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Full Paper

Antifungal and anti-biofilm activity of *Dictyophora indusiata* against *Candida albicans*

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Abstract: *Candida albicans* is an opportunistic fungal pathogen in humans. Its biofilm formation is the main factor in increasing virulence and antifungal resistance. *Dictyophora indusiata* is a medicinal mushroom with various pharmaceutical effects. This work aims to determine the anticandidal activity of *D. indusiata* extracts using agar diffusion assay and broth microdilution method to determine the minimal inhibitory concentration (MIC). The inhibition of biofilm formation was measured by crystal violet staining, and the cytotoxicity of extracts was measured in HaCaT cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay. Only the hexane extract of the mushroom can reduce *C. albicans* growth with an MIC of 1 mg/mL. For anti-biofilm activity, hexane and methanol extracts at concentrations of 0.2-0.8 mg/mL and 0.4-0.8 mg/mL respectively significantly show 50-75% biofilm reduction in 24 hr ($p<0.01$). Cell toxicity is observed for the hexane extract when the concentration is 0.1 mg/mL ($p<0.001$) or higher, while methanol extract is less toxic to human keratinocyte cells.

Keywords: *Dictyophora indusiata*, *Candida albicans*, anti-biofilm activity, antifungal activity

INTRODUCTION

Candida albicans is a communal fungus colonising on the skin and intestinal or genital mucosa in healthy individuals. The impairment of host immunity, such as in HIV, cancer, or organ transplant patients, leads to the pathology of *C. albicans*, which ranges from skin and mucosal

infection to candidemia (a bloodstream infection) and septic shock [1, 2]. The virulent process of *C. albicans* consists of three steps, viz. adhesion in yeast form on medical devices or host cells, hyphal transition, and biofilm formation. Biofilm, the complex structure of the hyphal, yeast cell and extracellular matrix, can resist the host immune response and antifungal drugs. Moreover, it is the yeast cell reservoir for dispersion to other sites of infection [1]. The inhibition of biofilm development is the main factor in drug susceptibility and increased clinical efficacy. Numerous studies have investigated the molecules or substances that possess anticandidal and anti-biofilm properties.

Dictyophora indusiata or *Phallus indusiatus* is an edible mushroom widely used in traditional medicine, functional foods and skincare agents [3]. Its pharmacological activities have been reported to include antioxidant, anti-inflammation, neuroprotection, hepatoprotection, anticancer and anti-obesity [3-5]. The major bioactive compounds of *D. indusiata* are water-soluble polysaccharides [6, 7] as well as less polar compounds in the methanolic, ethanolic, ethyl acetate and hexane extracts [8, 9]. The water extract of *D. indusiata*, which is stable at high temperatures and has a bacteriostatic effect in a wide pH range, inhibits food-borne pathogens' growth [10]. The ethyl acetate extract decreases bacterial growth better than the ether extract, and it demonstrates antibacterial properties against pathogenetic and spoilage bacteria under neutral and weakly basic conditions [11, 12]. Compared to the antibacterial property, the antifungal activity of *D. indusiata* water extract is weaker [13] while no effect on fungi growth is observed with the ether and ethyl acetate extracts [14]. There is currently no data on the antifungal activity of acid, alkaline, methanol and hexane extracts of *D. indusiata*. Furthermore, the ability of the mushroom to prevent *C. albicans* from forming biofilm, which is associated with antifungal resistance, has not been investigated. Therefore, this study aims to examine the anticandidal and anti-biofilm activities of *D. indusiata*'s extracts against *C. albicans*. We also determine their cytotoxicity to immortalised human keratinocytes (HaCaT cells) to determine concentrations for treatment purposes.

MATERIALS AND METHODS

Materials and Reagents

Lyophilised powder from the fruiting body of *D. indusiata* was provided by Thailand Institute of Scientific and Technological Research. *C. albicans* ATCC 10231 and HaCaT cells were purchased from the microbiology laboratory of Pharmaceutical Science, Ubon Ratchathani University (Thailand) and the American-Type Culture Collection, Rockville (USA) respectively. Sabouraud dextrose agar was purchased from Himedia (India). Yeast extract-peptone-dextrose broth was purchased from BD Difco (USA). Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's modified eagle's medium, penicillin, streptomycin, amphoterin B and foetal bovine serum were purchased from Fisher Scientific (Canada). All extraction reagents were of analytical grade.

