Anticandidal Effect of *Polygonum cuspidatum* on *C. albicans* Biofilm Formation

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Candida albicans is a common opportunistic pathogen and is frequently associated with biofilm formation occurring on the surfaces of host tissues and medical devices. On account of the distinct resistance of *C. albicans* biofilms to the conventional antifungal agents, new strategies are required to cope with these infections. The root of *Polygonum cuspidatum* has been used for medicinal purposes in East Asia. The aim of this study was to assess the anticandidal potential of the *P. cuspidatum* ethanol extract by evaluating biofilm formation, integrity of the cell membranes of *C. albicans* and adhesion of *C. albicans* cells to polystyrene surfaces. The growth and development of the biofilm was assessed using an XTT reduction assay, and the extract (0.39 mg/ml) significantly reduced (41.1 ± 17.8%) biofilm formation of 11 *C. albicans* strains. The extract damaged the cell membranes of *C. albicans* and remarkably inhibited cell adhesion to polystyrene surfaces. The plant extract displayed fungistatic activity without significant hemolytic activity. Based on the results of this study, the *P. cuspidatum* extract has promising potential for use in treating biofilm-associated *Candida* infection.

Key words : antifungal activity, biofilm, *Candida albicans*, cell adhesion, *Polygonum cuspidatum*

Introduction

Candida species are opportunistic pathogens that are the major cause of morbidity and mortality particularly in immunocompromised patients. *C. albicans* is still documented as a leading fungal pathogen, although pathogenic non-albicans species, such as *C. krusei, C. tropicalis, C. glabrata, C. parapsilosis* and *C. guilliermondii*, are continuously being discovered[1].

The virulence of *Candida* species has been attributed to several factors, including the formation of hyphae and biofilms, secreting hydrolases, adhering to host tissues and responding to environmental changes and morphogenesis[2]. One of the most important characteristics of *C. albicans* is the ability to colonize on both biological and inert surfaces including intravascular catheters allowing for the formation of a biofilm structure that can subsequently infect human hosts. Especially, microbial infections in humans have been estimated to be correlated with biofilm formation in 65% of cases[3]. These biofilms consist of matrix-enclosed microcolonies of yeast, hyphae and pseudohyphae, arranged in a complex structure. Fungal biofilms maintain their niche by evading host immune mechanisms, withstanding the competitive pressure from other organisms, and resisting antifungal treatment[4]. Biofilm formation of *C. albicans* is a highly complex phenomenon, distinct from fungal adhesion and dependent on many factors[4,5].

Treatment of *Candida* infections is complicated because of the eukaryotic nature of fungal cells. During the last decades, treatment has relied on fungicidal polyene drugs such as amphotericin B, which binds to the fungal ergosterol, the main component of fungal cell membranes, disrupts cell membrane permeability. More recently, fungistatic drugs such as the azoles have become more widely used to treat fungal infection due to the comparative ease of their use[6]. However, extensive use of these available drugs has led to the emergence of resistant strains and a variety of associated problems due to serious side effects at the therapeutic dosage[7]. Therefore, the discovery of novel antifungal agents with low toxicity and high therapeutic activity is urgently required. Recently, plant-derived compounds have attracted attention for use as alternatives to traditional microbial control strategies, since these compounds are widely believed to be safe and have a

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long history of use in folk medicine for the prevention and treatment of disease and infections[8].

Polygonum cuspidatum is a perennial with spreading rhizomes and numerous reddish-brown stems. The dried root of *P. cuspidatum* has been traditionally used for the treatment of menoxenia, skin burn, gallstone, hepatitis, inflammation, osteomyelitis, arthralgia, chronic bronchitis, jaundice, amenorrhea, and high blood pressure[9,10]. The plant has been reported to include various chemical compounds such as anthraquinones, stilbenes, flavonoids, and other phenols[11].

So far nobody has reported antifungal properties of the roots of *P. cuspidatum* against *Candida albicans*. In this paper, we investigated the *in vitro* inhibitory effects of the *P. cuspidatum* extract on *C. albicans* pathogens by assessing biofilm formation of *C. albicans* and its mode of action for antifungal activity against fungal pathogens, and demonstrated the potential of the extract for use in treating biofilm-associated *Candida* infection.

