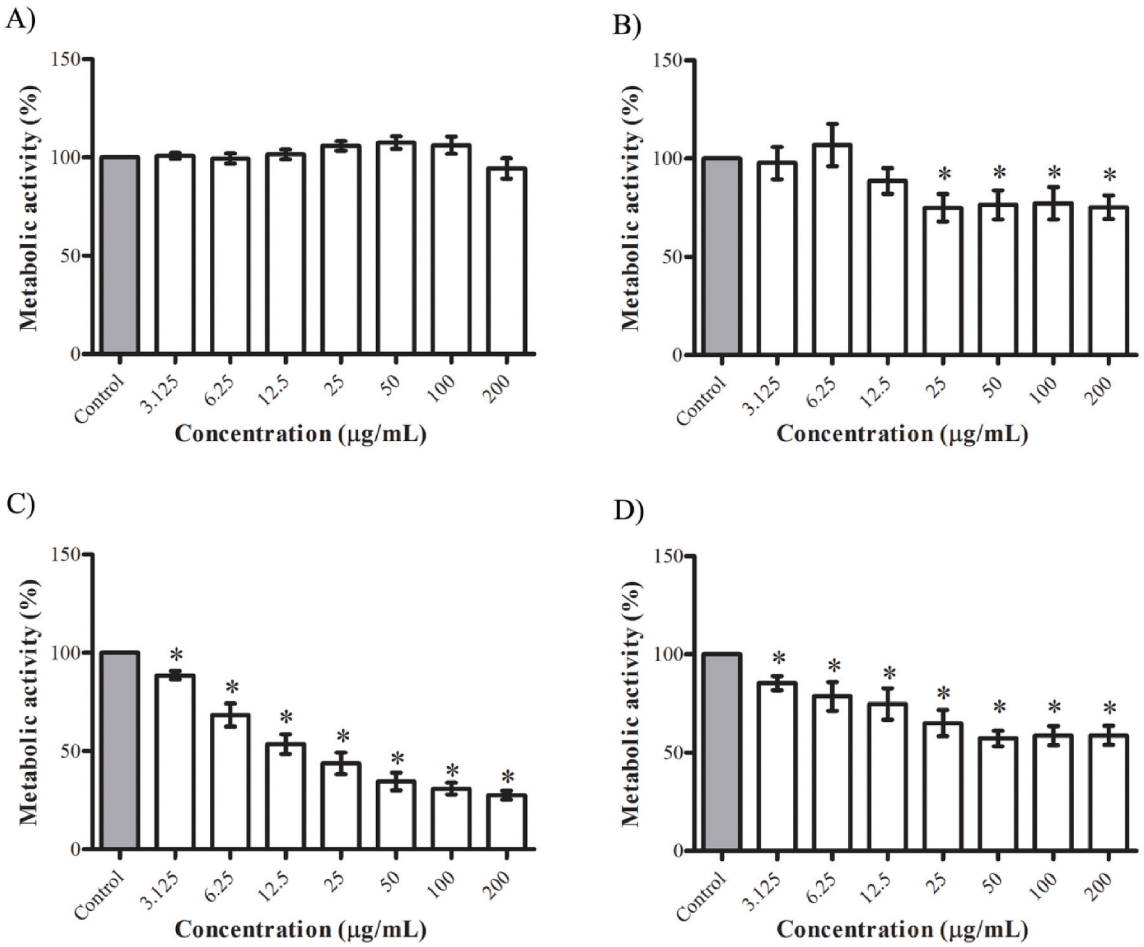

Fig. 2. Concentration response of the *B. rotunda* extract on *C. albicans* in planktonic (A), adhesion (B), biofilm development (C), and mature biofilm (D) stages. The metabolic activity, represented by the *C. albicans* biofilm, was compared with the untreated control. Each value is presented as the mean ± SEM (*p-value < 0.05, n = 4–8).