Dictyophora indusiata Extraction Process

Hot water, acid and alkaline extraction processes were carried out according to the modified method of Hua et al. and Wang et al. [15, 16]. Briefly, *D. indusiata* powder was mixed with distilled water at 1: 20 w/v and boiled at 95ºC for 2 hr. The solution was centrifuged and the residue was re-extracted twice. The combined supernatant was deproteinised using Savage reagent ($CHCl_3$: n-BuOH = 4:1 v/v). The deproteinised solution was precipitated by 80% cold ethanol at 4ºC for 24

hr. Centrifugation was employed to precipitate the residue and remove the access solution. Lyophilisation was carried out on the deproteinised solution and the residue was collected as the hot water extract. Acid and alkaline extracts were prepared by immersing the residue from the hot water extraction in 1M HCl or 1% NaOH at the ratio of 1: 20 w/v at 55ºC for 7 hr. The residue of acid and alkaline extracts were re-extracted twice. The acid and alkaline solutions after pooling were adjusted to neutral by using 8% NaOH and 1M HCl before being concentrated by vacuum evaporation. Dialysis was used to desalt the solutions for 3 days using a dialysis bag with a molecular weight cutoff of 6,000-8,000 kDa. Finally, lyophilisation was used to dry both the acid and alkaline solutions. Before biological testing, the powders of hot water, acid and alkaline extracts were resuspended in distilled water.

For water-insoluble substances, extractions with hexane and methanol were conducted according to the modified method of Lee et al. [17]. *D. indusiata* powder was macerated with hexane or methanol (1: 20 w/v) at 25°C and the powder and solvent mixed by an orbital shaker for 24 hr. The solution was filtered through filter paper, and the residue was re-extracted twice with fresh solvent. The combined filtered solution was evaporated to dryness by a vacuum evaporator at 45°C, 200 mbar for hexane extract and 45°C, 110 mbar for methanol extract. The hexane extract was resuspended in DMSO, and the methanol extract was resuspended in 50% aqueous DMSO before the biological test.

Agar Well Diffusion Method

C. albicans colonies were mixed with 0.9% NaCl solution for preparing cell suspension, which was adjusted to 0.5 McFarland. The cell suspension was plated onto a Sabouraud dextrose agar with a sterile cotton swab. A 6-mm sterile metallic cork-borer was used as sample holder in the agar. The hole was filled with 60 µL of mushroom extract at a concentration of 10 µg/L or amphoterin B at a concentration of 250 µg/mL. The inhibition zone was measured after the plate was incubated at 30ºC for 48 hr. Distilled water was used as a diluent control in all extracts except the hexane extract, which was dissolved with 50% DMSO [18].

Evaluation of Minimal Inhibitory Concentration (MIC)

The MIC was determined by the Clinical and Laboratory Standards Institute M27-A2 broth microdilution method [19, 20] with modification. Five colonies of *C. albicans* ATCC 10231 were picked up from Sabouraud dextrose agar and diluted with 0.9% NaCl to match the turbidity of a 0.5 McFarland standard. The cell suspension was adjusted to twofold strength with an RPMI 1640 medium at a concentration of $0.5\text{-}2.5\times10^3$ CFU/mL. The extracts and amphotericin B concentrations used were in ranges of 0.8-0.125 mg/mL and 25-0.196 µg/mL respectively. Microdilution plates were incubated at 35°C for 24 hr, and cell viability was determined by visual examination of growth inhibition. The MIC was the lowest concentration of treatment that showed no visible growth.