Materials and Methods

1. Plant material and extraction

Roots of *P. cuspidatum* were purchased from jchanbang.com, Korea. A voucher specimen was deposited at the Department of Oriental Medicine, Semyung University. Dried *P. cuspidatum* (30 g) were soaked in 0.6 liters of 70% ethanol for 2 hr and boiled at 70℃ for 2 hr. They were then centrifuged at 2,000 *g* for 20 min, and the supernatant was concentrated using a vacuum evaporator and lyophilized to yield an ethanol extract. The dried extract (3.9 g) was resuspended in DMSO to 50 mg/mℓ, and kept at -20℃ until used.

2. Strains and growth

Candida albicans (KCCM 50235, ATCC 18804), *C. glabrata* (KCCM 50044, ATCC 2001), *C. tropicalis* (KCCM 50075, ATCC 750) and *C. krusei* (KCCM 11426, ATCC 32196) were obtained from Korean Culture Center of Microorganisms (KCCM). A total of 10 clinical *C. albicans* isolates in 98 clinical isolates from candidemic patients[12] were used in this study, and kindly provided by Prof. K.-H. Lee (Yonsei University, Wonju, Korea). Biofilm formation was assessed with the XTT [2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] reduction assay using menadione. Isolates were retrieved from storage at -70℃ and subcultured twice on YM agar to ensure optimal growth.

3. Antifungal susceptibility test

Antifungal susceptibility was determined according the CLSI M27-A3 microdilution protocol[13] with minor modifications, which is based on the colorimetric broth microdilution method using resazurin[14]. All assays were repeated three times. The minimum inhibition concentration (MIC) was chosen using two criteria: the highest MIC of the three tests if they were different or the MIC that was reproduced in at least two assays. Sterility and growth controls in the presence of organic solvents employed in sample preparation were also included. No inhibitory effects were observed in the presence of the solvent control at the highest concentration used (1% v/v). The positive control drug amphotericin B was purchased from Sigma.

4. Determination of antibiofilm activity

For each strain tested, the *C. albicans* cell suspension was diluted in Yeast Nitrogen Base supplemented with 5% glucose (YNB) to a concentration of 1 x 10^7 cells/mℓ. Biofilm formation was achieved by aliquoting 0.1 mℓ of *C. albicans* culture into a 96-well microtiter plate, and the plate was incubated at 37℃ for 3 hr to allow *C. albicans* to form biofilms. The plate was washed twice with YNB to remove planktonic yeasts, and the biofilms were treated with 0.2 mℓ of 0, 1x MIC and 2x MIC of the *P. cuspidatum* extract to investigate the inhibitory activity during the initial steps of biofilm formation. The plate was incubated for an additional 24 hr at 37℃ with moist air. The viability of the biofilm remaining on the surfaces of the wells was quantified using the XTT reduction assay[15]. All the tests were performed in triplicate. The baseline values were normalized to 100 and the results were expressed in percentages of inhibition. The data from one of three independent experiments are presented.

5. Microscopic evaluation of the effect of *P. cuspidatum* on cell adhesion

To investigate the inhibitory effect of *P. cuspidatum* on cell adhesion to polystyrene surfaces, 0.1 mℓ of *C. albicans* cells at the exponential growth phase (1 x 10^7 CFU/mℓ) were aliquoted into a 96-well polystyrene microtiter plate (BD Falcon, USA) and the plate was incubated with 0.39 mg/mℓ of the *P. cuspidatum* extract along with a negative control at 37℃ for 3 hr to allow *C. albicans* to adhere to the plate. The plate was washed twice with PBS to remove planktonic or weakly attached yeasts. The adhered cells were examined using an inverted phase contrast microscope.

6. Effect of the *P.cuspidatum* extract on cytoplasmic membrane

The *C. albicans* cell suspension was diluted in RPMI 1640

buffered to pH7.0 with 0.165 M MOPS to give a final inoculum concentration of 1 x 10^6 CFU/mℓ. Experimental cultures were grown without or with 0.39 mg/mℓ of the *P. cuspidatum* extract at 37℃ for 4 hr, and the cells were washed with PBS by centrifuging at 2,000 rpm for 5 min. The positive control drug amphotericin B was included. In order to examine whether the *P. cuspidatum* extract damages yeast cell membranes, propidium iodide (PI), which penetrates cells with membrane lesions or nonviable cells, was added to a final concentration of 1 μg/mℓ and incubated for 30 min at room temperature. Confocal laser microscopy (Olympus) was used to evaluate the effect of the plant extract.

