Pharmacogn. Res.
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ORIGINAL ARTICLE

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

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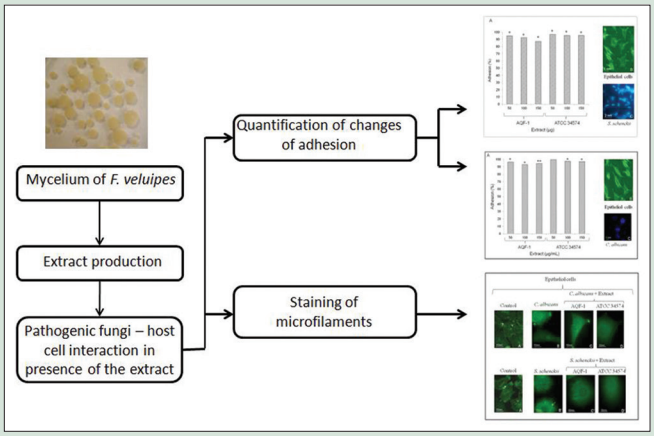
ABSTRACT

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.



Abbreviation Used: YPG: Yeast peptone glucose, DMEM: Dulbecco's Modified Eagle's medium, FITC: Fluorescein isothiocyanate

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DOI: 10.4103/0974-8490.178648

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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.[1] 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.[2] Similarly, the attachment of *Helicobacter pylori* to human gastric cells can be inhibited by water extracts of *Vernonia kotschyana* or *Cochlospermum tinctorium*.[3] Moreover, *Rhodomyrtus 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

Cite this article as: Kashina S, Villavicencio LL, Balleza M, Sabanero GB, Tsutsumi V, Lopez MS. Extracts from *Flammulina velutipes* inhibit the adhesion of pathogenic fungi to epithelial cells. Phcog Res 2016;8:S56-60.

instance, has demonstrated the capacity to inhibit the adhesion of *E. coli* and *Candida albicans* to host cells.[5,6]

Flammulina velutipes is a culinary mushroom known for its medicinal proprieties. This mushroom has being used for centuries in Asian countries for their to anti-cancerous, antioxidant and immuno stimulant activities.[7] In this regard, *F. velutipes* water extracts can be obtained by different methods, it is report that contain approximately 20% mannose.[8] 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 México) 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% CO_2 atmosphere, until confluence (80%).

F. velutipes extracts production. The aqueous extracts from both strains were obtained by sonication according to.[8]

Total carbohydrate and protein determination

Total carbohydrate content was assessed by Dubois method.[10] Protein was determined by Lowry method.

Effect of the extracts on fungi – host cells interaction

Yeast were centrifuged and suspended in DMEM in concentration 2 x 10^8/mL for *S. schenckii* and 1.5 x 10^{10}/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:

Quantity of attached yeast = Inicial quantity of yeast - Quantity of no attached yeast

The percentage of adhesion comparing with control was calculated as:

*Percentage of adhesion = (Quantity of attached yeast/Quantity of attached yeast in control)*100*

Effect of mannose on fungi – host cells interaction

Yeast were centrifuged and suspended in DMEM in concentration 2 x 10^8/mL for *S. schenckii* and 1.5 x 10^{10}/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/1 h, 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, U.S.A; 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. schenkii* 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