Biofilm Formation

The biofilm formation of *C. albicans* was produced in 24-well plates, following the previous method [21, 22] with some modification. Yeast cells were inoculated in 25 mL of yeast extract peptone dextrose medium and incubated overnight on a 180-rpm orbital shaker at 30°C. The culture medium was centrifuged at 3,000 rpm for 10 min. and washed twice with sterile PBS. The pellets

were resuspended and adjusted with RPMI 1640 to a final density of 10^6 cells/mL by hemacytometer counting. The medium containing the adjusted yeast cells was treated with 0.1-0.8 mg/mL of *D. indusiata* extract and incubated at 37°C for 6 and 24 hr. At the specified time, the medium and nonadherent cells were removed by washing with phosphate buffered saline, and biofilms were stained with 0.4% crystal violet for 45 min. Biofilm formation after 6 and 24 hr of incubation was observed by light microscopy and destained with 95% ethanol for 10 min. The absorbance of the destaining solution was measured with a spectrophotometer plate reader at 595 nm.

Cell Cytotoxicity

The immortalised human keratinocyte cell line was used to determine the cytotoxicity of the hexane and methanol extracts of *D. indusiata*. The cells were cultured in Dulbecco's Modified Eagle medium containing 10% foetal bovine serum, 100 units/mL of penicillin, 100 µg/mL of streptomycin and 0.25 µg/mL of amphotericin B. Cells were detached with 1 × trypsin/EDTA solution and added to the 96-well microtiter plate at a density of 5×10^3 cells/100 µL per well. The plate was incubated at 37°C with 5% CO_2 for 24 hr. The methanol and hexane extracts were diluted from a maximum concentration of 40 µg/mL to a minimum concentration of 0.16 µg/mL and incubated for 24 hr. The number of live cells was measured by the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide from yellow to blue colour, which was measured at 540 nm. Distilled water and 50% DMSO were the diluent controls of hexane and methanol extracts respectively.

Statistical Analysis

Data were collected in triplicate and analysed with variance (ANOVA) using SPSS 11.0 software. Significant differences among means were differentiated by Scheffe's test at a statistical significance of 95%.

RESULTS AND DISCUSSION

Anticandidal Activity of *D. indusiata*

In agar diffusion assay the commercially available amphotericin B concentration of 250 µg/mL can inhibit the growth of *C. albicans* with an inhibition zone diameter of 14.18 ± 0.57 mm, while diluent treatment control has no inhibitory effect. Only the hexane extract of *D. indusiata* shows anticandidal activity against *C. albicans*, with an inhibition zone diameter of 20.36 ± 1.66 mm (Figure 1 and Table 1). The MIC values of the hexane extract and amphotericin B are 1 mg/mL and 1.56 µg/mL respectively (Table 1). The water, alkaline, acid and methanol extracts do not show anticandidal activity against *C. albicans* even at concentrations up to 3 mg/mL.

While the main components of *D. indusiata* (polysaccharides) have pharmacological activities such as immunological, antitumour, antioxidant, hypoglycaemic and hepatoprotective effects [4], few studies have documented antifungal activity. The study from Oyetayo et al. [13] showed antimicrobial properties against *C. albicans* in the water-soluble extract of *D. indusiata*. However, the concentration required for this effect was exceedingly high (200 mg/mL) for therapeutic use. Egra et al. [23] reported the anticandidal effect of ethanol, ethyl acetate and hexane extracts of white-oyster mushroom. Treatment of 25-100 µg of white-oyster mushroom extracts resulted in a clear zone with a width of 8-10 mm in an agar plate diffusion assay. Acne bacteria

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were unaffected by these extracts. Previous studies have reported that essential oils, lipids and aromatic molecules from herbs and fruits have potent anticandidal activity [24, 25]. Phan et al. [26] determined anticandidal activity of methanol, ethyl acetate and water extracts of *Pleurotus giganteus*. Only the ethyl acetate extract above 50 g/mL could inhibit the growth of *Candida* species.