7. Time-kill assay

The *C. albicans* cell suspension was diluted in RPMI 1640 buffered to pH7.0 with 0.165M MOPS to give a final inoculum concentration of 2 x 10^4 CFU/mℓ, which was used in a broth macrodilution. Experimental cultures were grown in the absence and presence of the plant extract (0.39 mg/mℓ) at 37℃ with shaking. At predetermined time points, a 100 μℓ aliquot was removed from the cell suspension and diluted with distilled water. A 100 μℓ aliquot from each dilution was streaked on YM agar plates, and colony counts were determined after incubation at 37℃ for 24 hr. The colony counts represented the average measurements conducted in triplicate of one of the three independent assays. It was considered fungicidal activity when there was a decrease greater than or equal to $3\log_{10}$CFU/mℓ of the initial inoculums, resulting in reduction of 99.9% or more CFU/mℓ in 24 hours compared with the initial inoculum. Activity lower than that described was considered fungistatic[16].

8. Germ-tube formation assays

Serial dilutions of the *P. cuspidatum* extract ranging from 0.78 to 0.19 mg/mℓ were made in a 96-well microtiter plate containing 0.1 mℓ of sterile fetal bovine serum. Ten μℓ of a *C. albicans* suspension (1 x 10^6 CFU/mℓ) was added to each well. After incubation at 37℃ for 18 hr, the microtiter plate was evaluated for the formation of germ tubes, using an inverted phase contrast microscope.

9. *In vitro* hemolysis with the *P. cuspidatum* extract

To assess the cytotoxicity effects of the plant extract against human blood cells, hemolytic activity was evaluated by the percentage of hemolysis[17]. The hemolysis percentage was calculated using the following equation: % hemolysis = [(A_{450} of test compound treated sample – A_{450} of buffer treated sample)/(A_{450} of 1% Triton X - 100 treated sample – A_{450} of buffer treated sample)]x100. Hemolysis was also double-checked using an inverted contrast microscope.

10. Statistical analysis

All experiments were repeated at least twice in triplicate or quadruplicate. For each outcome, data were summarized as mean ± standard deviation. The effect of *P. cuspidatum*-treatments when compared with controls was assessed by using Student's t-test. One-way analysis of variance was performed for the comparison of pairs of groups followed by Turkey's test. A p value of <0.05 was considered statistically significant.

Results

1. Antifungal susceptibility test

The antifungal activity of the *P. cuspidatum* extract was evaluated against *C. albicans, C. glabrata, C. krusei* and *C. tropicalis* according the modified CLSI M27-A3 microdilution protocol, which is based on the colorimetric broth microdilution method using resazurin. The amount of cell growth in the wells was compared with spectrophotometric and colorimetric readings to ensure reproducibility. The ethanol extract of *P. cuspidatum* showed the antifungal activity against *C. albicans* ATCC 18804 with the MIC of 391 μg/mℓ (Table 1). The *P. cuspidatum* extract was also active against *C. glabrata* ATCC 2001, *C. krusei* ATCC 750 and *C. tropicalis* ATCC 32196, which are clinically important pathogen.

Table 1. Minimum inhibition concentrations of the *P. cuspidatum* ethanol extract

Candida species	MIC (μg/ml)	
	P. cuspidatum	Amphotericin B
C. albicans ATCC 18804	391	0.25
C. glabrata ATCC 2001	6,250	0.25
C. krusei ATCC 750	98	1.00
C. tropicalis ATCC 32196	391	0.25

2. Determination of antibiofilm activity

A quantitative measure of biofilm formation and viability was detected by XTT reduction assay using menadione in a standard strain of *C. albicans* ATCC 18804 and 10 clinical *C. albicans* isolates from candidemic patients. Adherence is a key step inbiofilm formation. In order to investigate the effect of the *P. cuspidatum* extract on the early phase of biofilm formation, the metabolic activity of biofilms aged 3 hr, which is considered the initial phase of biofilm formation, was measured after exposure to the plant extract for 24 hr. As shown in Table 2, the reduction in metabolic activity of 10 out of 11 *C. albicans* strains was statistically significant ($p \leq 0.05$ or

0.01) after treatment with 0.39 or 0.78 mg/mℓ of the plant extract. Relative inhibition of biofilm formation is shown in Fig. 1. Compared with the untreated 11 *C. albicans* controls, relative inhibition of biofilms showed ranging from 9.8 to 62.2% (41.1 ± 17.8%) after treating with 0.39 mg/mℓ of the plant extract.