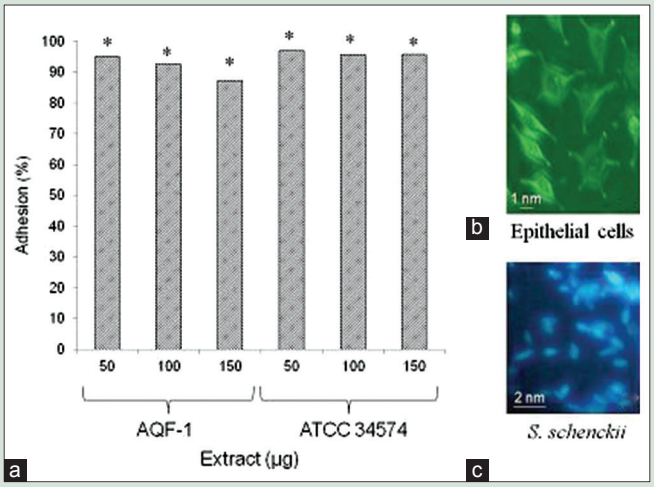

Figure 1: Adhesion of *Sporothrix schenckii* to epthelial cells (a). Influence of extracts of *Flammulina velutipes* on the process of adhesion of *Sporothrix schenckii* yeast cells (c) to epithelial cells (b), shown the inhibition of adhesion at 1 h. The inhibition of adhesion indicates the efficacy of both extracts of *Flammulina velutipes*. All experiments were performed independently at least 3 times for triplicate. Significance was analyzed using Mann-Whitney test. Difference was considered significant *p < 0.05

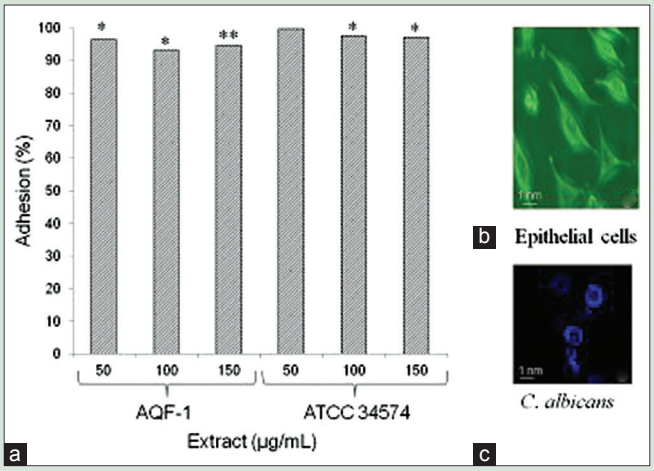

Figure 2: Adhesion of *Candida albicans* to epthelial cells (a). Influence of extracts of *Flammulina velutipes* on the process of adhesion of *Candida albicans* yeast cells (c) to epithelial cells (b), inhibition of adhesion at 1 h of *Candida albicans* to host cells was dose independent for both extracts of *Flammulina velutipes*. All experiments were performed independently at least 3 times for triplicate. Significance was analyzed using Mann-Whitney test (* - p < 0.05, ** - p < 0.01).

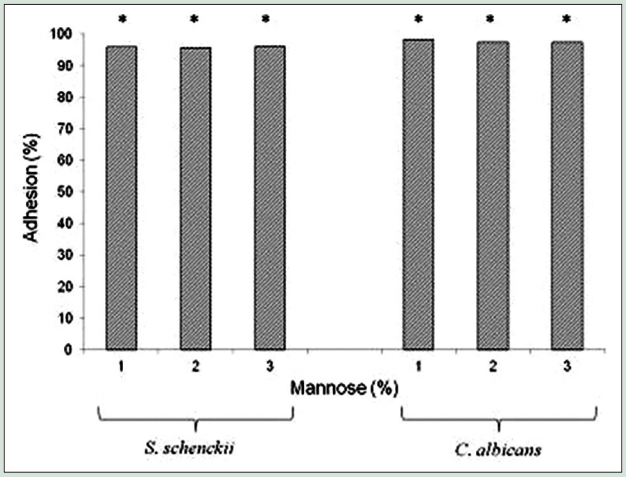

Figure 3: Effect of mannose in the fungi-epithelial cells interaction. The effect of presence of mannose in medium on inhibition of *Sporothrix schenckii* and *Candida albicans* yeast cells on the process of adhesion to epithelial cells was realized at 1h of interaction. The results have shown that inhibition of adhesion is dose independent. 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

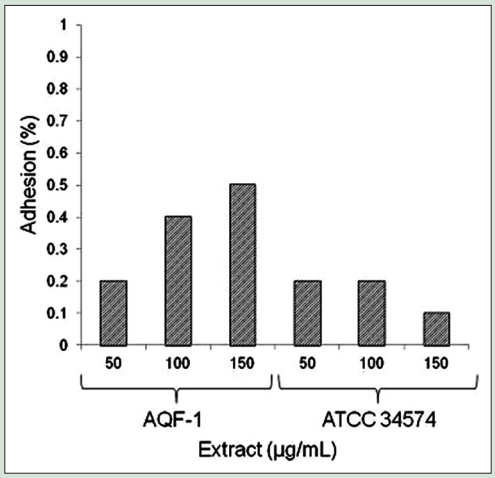

Figure 4: Adhesion of *Saccharomyces cerevisiae* to epithelial cells. *Saccharomyces cerevisiae* was implemented as a control of adhesion. Extracts of *Flammulina velutipes* did not produce a statistically significant difference in the percentage of adhesion (1 h), 0.37 and 0.17 for AQF-1 and ATCC 34574 respectively ($P > 0.05$). 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.

