Extracts from *Flammulina velutipes* Inhibit the Adhesion of Pathogenic Fungi to Epithelial Cells

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ABSTRACT

**Background:** Recently, extracts from natural sources have been tested for their antifungal properties. In this aspect, *Flammulina velutipes* extracts possess a significant amount of branch-chained carbohydrates with mannose moieties that, hypothetically, can reduce the adhesion. **Objective:** In this study, we assessed the capacity of extracts from *F. velutipes* (wild-type AQF-1 and ATCC 34574 as the reference strain) to inhibit the adhesion of *S. schenkii* and *C. albicans* to epithelial cells. **Materials and Methods:** The aqueous extracts from *F. velutipes* strains were obtained by sonication, total carbohydrate and protein was analyzed by Dubois and Lowry methods respectively. Effect of the extracts (50, 100 and 150 µg/mL) on the fungi adhesion to host cells was evaluated after 1 h interaction, and the percentage of inhibition of adhesion was measured. After of interaction the cytoskeleton from cell was analyzed with phalloidin-FITC. **Results:** The extract from strain AQF-1 (50, 100 and 150 µg/mL) inhibited the adhesion of: *S. schenkii* in a dose-dependent manner (4.9, 7.5 and 12.7%, respectively) and *C. albicans* in a dose-independent manner (5.2%). The percentage of inhibition by extracts from the strain ATCC34574 at the same concentrations, shown that are dose independent for both fungi: 3.9% for *S. schenkii* and 2.6% for *C. albicans*. **Conclusion:** The extracts from *F. velutipes* inhibit the adhesion of pathogenic fungi to host cells. The mechanism molecular is unknown; however, is probably an interaction between the polysaccharides from extracts with the fungi receptors. This aspect is currently analyzed.

**Key words:** Adhesion, basidiomycets extracts, epithelial cells, *Flammulina velutipes*, fungi

**SUMMARY**
- The yields of mycelium from two strains of *F. velutipes* and the extract from it were similar.
- Extracts from both strains have inhibited adhesion of *S. schenkii* and *C. albicans* to epithelial cells in vitro, but the extract from strain AQF-1 was more effective.
- The extracts have not prevented damage to epithelial cells caused by pathogenic fungi.

ABRÉVITATION USED: YPG: Yeast peptone glucose, DMEM: Dulbecco’s Modified Eagle’s medium, FITC: Fluorescein isothiocyanate

INTRODUCTION

Mycosis caused by opportunistic pathogenic fungi has augmented due to the increased and uncontrolled use of antibiotics and the increase in immuno compromised patients. The antifungal therapy is not efficient in some cases because of the high resistance of pathogens and the side effects of drugs. Most currently used treatments directly affect the viability of fungi at different stages of differentiation, but their adhesion to the host cell—the first step of infection—can be a target too. One of the promising lines of research in this field is the use of different natural substances and extracts from plants. Cranberry juice, a well-known traditional method against urinary infections, has demonstrated the suppression of adhesion of *Escherichia coli* to host cells by the inhibition of adhesins molecules.[4] Similarly, the attachment of *Helicobacter pylori* to human gastric cells can be inhibited by water extracts of *Vernonia tomentosa* leaf extract has demonstrated inhibitory activity of the adhesion of pathogen microorganisms to buccal cells.[4] Complex mixtures of substances from plants are not the only strategy used; some isolated compounds present similar effects. Mannose, for

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instance, has demonstrated the capacity to inhibit the adhesion of E. coli and Candida albicans to host cells.[8,9] Flammulina velutipes is a culinary mushroom known for its medicinal proprieties. This mushroom has been used for centuries in Asian countries for their to anti-cancerous, antioxidant and immune stimulant activities.[2] In this regard, F. velutipes water extracts can be obtained by different methods, it is reported that contain approximately 20% mannose.[9] The aim of this study was to evaluate the effect of two extracts from F. velutipes (AQF-1 wild type and ATCC 34574 collection strain) on the adhesion of yeast of pathogenic fungi to epithelial cells as well as Candida albicans and Sporothrix schenckii,[9] ethological agents of candidiasis and sporotrichosis, respectively.

MATERIALS AND METHODS

Strains and conditions of cultivation

The environmental strain of F. velutipes AQF-1 was a kind gift from PhD Elena P. Ananyeva, Saint Petersburg State Chemical Pharmaceutical Academy, Saint Petersburg, Russia. The strain ATCC 34574 was purchased from American Type Culture Collection. Both strains were grown in YPG medium for 14 days, 24°C, 150 rpm.

The yeast of S. schenckii MP103 (the clinical isolate was kindly provided by PhD Haydee Torres Guerrero, Universidad Autonoma de Mexico) was grown in YPG medium, pH 7.2, at 37°C during 5 days at 120 rpm.