Table 2. Metabolic activity of *C. albicans* biofilms after treating with 0, 1x MIC or 2x MIC of the *P. cuspidatum* extract

C. albicans strains	Concentration of *P. cuspidatum* (mg/ml)		
	0	0.39	0.78
	Metabolic activity[a]		
Clinical Isolates			
1	0.549 ± 0.036	0.214 ± 0.052**	0.083 ± 0.012**
2	0.542 ± 0.044	0.493 ± 0.036	0.488 ± 0.041
3	0.535 ± 0.061	0.240 ± 0.027**	0.108 ± 0.017**
4	0.448 ± 0.055	0.169 ± 0.038**	0.075 ± 0.005**
5	0.440 ± 0.051	0.317 ± 0.038*	0.152 ± 0.008**
6	0.386 ± 0.094	0.233 ± 0.061*	0.117 ± 0.012**
7	0.351 ± 0.025	0.198 ± 0.062**	0.179 ± 0.021**
8	0.289 ± 0.016	0.218 ± 0.033*	0.189 ± 0.020**
9	0.248 ± 0.021	0.175 ± 0.006**	0.139 ± 0.013**
10	0.202 ± 0.040	0.127 ± 0.036**	0.055 ± 0.002**
C. albicans ATCC 18804	0.280 ± 0.043	0.104 ± 0.020**	0.059 ± 0.002**
Mean	0.388	0.226	0.149

[a] Metabolic activity was assessed using the XTT assay measuring the absorbance at 492 nm. The differences in the optical density (mean ± standard deviation) of individual biofilms incubated with *P. cuspidatum* extract and control values were calculated using Paired Student's t-test, and were considered statistically significant when p-value was ≤ 0.05. All experiments were performed three times with four replicates. *$p \leq 0.05$,**$p \leq 0.01$.

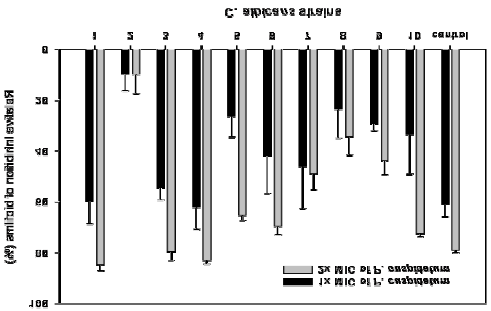

Fig. 1. Relative inhibition (mean ± standard deviation) of biofilm formation in a control strain and 10 clinical isolates of *C. albicans* after treating with 0, 1x MIC or 2x MIC of the *P. cuspidatum* extract. Metabolic activity was assessed using the XTT assay measuring the absorbance at 492 nm. Relative inhibition of biofilm formation were calculated as $100\times(1-A_{492}$ with *P. cuspidatum*$/A_{492}$ with out *P. cuspidatum*$)$.1x MIC: 0.39 mg/ml; 2x MIC: 0.78 mg/ml.

3. Effect of the *P. cuspidatum* extract on cell adhesion

To examine the inhibitory effect of the plant extract on cell adhesion to polystyrene surfaces, *C. albicans* cells (1 x 10^7 CFU/mℓ) were incubated at 37℃ with 0.39 mg/mℓ of the extract in wells of a microtiter plate to allow *C. albicans* to form biofilms. After 3 hours, the planktonic or weakly attached yeasts were removed by aspirating the supernatant, and the adhered cells were examined using an inverted phase contrast microscope (Fig. 2). Compared with the control cells, proportion of attached cells was extremely low, indicating that the *P. cuspidatum* extract has a strong inhibitory activity against *C. albicans* cell adhesion to the polystyrene plate.

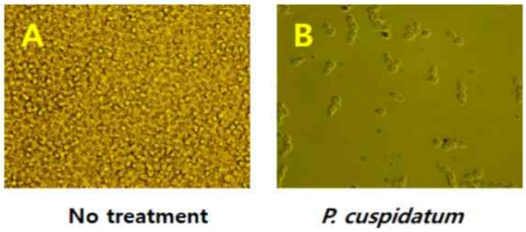

Fig. 2. Effect of the *P. cuspidtum* extract on cell adhesion. *C. albicans* culture was incubated without (A) and with 0.39 mg/ml (B) of the plant extract for 3 hr, and washed with PBS. Images of the adhered cells to polystyrene surfaces are shown using an inverted phase contrast microscope.