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

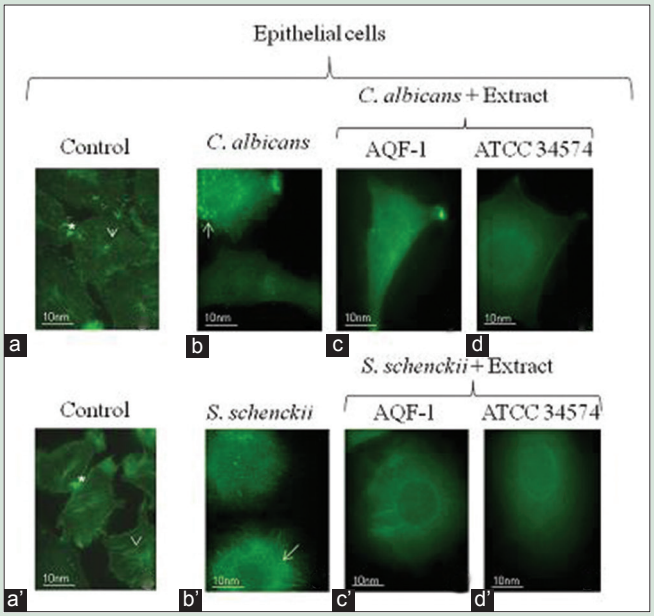

Figure 5: Micrographs of the cytoskeleton of epithelial cells. The interaction of epithelial cells with *Candida albicans* or *Sporothrix schenckii* with or without exposed to extracts of *Flammulina velutipes* (150 µg/mL) was realized at 1 h. The microfilaments was stained with Phalloidin-FITC. The cytoskeleton from host cells began to depolymerize (arrow) in the interaction with fungi (b, b'), in contrast, control epithelial cells (a, a') present pronounced actin stress fibers (arrowhead) and concentrated actin in the union cells (asterix). The fungi pathogens exposition to extracts of *F. velutipes* (c, d, c' and d') reduce the damage cell, the morphology is conserved although the microfilaments of host cells shown a alteration

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,[13,14] 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.[15] In this context, we investigated the inhibition of adhesion of *C. albicans* and *S. schenkii* 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.[16] 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 secretions,[17,18] 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,[19,20] quantitatively the difference in the adhesion of pathogenic fungi probability is due by the differents receptors in the superficies 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,[18,21] this 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.

Acknowledgements

The authors acknowledge support from the clinical isolate of *C. albicans* was kindly provided by QFB. Gloria Sabanero López and yeast of *S. cerevisiae* BY4741 by Dra. G. Angelica Gonzalez Hernandez (DCNE, UG).