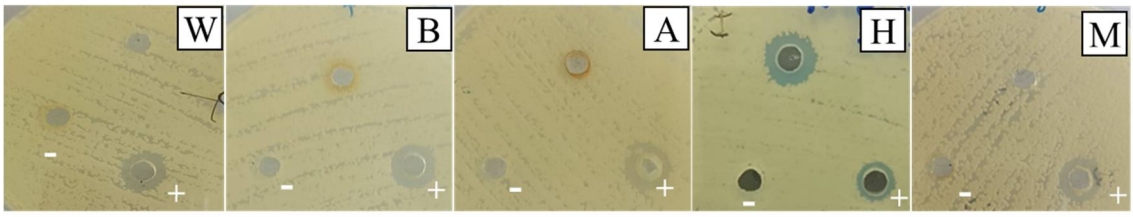

Figure 1. Clear zones after incubating with extracts for 48 hr at 30 ºC: W = hot water extract, B = alkaline extract, A = acid extract, H = hexane extract, M = methanol extract. Positive control = amphotericin B 250 µg/mL, negative control = distilled water (diluent control) or 50% DMSO for hexane extract

Table 1. Diameters of inhibition zone of *D. indusiata* extracts against *C. albicans*

***D. indusiata* extract**	**Clear zone (mm) ± SD** (Extract conc.10 mg/mL)	**MIC** (mg/mL)
Hot water	0	ND
Alkaline extract	0	ND
Acid extract	0	ND
Hexane extract	20.36 ± 1.66	1
Methanol extract	0	ND
(Amphotericin B)	14.18 ± 0.57	1.56×10^{-3}

Note: ND = not determined. Extracts show no anticandidal activity up to 3 mg/mL

Inhibition of *C. albicans* Biofilm Formation

The ability of *D. indusiata* extracts to affect biofilm development in *C. albicans* was determined after incubation with the extracts for 6 and 24 hr. From Figure 2, after 6 hr, the hot water, alkaline and acid extracts at 0.1-0.8 mg/mL do not affect biofilm formation. In contrast, hexane and methanol extracts decrease biofilm at concentrations higher than 0.1 mg/mL. Thus, we focused on methanol and hexane extracts at 24 hr of incubation. As observed in Figure 3, concentrations higher than 0.1 mg/mL for hexane extract or 0.2 mg/mL for methanol extract significantly decrease biofilm formation ($p<0.01$), and the hexane extract seems to have a higher biofilm inhibition effect than the methanol extract. Biofilm formation involves adhering to the surface, hyphal development and extracellular matrix production to mature biofilm. From Figure 3a, methanol extract reduces biofilm formation, whereas hexane extract also inhibits hyphal growth. More than twenty compounds were found in the hexane extract [27]. At least two compounds, hexanoic acid and nonanoic acid, effectively inhibit *C. albicans* biofilm formation [28]. Sharma et al. [29] reported 5-hydroxymethyl-2-furfural in the methanol fraction of *D. indusiata*, which was the same compound as that isolated from the marine bacterium *Bacillus subtilis.* Treatment with 0.4

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mg/mL of 5-hydroxymethyl-2-furfural from *Bacillus subtilis* resulted in nearly 50% and 80% inhibition of *C. albicans* adhesion and auto-aggregation respectively [30]. This supports our results that the methanol extract from *D. indusiata* interferes with the biofilm formation.