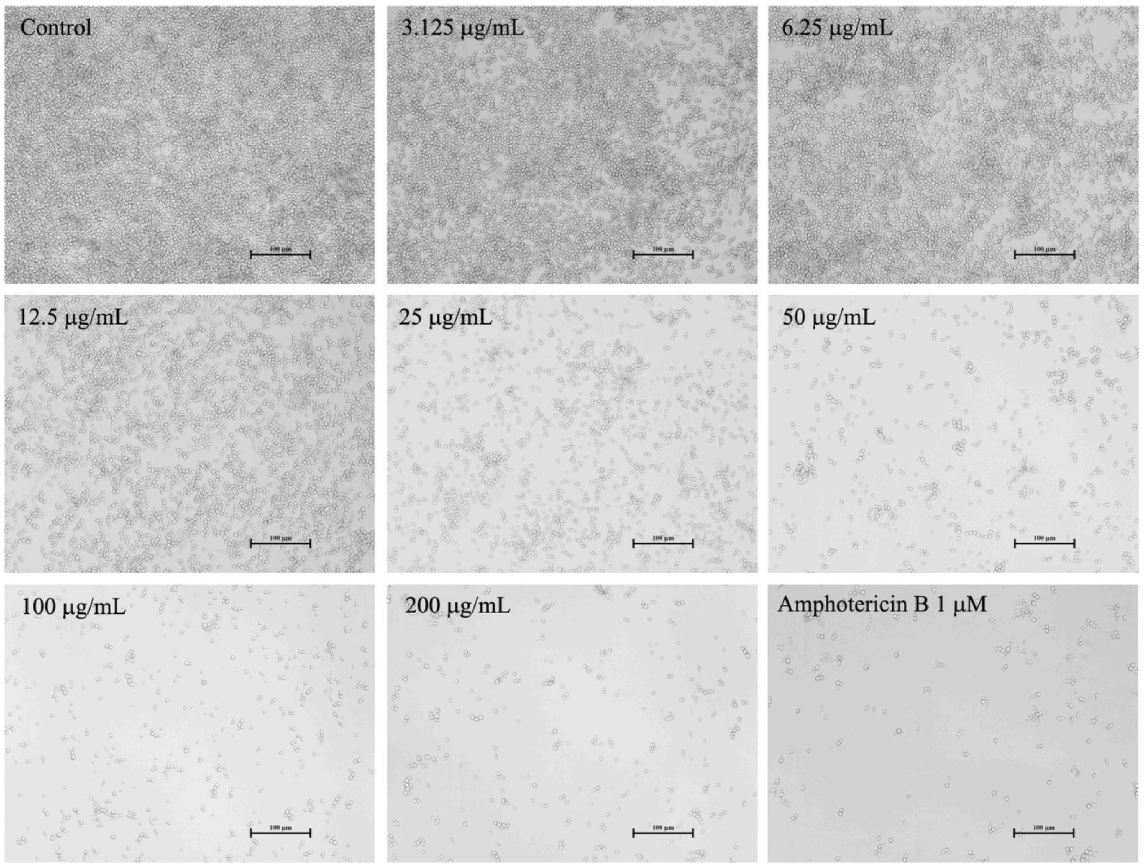

Fig. 3. Effects of the *B. rotunda* extract on the biofilm development stage. Formation of the *C. albicans* biofilm in the absence (control) or presence of the extract at a concentration of 3.125–200 µg/mL. The biofilm morphology was photographed using a light microscope. The positive control was 1 µM amphotericin B. The scale bar represents 100 µm.

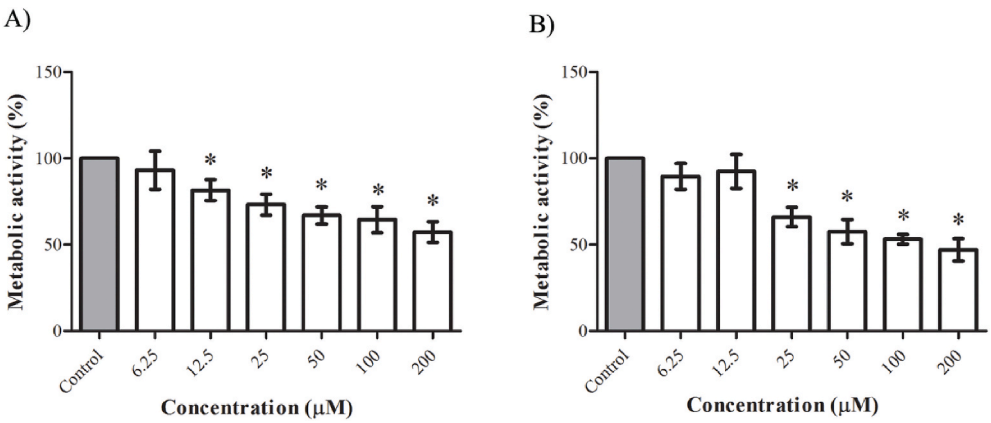

Fig. 4. Concentration-dependent response during biofilm development stages on pinostrobin (A) and pinocembrin (B). The metabolic activity, represented by the *C. albicans* biofilm, was compared with the untreated control. Each value is presented as the mean ± SEM (*p-value < 0.05, n = 5–7).