4. Effect of the *P. cuspidatum* extract on cytoplasmic membrane

PI is a fluorescent probe which penetrates cells with severe membrane lesions or nonviable cells, resulting in increased red fluorescence[18]. Confocal laser microscopy (Olympus) was used to assess the effect of the extract. Like the cells treated with amphotericin B (Fig. 3B), which disrupts cell membrane permeability, *C. albicans* cells treated with the plant extract were stained red with the fluorescent dye PI (Fig. 3C), representing that the *P. cuspidatum* extract damaged the *C. albicans* cell membrane. Moreover, some of the ruptured cell debris and the darkened cells were to be seen (Fig. 2B and Fig. 3C) in the extract-treated cells, suggesting that the *P. cuspidatum* extract causes cell lysis or death. The darkened cells seem to be sick or dead, because they were stained red in Fig. 3C.

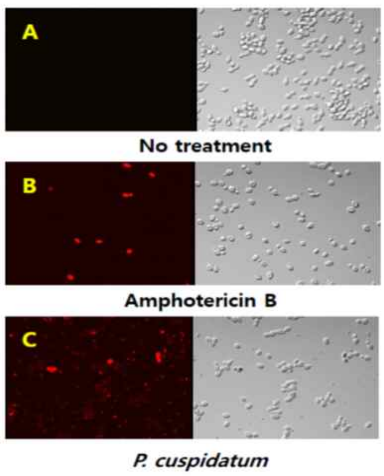

Fig. 3. Images of *C. albicans* cells using a confocal laser microscope. Cells with membrane damage were stained with PI (red signals): A; untreated control cells, B; cells treated with 1 μg/ml of amphotericin B, and C; cells treated with 0.39 mg/ml *P. cuspidatum.*

5. Time-kill assay

The antifungal activity of the *P. cuspidatum* extract against

C. albicans was determined using a colony count assay. The results of time-kill curve analysis are presented in Fig. 4. The *P. cuspidatum* extract at a concentration of 0.39 mg/mℓ weakly inhibited growth compared to the control. The relative viability was less than 10% at 22 hr, but more than 0.01% at 24 hr[16], meaning that the activity of the extract is fungistatic.

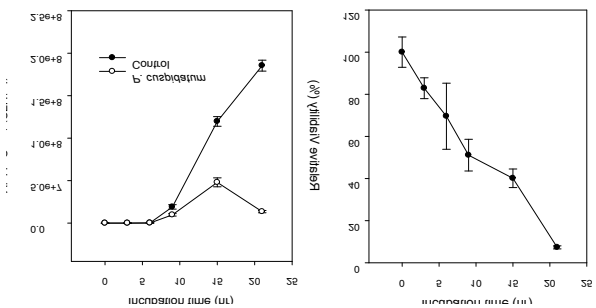

Fig. 4. Effect of the *P. cuspidatum* extract on the viability of *C. albicans*. *C. albicans* cells (2 x 10^4 CFU/ml) were incubated in the absence (control) and presence of 0.39 mg/ml of the extract at 37℃, and a colony count assay was used to determine the candidacidal activity of the extract against *C. albicans*.

6. Germ-tube formation assays

Biofilm formation of *C. albicans* is associated with mycelial formation. After the exposure of the *P. cuspidatum* extract to the *C. albicans* cells, the cell density was remarkably decreased, but no good effect on germ tube formation was detected (Fig. 5B).

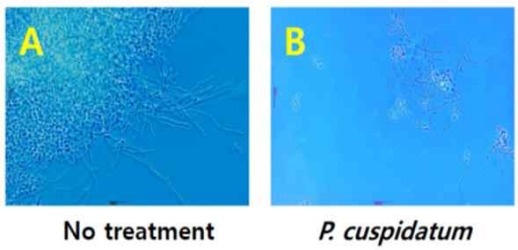

Fig. 5. Effect of the *P. cuspidatum* extract on mycelial formation. *C. albicans* (1 x10^4 CFU/ml) was incubated without (A) and with 0.39 mg/ml of the plant extract (B) in fetal bovine serum for 18 hr at 37℃ and microscopic images are shown.