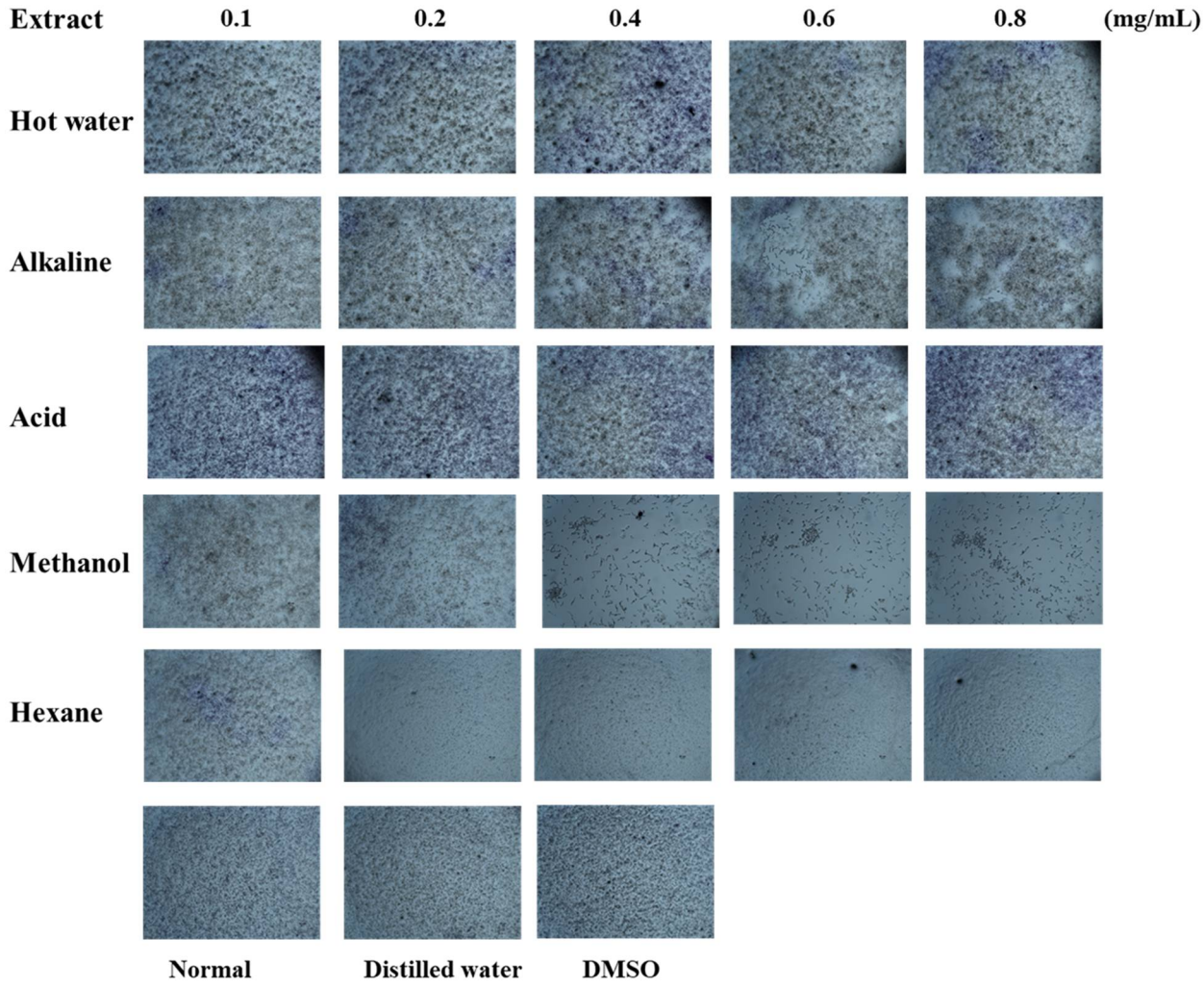

Figure 2. Biofilm development of *C. albicans* after incubation with different concentrations of *D. indusiata* extracts for 6 hr. Normal = without extract; distilled water = diluent control; DMSO = 50% DMSO as control for hexane extract.