The yeast of C. albicans (the clinical isolate was kindly provided by QFB, Gloria Sabanero López) and the yeast of S. cerevisiae BY4741 were grown in YPG medium, pH 7, at 37°C during 2 days at 120 rpm.

Culture of epithelial cells

Epithelial cells from cell line L929 (ATCC CCL-1) were grown in Dulbecco's Modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% of Fetal Bovine Serum (FBS, Gibco) and antibiotic Penicillin – Streptomycin (Gibco) in 96 well cell culture plates (Corning, NY, USA). For interaction assay, cells were incubated with DMEM without FBS. Cells were incubated at 37°C, in a humidified and 5% CO₂ atmosphere, until confluence (80%).

F. velutipes extracts production. The aqueous extracts from both strains were obtained by sonication according to.[9]

Total carbohydrate and protein determination

Total carbohydrate content was assessed by Dubois method.[9] Protein was determined by Lowry method.

Effect of the extracts on fungi – host cells interaction

Yeasts were centrifuged and suspended in DMEM in concentration 2 x 10⁹/mL for S. schenckii and 1.5 x 10⁹/mL for C. albicans and S. cerevisiae. Immediately after, extracts from F. velutipes were added in final concentration 50, 100 and 150 µg/mL. After 1h of incubation, the quantity of no attached yeast was determined by spectroscopy at 280 nm (Epoch Biotek). Quantity of attached yeast was calculated as:

\[
\text{Quantity of attached yeast} = \text{Inicial quantity of yeast} - \text{Quantity of no attached yeast}
\]

The percentage of adhesion with control was calculated as:

\[
\text{Percentage of adhesion} = \left(\frac{\text{Quantity of attached yeast}}{\text{Quantity of attached yeast in control}}\right) \times 100
\]

Effect of mannose on fungi – host cells interaction

Yeasts were centrifuged and suspended in DMEM in concentration 2 x 10⁹/mL for S. schenckii and 1.5 x 10⁹/mL for C. albicans. Epithelial cell was washed with DMEM and the yeast was added at cells culture. Afterwards, mannose was added in final concentration 1, 2 or 3%. After 1 h of incubation, the quantity of no attached yeast was determined by spectroscopy. Quantity of attached yeast and the percentage of adhesion comparing with control were calculated as it described above.

Staining of microfilaments

The epithelial cells – fungi interaction without/with extracts of F. velutipes exposed at 150 µg/mL, were fixed with 4% (w/v) formaldehyde (Polysciences, USA), for 15 min at room temperature, permeabilized with 0.5% (v/v) Triton X-100 for 3 min. Actin filaments were stained with phalloidin-FITC (Sigma, St. Louis, MO, USA; 1:100 dil) for 20 min at room temperature. The samples were mounted on cover slips using VECTASHIELD (Vector Laboratories Inc., Burlingame, CA). The preparations were analyzed with a fluorescence microscope (Nikon HFX-II, Japan) equipped with a UV filter (Exc = 400-420 nm).

Statistical analysis

All experiments were performed independently at least 3 times for triplicate. Significance was analyzed using Mann-Whitney test. Difference was considered significant where *p < 0.05.

RESULTS

Extracts [Table 1] from the wild-type strain (AQF-1) and collection strain (ATCC 34574) were found to possess a high content of carbohydrates (95% and 96.7%, respectively) and low content of proteins (2.9 and 3.1%, respectively).

The assay of the interaction with epithelial cells - S. schenckii [Figure 1] shown for extract wild type strain (AQF-1), that increased in the inhibition of adhesion was dose dependent: 4.9, 7.5 and 12.7% for the concentrations of 50, 100 and 150 µg/mL, respectively. However, for the extract from collection strain (ATCC 34574), the inhibition was dose independent (3.9%). The results indicate that both extracts present activity to inhibit adhesion of S. schenckii to epithelial cells.

In contrast, the interaction of epithelial cells with C. albicans [Figure 2] showed that the extracts from F. velutipes decreased the adhesion of yeast compared with the control, and it was dose independent for both extracts. Nevertheless, significant changes for extract from collection strain (ATCC 34574) were observed for the high concentrations (100 and 150 µg/mL). The percentage of the inhibition of adhesion for extract from wild type (AQF-1) was 5.2% and that for collection strain (ATCC 34574) was 2.6%, indicating the efficacy of the wild type extract in the adhesion inhibition of C. albicans to epithelial cells.

Mannose was used as a positive control of the inhibition of adhesion of fungi to epithelial cells [Figure 3]. The results showed a 4.1% and 2.3% inhibition of adhesion for S. schenckii and C. albicans, respectively, that was dose independent (1-3% mannose).