7. *In vitro* hemolysis with the *P. cuspidatum* extract

The cytotoxic effects of the extract were evaluated by hemolytic activity against human blood cells(Table 3). Hemolytic activity (34.5 ± 2.4%) was observed after treating 2x MIC (0.78 mg/mℓ) of the *P. cuspidatum* extract, but 0.39 mg of the extract did not cause any hemolysis, meaning the extract is safe at minimum inhibition concentration. Hemolysis was also double-checked using an inverted contrast microscope, and 0.39 mg/mℓ of the extract did not cause to form crenated erythrocytes(data now shown).

Table 3. Hemolytic effects of 2x, 1x, 0.5x, 0.25x and 0.13x MIC of the *P. cuspidatum* extract or amphotericin B at indicated concentration

	P. cuspidatum				
Concentration (μg/ml)	782	391	196	98	49
Hemolysis (%)	34.5 ± 2.4	0.1 ± 0.0	0.1 ± 0.2	0.0 ± 0.0	0.5 ± 0.0

	Amphotericin B				
Concentration (μg/ml)	100	50	25	12.5	6.3
Hemolysis (%)	1.4 ± 0.0	1.3 ± 0.0	1.2 ± 0.1	0.5 ± 0.2	0.2 ± 0.3

The hemolysis percentage was calculated as follows: % hemolysis = [(A_{450} of test compound treated sample - A_{450} of buffer treated sample)/(A_{450} of 1% Triton X-100 treated sample - A_{450} of buffer treated sample)] x 100.

Discussion

C. albicans is known to be the most common species associated with candidiasis and the most frequently recovered species from hospitalized patients. *C. albicans* forms biofilms that are attached to surfaces. Fungal biofilms maintain their niche by evading host immune mechanisms, and resisting antifungal treatment. Medicinal plants have been used in traditional folk medicines for thousands of years, and have shown promise as a source of components for the development of new antibacterial, antifungal, antiviral, anticancer, and antihypertensive drugs[19]. In this context, we have focused on the extract of the root of *P. cuspidatum*.

The opportunistic fungal pathogen *C. albicans* can grow in a variety of morphological forms depending on environmental conditions. C. albicans typically forms buds, but under different conditions elongated pseudohyphae, filamentous chains of cells termed hyphae, or large rounded cells termed chlamydospores can be induced to form[20,21]. The dimorphic transition of *C. albicans* from yeast to the mycelial form is responsible for pathogenicity, where mycelial shapes are predominantly found during host tissue invasion and are thought to be important for biofilm formation[22].

The present study showed that the *P. cuspidatum* extract did not exert antifungal activity towards the serum-induced mycelial structure of *C. albicans*. However, the extract significantly reduced biofilm formation at an early stage of biofilms. Especially, the *P. cuspidatum* extract was very effective at the initial step of cell adhesion to the plate. Based on the result of PI stains of *P. cuspidatum*-treated *C. albicans* cells, the extract impaired the cell membrane of *C. albicans* and caused cell lysis to death. Data of the time-kill assays represent that the *P. cuspidatum* extract was fungistatic. It seems that the antifungal activity of the effective component(s) of *P. cuspidatum* is moderately strong, and it can be deduced that the extract damages *C. albicans* cell membrane, and makes the cells be ruptured or sick. Due to the damaged cell membranes,

the cells seemed to be difficult to attach to the polystyrene surfaces. Those combined effects of the extract appear to be involved in inhibiting biofilm formation. During biofilm formation, *C. albicans* cells express several genes that are involved in adhesion including the ALS protein family[23], carbohydrate synthesis, drug resistance, such as efflux pumps, and quorum sensing[4,24]. Adhesion of *C. albicans* to host cells depends on the interactions between mannoproteins with lectin-like properties and fucosyl or glucosaminyl glycosides on the surface of epithelial cells[25]. Many other factors seem to be involved in reducing biofilm formation of *C. albicans.* In order to elucidate the mechanism of action, further purification of the active components from the root of *P. cuspidatum* is needed.

Many antifungal agents have limited clinical applications, because they also cause cytolysis of human erythrocytes. The results of the hemolysis test showed that 0.39 mg/mℓ of the *P. cuspidatum* extract did not produce cytolysis of human erythrocytes nor deform the cells.

In summary, the results of this paper indicate that the antifungal activity of the *P. cuspidatum* extract against *C. albicans* is due to multiple effects including inhibiting biofilm formation, damaging the cell membranes and reducing the cell adhesion to polystyrene surfaces. The findings indicate that the *P. cuspidatum* extract holds a great promise for use in the treatment of biofilm-associated *Candida* infection and further purification is required to obtain potential antifungal compounds.

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