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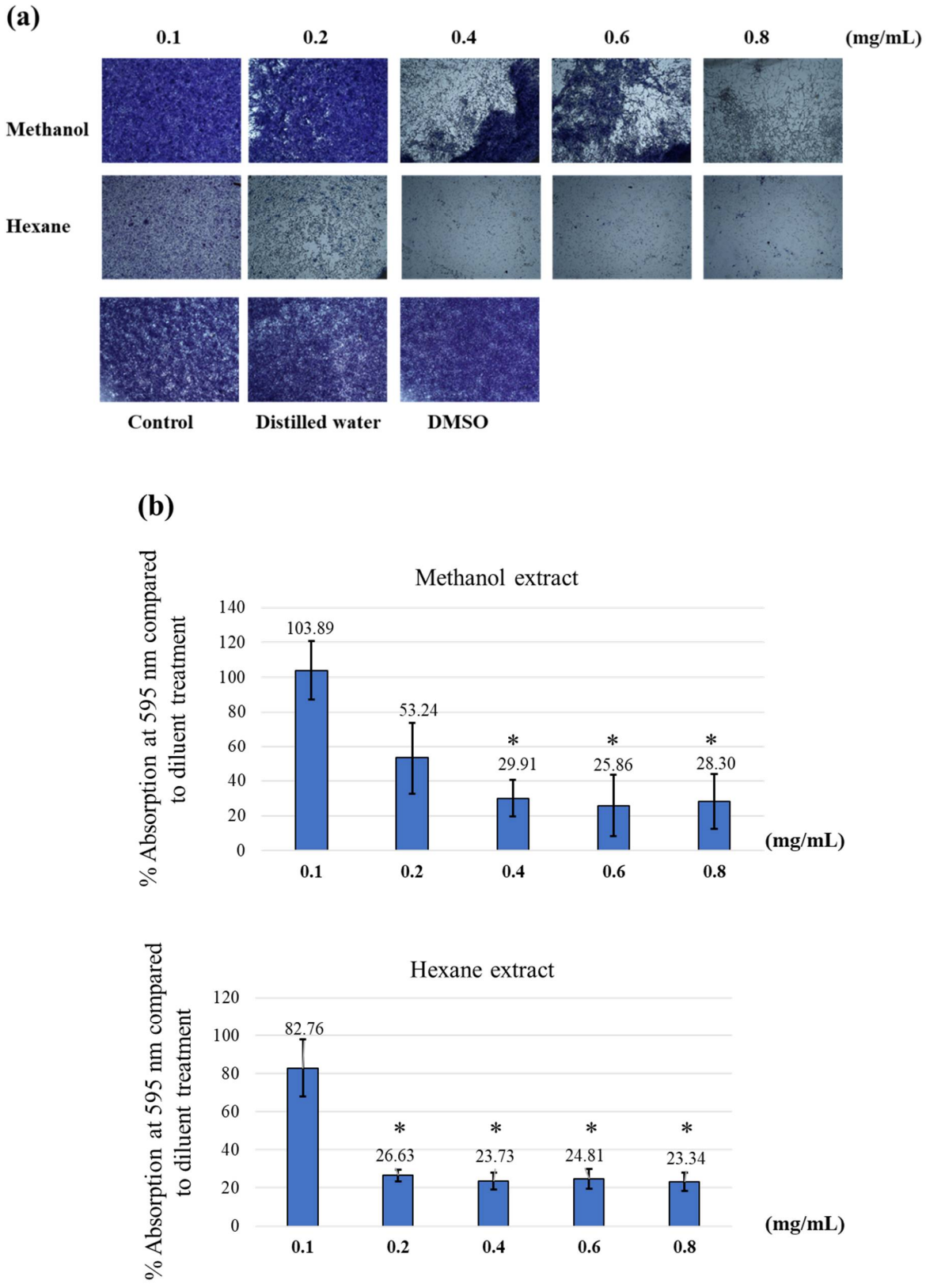

Figure 3. Formation of biofilm of *C. albicans* after incubation with hexane and methanol extracts of *D. indusiata* for 24 hr: (a) Biofilm formation after staining with crystal violet; (b) Percentages of biofilm formation by crystal violet staining.
* $p < 0.01$ compared to diluent treatment control

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Cell Cytotoxicity of *D. indusiata* Extracts

Hexane and methanol extracts of *D. indusiata* at concentrations of 0.0125, 0.025, 0.05, 0.1, 0.2 and 0.4 mg/mL were applied to HaCaT cells. The cytotoxicity results are shown in Figure 4. After 24 hr, all concentrations of the methanol extract give cell viability of 77.52-94.43%, which are not significantly different compared to distilled water treatment. Similar results were reported by Nguyen et al. [31]. For hexane extract, the high concentration range of 0.1-0.4 mg/mL significantly decreases cell viability to 19.16-12.83% ($p<0.001$). In another study, hexane extract at 0.0125-0.05 mg/mL was found to reduce HCT116 cell viability by 80-90% [27]. Our findings indicate that, compared to the hexane extract, the methanol extract is less toxic to cells but still has biofilm inhibition effect on *C. albicans*.