3.5. RT-qPCR: effect on ALS1, ALS3, and ACT1 gene expression

The *ALS3* mRNA level decreased significantly with 12.5 µg/mL of the *B. rotunda* extract (Fig. 7A). The expression of *ALS3* mRNA was also inhibited by pinostrobin and pinocembrin at concentrations of 100 µM. Pinocembrin showed the strongest suppression (0.18-fold) when compared to the untreated control. The *ACT1* mRNA level decreased significantly with 100 µM pinocembrin only (Fig. 7B). However, the *ALS1* mRNA level was not altered with any of the treatments (data not shown).

4. Discussion

The development of a *C. albicans* biofilm is associated with resistance to available antifungal drugs (Gulati and Nobile, 2016; Wall et al., 2019). After biofilm formation, the susceptibility of *C. albicans* to amphotericin B, fluconazole, nystatin, and chlorhexidine has been observed to noticeable decrease (Chandra et al., 2001). This phenomenon was clearly observed in the study. The susceptibility of the *C. albicans* biofilm to amphotericin B decreased 4.6-fold when compared to the planktonic condition. These results confirm that the ability to

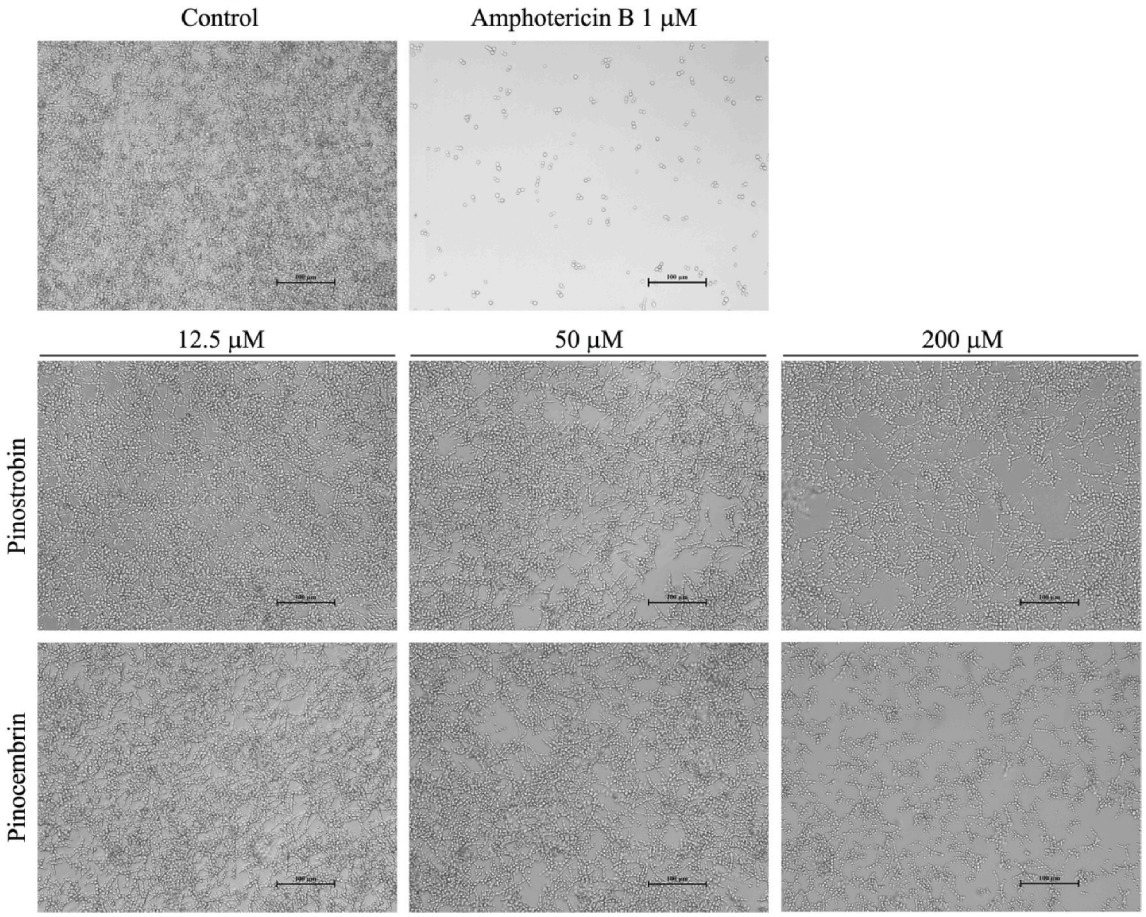

Fig. 5. Effects of pinostrobin and pinocembrin on the biofilm development stage. *C. albicans* biofilm formation in the absence (control) or presence of the compounds at concentrations of 12.5, 50, and 200 μM. The biofilm morphology was photographed using a light microscope. The positive control was 1 μM amphotericin B. The scale bar represents 100 μm.

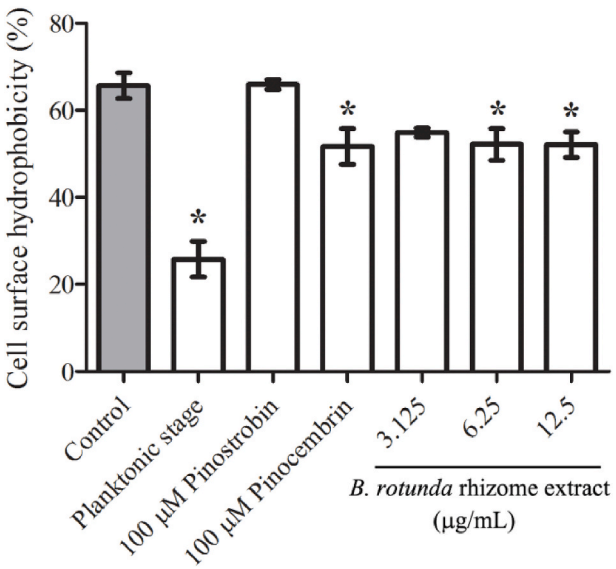

Fig. 6. Effects of the *B. rotunda* extract, pinostrobin, and pinocembrin on the percentage of cell surface hydrophobicity. *C. albicans* biofilm formation in the absence (control) or presence of the treatments. For the planktonic stage, *C. albicans* was cultured without treatments at 37 °C for 24 h and agitated at 120 rpm. Each value is presented as the mean ± SEM (*p-value < 0.05, n = 3–5).