S. cerevisiae was implemented as a control of adhesion [Figure 4] because it is not a pathogen for humans, and it was not expected to adhere to epithelial cells. Effectively, extracts from both strains of F. velutipes did not produce a statistically significant difference in the percentage

| Table 1: Yield of mycelium and extracts from Flammulina velutipes, quantification of carbohydrates and proteins |
|-------------------------------------------------|-----------------|-----------------|
| Strain                                          | AQF-1           | ATCC 34574      |
| Yield of the mycelium(g dried mycelium/L)       | 2.5±0.3         | 1.80±0.27       |
| Yield of extract(percentage of dried mycelium)  | 4.8±0.5         | 3.0±0.6         |
| Total carbohydrates(percentage of the extract)  | 95±0.7          | 96.7±1.2        |
| Protein(percentage of the extract)              | 2.9±0.2         | 3.1±0.3         |
of adhesion: 0.37 and 0.17 for extracts from wild type (AQF-1) and collection strain (ATCC 34574), respectively.

Furthermore, the interaction of epithelial cells with *C. albicans* or *S. schenckii* shown [Figure 5b and b’], the microfilaments were markedly decreased and the depolymerized cytoskeleton produced spherical-shaped epithelial cells and disruption of cell-cell junction. In contrast, with the control epithelial cells, that present actin stress fibers (microfilaments) and concentrated F-actin in the junction cells [Figure 5a and a’].

When the fungi pathogens were exposed at extracts of *F. velutipes* [Figure 5] and then the epithelial cells interaction, the microfilaments of the epithelial cells shown a alteration [Figure 5c, d and c’, d’], however, the morphology of the epithelial cells is conserved, particularly with *C. albicans*; in this regard, *S. schenckii* shown more damage on the epithelial cell [Figure 5c’and d’]. This result indicates that the extracts of *F. velutipes* have a potential activity versus the fungi pathogens.

**DISCUSSION**

The crucial step of the interaction of pathogen fungi to host cells is adhesion. The superficial receptors of fungi recognize external molecules of host cells, and then adhesion occurs.[11,12] Some extracts from natural...
sources can inhibit the adhesion of pathogens to host cells. In this context, the extracts two strain (wild type AQF-1 and collection strain ATCC 34574) from F. velutipes were tested for study, their capacity to inhibit adhesion of fungi to host cells; this hypothesis is based on the high content of carbohydrates to indicate that it may possess such ability. It is evident that AQF-1 and ATCC 34574 extracts contain an amount of carbohydrates similar to other fungi as well as Ganoderma lucidum and Pleurotus sajor-caju and present anti-oxidant activity. Another hands, Inngjerdingen et al. demonstrated that carbohydrates possess the ability to inhibit the adhesion of pathogens to host cells.

Furthermore, other groups have previously reported that surface glycoproteins of fungi with mannose and glucose residues can participate in adhesion. In this context, we investigated the inhibition of adhesion of C. albicans and S. schenckii to epithelial cells in the presence of the extracts from F. velutipes from AQF-1 and ATCC 34574. The extracts from F. velutipes inhibit the adhesion of pathogenic fungi to host cells. However, the exact mechanism of inhibition of adhesion by the extracts is unknown; probability, it is due to a non-specific interaction between the polysaccharides and fungi.

Another hands, the cytoskeleton is vital for the formation and maintenance of a functional physical barrier between the cell and its surroundings, and some fungal pathogenic induce a host actin-based cytoskeleton reorganization, which directs membrane engulfment of the pathogen. However, the alteration of the act in cytoskeleton of epithelial cells after of adhesion of pathogen fungi to the cell surface has not yet been investigated in C. albicans and S. schenckii exposed to extracts F. velutipes. Our results shown, changes in the microfilaments, probability for the proteases fungi secretion, consequently is affect the morphology.

In C. albicans and S. schenckii, has been described that in the adhesion process, there is participation of surface proteins from the epithelial cells that bind to polysaccharide and protein complexes from the fungal cell wall, resulting in the bind of the pathogen to the host the receptors. Quantitatively the difference in the adhesion of pathogenic fungi probability is due by the differents receptors in the supericies of C. albicans and S. schenckii.

We observed a marked morphological alteration of the epithelial cells in interaction with S. schenckii this alteration is minor for C. albicans although were exposed to extracts of F. velutipes, this is probably due to the proteolytic activity of fungi aspect, should be studied.

**CONCLUSION**

The extracts of F. velutipes are antifungal potentials, particularly the extracts AQF-1 (wild type strain) presented major activity. Our predicted is that all components of the extract contribute for activity.

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**Conflicts of interest**

There are no conflicts of interest.

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