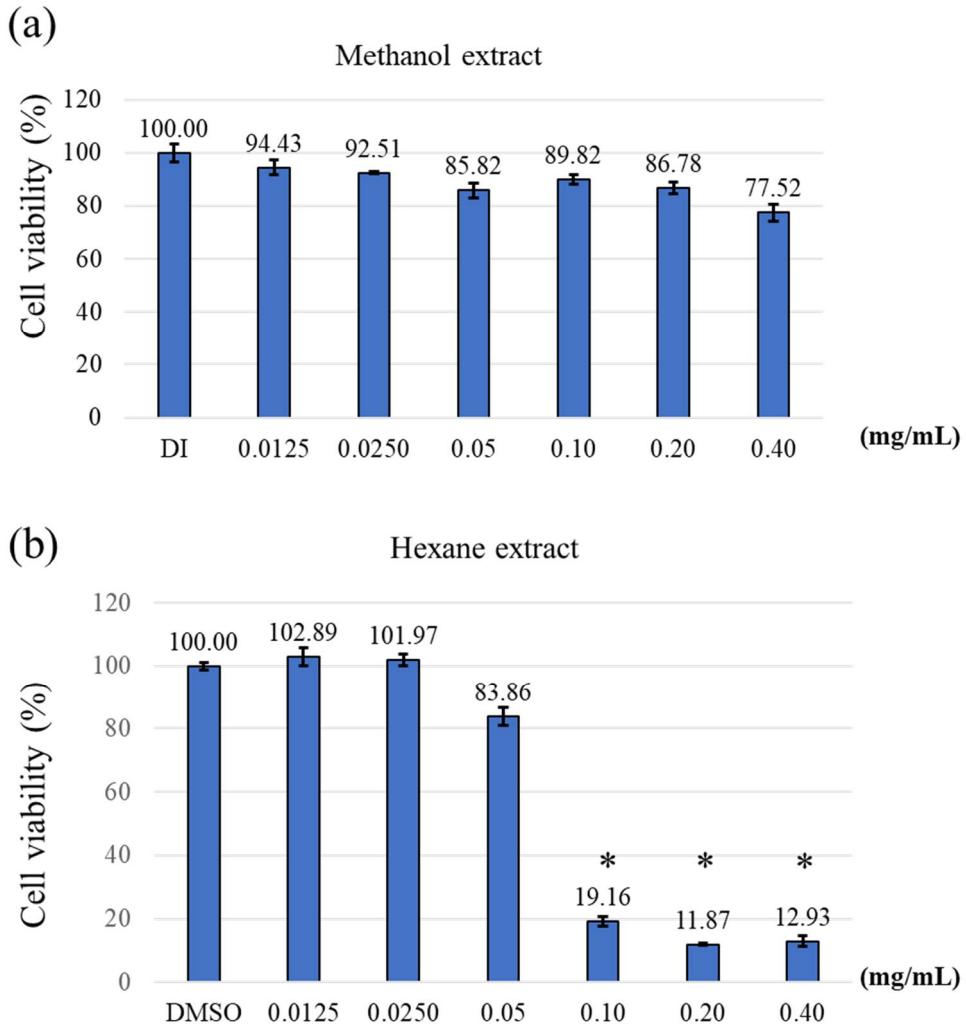

Figure 4. Percentage of cell viability of HaCaT cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell viability was assessed after exposure for 24 hr to (a) methanol extract and (b) hexane extract.
*p <0.001 compared to diluent treatment control (DI = distilled water, DMSO = 50% DMSO)

CONCLUSIONS

The hexane extract of *D. indusiata* has anticandidal activity against *C. albicans* while both hexane and methanol extracts can suppress biofilm formation of the fungus. However, the methanol

extract exhibits less cell toxicity compared to the hexane extract. We propose that the lipophilic components of *D. indusiata* should be preferentially investigated for their antifungal properties against *C. albicans*.

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