form a biofilm could be a limitation of antifungal therapy.

Recently, the extract of *B. rotunda* was reported to be a moderate anti-*Candida* species agent. At 10 mg/mL, the extract inhibited the growth of *C. albicans*, *C. guilliermondii*, *C. parapsilosis*, and *C. tropicalis* according to the disc diffusion assay (Rukayadi et al., 2008). The MIC and MFC of the *B. rotunda* extract for *C. albicans* were reported to be 2.5 mg/mL and 5 mg/mL, respectively (Jitvaropas et al., 2012). Considering the antifungal effect on *C. albicans* in our study, the *B. rotunda* extract had no effect at concentrations of 3.125–200 μg/mL. This result was not totally unexpected because the test was performed at much lower concentrations than those used in previous reports. In contrast, all stages of *C. albicans* biofilm formation, including adhesion, biofilm development, and mature biofilm, were significantly inhibited. These results suggest that the *B. rotunda* extract could inhibit biofilm formation of *C. albicans* without an effect on fungal growth. Therefore, the mechanism of action could be related to regulation of biofilm formation rather than interruption of the structure of *C. albicans*. Unlike available antifungal drugs, it is important to find a selective biofilm inhibitor that lessens the chance of the development of drug resistance.

As seen in Fig. 2B, the ability of *C. albicans* to adhere on the polystyrene surface was significantly inhibited by the extract. Sroisiri and Boonyanit (2010) also demonstrated that a pre-treated acrylic strip with the extract of *B. rotunda* could decrease *C. albicans* adhesion. This observation indicated that the contributing factors might be due to interference with the charge of the surface, cell surface hydrophobicity, or adhesin proteins on the cell wall.