Financial support and sponsorship

The authors acknowledge financial support from University of Guanajuato (DAIP - Grant 523/2015) and CONACYT by a student fellowship to Kashina S.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Miceli MH, Díaz JA, Lee SA. Emerging opportunistic yeast infections. Lancet Infect Dis 2011;11:142-51.
2. Zafriri D, Ofek I, Adar R, Pocino M, Sharon N. Inhibitory activity of cranberry juice on adherence of type 1 and type P fimbriated *Escherichia coli* to eucaryotic cells. Antimicrob Agents Chemother 1989;33:92-8.
3. Inngjerdingen KT, Thöle C, Diallo D, Paulsen BS, Hensel A. Inhibition of *Helicobacter pylori* adhesion to human gastric adenocarcinoma epithelial cells by aqueous extracts and pectic polysaccharides from the roots of *Cochlospermum tinctorium* A. Rich. and *Vernonia kotschyana* Sch. Bip. ex Walp. Fitoterapia 2014;95:127-32.
4. Limsuwan S, Homlaead S, Watcharakul S, Chusri S, Moosigapong K, Saising J, *et al.* Inhibition of microbial adhesion to plastic surface and human buccal epithelial cells by *Rhodomyrtus tomentosa* leaf extract. Arch Oral Biol 2014;59:1256-65.
5. Li K, Zhou W, Hong Y, Sacks SH, Sheerin NS. Synergy between type 1 fimbriae expression and C3 opsonisation increases internalisation of *E. coli* by human tubular epithelial cells. BMC Microbiol 2009;9:64.
6. Collins-Lech C, Kalbfleisch JH, Franson TR, Sohnle PG. Inhibition by sugars of *Candida albicans* adherence to human buccal mucosal cells and corneocytes *in vitro*. Infect Immun 1984;46:831-4.
7. Leung MY, Fung KP, Choy YM. The isolation and characterization of an immunomodulatory and anti-tumor polysaccharide preparation from *Flammulina velutipes*. Immunopharmacology 1997;35:255-63.
8. Zhang Z, Lv G, He W, Shi L, Pan H, Fan L. Effects of extraction methods on the antioxidant activities of polysaccharides obtained from *Flammulina velutipes*. Carbohydr Polym 2013;98:1524-31.
9. Hostetter MK. Adherente molecules in pathogenic *Fungi*. Curr Opin Infect Dis 1996;9:141-5.
10. Dubois M. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350-6.
11. Mendes-Giannini MJ, Taylor ML, Bouchara JB, Burger E, Calich VL, Escalante ED, *et al.* Pathogenesis II: Fungal responses to host responses: Interaction of host cells with *Fungi*. Med Mycol 2000;38 Suppl 1:113-23.

12. Sandin RL, Rogers AL, Patterson RJ, Beneke ES. Evidence for mannose-mediated adherence of *Candida albicans* to human buccal cells *in vitro*. Infect Immun 1982;35:79-85.
13. Wagner R, Mitchell DA, Lanzi G, de Almeida MA, Berovic M. Current techniques for the cultivation of *Ganoderma lucidum* for the production of biomass, ganoderic acid and polysaccharides. Food Technol Biotechnol 2003;41:371-82.
14. Jantaramanant P, Sermwittayawong D, Noipha K, Hutadilok-Towatana N, Wititsuwannakul R. β-glucan-containing polysaccharide extract from the grey oyster mushroom [*Pleurotus sajor-caju* (Fr.) Sing.] stimulates glucose uptake by the L6 myotubes. Int Food Res J 2014;21:779-84.
15. Sandoval-Bernal G, Barbosa-Sabanero G, Shibayama M, Perez-Torres A, Tsutsumi V, Sabanero M. Cell wall glycoproteins participate in the adhesion of *Sporothrix schenckii* to epithelial cells. Mycopathologia 2011;171:251-9.
16. Meza I, Sabanero M, Stefani E, Cereijido M. Occluding junctions in MDCK cells: Modulation of transepithelial permeability by the cytoskeleton. J Cell Biochem 1982;18:407-21.
17. Schindler B, Segal E. *Candida albicans* metabolite affects the cytoskeleton and phagocytic activity of murine macrophages. Med Mycol 2008;46:251-8.
18. Lei PC, Yoshiike T, Ogawa H. Effects of proteinase inhibitors on the cutaneous lesion of *Sporothrix schenckii* inoculated hairless mice. Mycopathologia 1993;123:81-5.
19. Netea MG, Gow NA, Munro CA, Bates S, Collins C, Ferwerda G, *et al*. Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and toll-like receptors. J Clin Invest 2006;116:1642-50.
20. Previato JO, Gorin P, Travassos LR. Cell wall composition in different 393 cell types of the dimorphic species *Sporothrix schenckii*. Exp Mycol 1979;3:83-91.
21. Staniszewska M, Bondaryk M, Malewski T, Kurzatkowski W. Quantitative expression of *Candida albicans* aspartyl proteinase genes SAP7, SAP8, SAP9, SAP10 in human serum *in vitro*. Pol J Microbiol 2014;63:15-20.

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