The results suggest that the biofilm development and mature biofilm stages were dramatically inhibited by the extract in a concentration-

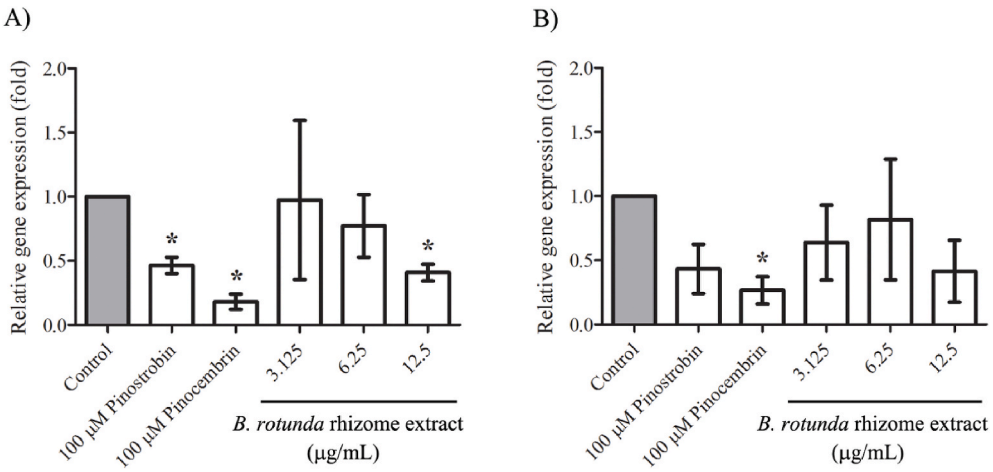

Fig. 7. Effects of the *B. rotunda* extract, pinostrobin, and pinocembrin on the levels of *ALS3* mRNA (A) and *ACT1* mRNA (B). *C. albicans* biofilm formation in the absence (control) or presence of the treatments. Fold changes represent the alteration in mRNA level compared to the control. Each value is presented as the mean ± SEM (*p-value < 0.05, n = 3–4).

dependent manner. Although the extract could decimate the maintenance of the mature biofilm, it was shown to be more effective in the stage of biofilm development than in the mature biofilm stage. Biofilm development consists of cell proliferation and filamentation of the adhered cells (Gulati and Nobile, 2016). Light microscopy results support the decrease in filamentous forms due to the *B. rotunda* extract (Fig. 3). This finding was similar to alizarin, a natural anthraquinone. It could not inhibit *C. albicans* under planktonic conditions, whereas biofilm formation and hyphal growth decreased through down-regulation of *ALS3, HWP1*, *ECE1*, and *RBT1* genes (Manoharan et al., 2017).

This study indicates that biofilm development of *C. albicans* decreases significantly with the *B. rotunda* extract. Interestingly, *C. albicans* biofilm development was significantly inhibited by pinostrobin and pinocembrin according to metabolic activity and light microscopy evaluations (Figs. 4 and 5). Each of pinostrobin and pinocembrin, at the equal amount presented in the *B. rotunda* extract, affected biofilm development at approximately 50% inhibition when compared with the extract. This result showed that the inhibitory effect of the extract greater than that of each compound, indicating possible synergistic effect. In addition, the filamentous forms were decreased with pinocembrin rather than pinostrobin. The structure-activity relationship of two compounds and alizarin were proposed (Fig. 8). The six-member ring condensed with benzene, a ketone group at 4-position and a hydroxy group at 5-position of pinostrobin and pinocembrin, a ketone group at 9-position and a hydroxy group at 1-position of alizarin were responsible for anti-biofilm formation activity. The substitution of hydroxy group on benzene ring improved the activity (Manoharan et al., 2017). Moreover, the methylation of hydroxy group decreased the activity. A previous study reported that the hyphae of *Penicillium italicum* disappeared and collapsed after treatment for 24 h, and the cell membrane was disturbed by pinocembrin. These results could be due to respiratory inhibition and energy homeostasis interference of the pathogen (Peng et al., 2012). Pinocembrin could effectively decrease the biofilm of many bacteria,

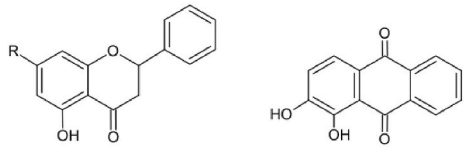

Fig. 8. Chemical structures of pinostrobin, pinocembrin, and alizarin.

such as *E. faecalis* and *S. mutans* (Ong et al., 2017; Veloz et al., 2019). This study indicated that pinocembrin might be an active compound for eradicating biofilm formation. Minor compounds in the *B. rotunda* extract might also affect the testing activity; therefore, further investigation should be pursued to achieve additional understanding on its property and mechanism.

Cell surface hydrophobicity contributes to biofilm formation and dispersion of *C. albicans* (Bujdáková et al., 2013). The *B. rotunda* extract and pinocembrin decreased the cell surface hydrophobicity of *C. albicans* when compared to the untreated biofilm. Similar to the study, a lectin from *Helianthus annuus* decreased the cell surface hydrophobicity of *C. albicans* and inhibited biofilm formation, the morphological transition, and cell viability (Rio et al., 2019). The alteration in hydrophobicity might depend on protein expression, such as expression of ALS3 and CSH1, on the cell surface (Bujdáková et al., 2013; Zhao et al., 2006).

The ALS family plays an important role in the interaction between pathogen and host cells. Today, the ALS gene family of *C. albicans* is classified into 9 types (Hoyer et al., 2008). It has been reported that alterations in *ALS1* and *ALS3* genes have an influence on biofilm formation (Chandra et al., 2001; O'Connor et al., 2005; Zhao et al., 2006). However, the *ALS1* mRNA level was not changed in our experiments; therefore, it might not be a target for the *B. rotunda* extract and two major constituents. For the *ALS3* mRNA level, it was significantly decreased by 12.5 μg/mL of the extract, 100 μM of pinostrobin, and 100 μM of pinocembrin. Therefore, our finding indicated that the *B. rotunda* extract inhibited the biofilm formation of *C. albicans* via pinostrobin and pinocembrin by down-regulating the *ALS3* mRNA. In a previous study, alizarin inhibited biofilm formation and confined the transition from yeast to the filamentous form. It decreased *ALS3* mRNA but increased *ALS1* mRNA, proving that these two types of genes possess distinct roles (Manoharan et al., 2017). ALS3 also contributed to invasion of the pathogen. It was demonstrated that ALS3 binds to cadherin on the host cells and induces endocytosis in the cells. The likelihood of endocytosis was lowered in the deleted *als3/als3* mutation (Phan et al., 2007). These observations provide a possible explanation for the decreased invasion of *C. albicans* in epithelial and endothelial cells with the *B. rotunda* extract and the two major compounds. Unexpectedly, the *ACT1* gene-encoding actin protein, a common housekeeping gene for *C. albicans* (Nailis et al., 2006), was significantly down-regulated by pinocembrin (Fig. 7B). Alteration in the actin level might be another contributing factor because hyphal-inducing signals in *C. albicans* were involved in complexation of adenylyl cyclase Cyr1, cyclase-associated protein Cap1, and actin. In the presence of cellular actin, the adenylyl cyclase could be fully activated to generate cAMP, resulting in protein

kinase A activation and hyphal-specific gene transcription (Zou et al., 2010). Therefore, the hyphal transition of *C. albicans* with pinocembrin treatment might be regulated by a reduction in the *ACT1* gene. However, this still needs to be investigated further.

5. Conclusion

Biofilm formation is a virulence factor of *C. albicans*, which is an opportunistic fungal pathogen. In this study, *B. rotunda* exhibited an anti-biofilm formation effect on *C. albicans*. Pinostrobin and pinocembrin, which are major constituents of the extract, noticeably inhibited the biofilm development stage. The extract and pinocembrin decreased the cell surface hydrophobicity and the level of *ALS3* mRNA. Therefore, the *B. rotunda* extract inhibited biofilm formation of *C. albicans* by reducing cell surface hydrophobicity and *ALS3* mRNA expression. Pinocembrin had a stronger effect on *ALS3* mRNA expression than pinostrobin. Only pinocembrin significantly decreased the *ACT1* mRNA expression.

Author contributions

Jamras Kanchanapiboon designed research studies, participated in all experiments, analyzed data and drafted the manuscript. Ubonphan Kongsa and Sunisa Kamponchaidet performed microdilution assay and anti-biofilm formation assay. Duangpen Pattamadilok prepared the extract and revised drafts of the manuscript. Detmontree Wachisunthon performed the UPLC analysis. Subhadhcha Poonsatha revised drafts of the manuscript and performed cell surface hydrophobicity assay and RT-qPCR together with Sasiwan Tuntoaw.

Declaration of competing interest

We wish to confirm that there are no known conflict of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgments

The authors would like to thank Mr. Sakwichai Ontong for authenticating the plant material. The authors would also like to acknowledge the financial supported from the Department of Medical Sciences, Ministry of Public Health, Thailand.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2020.113193.

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