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ANTIVIRAL ACTIVITY OF *AGARICUS BLAZEI* MURRILL SS. HEINEM EXTRACT AGAINST HUMAN AND BOVINE HERPESVIRUSES IN CELL CULTURE

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ABSTRACT

The aqueous extract of *Agaricus blazei* Murill ss. Heinem, a basidiomycete native from Brazil, frequently used by popular medicine, mainly in the form of tea, was assessed to its antiviral action against herpes simplex type 1 (HSV-1) and bovine herpes type 1 (BoHV-1) in HEp-2 cell culture. Viral replication inhibition was evaluated by plaque assay and immunofluorescence test. The extract demonstrated virucide action for both viruses, being more effective against HSV-1, inhibiting its infectivity in 78.4 and 73.9% at the concentrations of 50 and 100 µg/mL, respectively moreover, reduction in 47% the number of fluorescent cells was observed for both concentrations. The extract also showed discrete therapeutic activity. These results suggest that *A. blazei* extract acts mainly in the viral particle, however, the effect during virus replication can not be ruled out.

Key words: *Agaricus blazei*, BoHV-1, HSV-1, antiviral activity

INTRODUCTION

Herpes simplex virus type 1 (HVS-1) and bovine herpesvirus type 1 (BoHV-1) are members of the subfamily *Alphaherpesvirinae* and possess a double stranded DNA genome, which is involved by an icosahedric capsid and a lipidic envelope with glicoproteins spikes on it. HSV-1 is associated to human oro-facial, ocular infections and encephalitis. BoHV-1 is responsible for infections in bovines, such as those involved with the upper respiratory (rhinotracheitis) and genital tracts (vulvovaginitis) (9,13,16). To date there are approximately 40 drugs which have been officially approved for the chemotherapy of viral infections. Among anti-herpesvirus, acyclovir, pencyclovir, valacyclovir, famcyclovir, idoxuridine, brivudin and trifluridine are used (2,4,7).

The appearance of resistant strains and the toxicity presented by some drugs have stimulated the research for new substances, synthetic or natural, with antiviral activity (6). Fungi secondary metabolites exhibit a large number of biologic activities, making basidiomycetes an alternative target for antiviral research (18). Many fungi have been studied for their nutritional and medicinal properties (3).

Agaricus blazei is a basidiomycete, native from Brazil, and has been frequently used in popular medicine, mainly in the form of tea (14). The fructification body of *A. blazei* contains 85-87% of water. When dehydrated, this fungus is rich in proteins (40-45%), carbohydrates (38-45%), fibers (6-8%), lipids (3-4%) and vitamins, such as B1, B2 and niacin. It also contains ergosterol, which is converted into D2 vitamin. Potassium is the main mineral content of *Agaricus blazei* (14). Popularly, this fungus is used against physical and mental stress, osteoporosis and gastric ulcer, as stimulatory of immunity and for cholesterol reduction. It is also used as antioxidant, antimutagenic, and anticarcinogenic (5,8,10,12,19). Sorimachi *et al.* (19) also demonstrated that *A blazei* inhibited the cytopathic effect of Western Equine Encephalitis Virus.

Apart from *Agaricus blazei*, compounds from the basidiomycetes *Ganoderma pfeifferi* and *Rozites caperata* demonstrated antiviral activity for many viruses, such as herpes simplex (11,15). In this study, we evaluated the action of aqueous

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extract of *A. blazei* in the replication of HSV-1 and BoHV-1, in HEp-2 cell culture.

MATERIALS AND METHODS

Preparation of fungus aqueous extract

The aqueous extract of *A. blazei Murill* lineage 99/26 basidiocarp (produced and supplied by Faculdade de Ciências Agronômicas, UNESP/Botucatu – SP, Brazil) was prepared by dissolving 20g of the fungus, ground in 200 mL of distilled water, at room temperature (± 25ºC), under agitation for one hour. The solution was pre-filtered and followed by filtration in 0.22 µm membrane.

Cell line and viruses

HEp-2 cells were grown in DMEM medium, supplemented with 10% of bovine fetal serum (BFS), 100 µg/mL of streptomycin, 100 UI/mL of penicillin, and 2.5 µg/mL of fungizone. The HSV-1 strain was supplied by LVEO- IMPPG- UFRJ, Rio de Janeiro, Brazil. The BoHV-1 strain was supplied by Dr. A. Alfieri, LVA, DMVP, UEL, Londrina, Brazil. Virus stocks were prepared in HEp-2 cells and stored at -80ºC.

Citotoxicity assay

HEp-2 cells grown in 96-well plates for 48 hours, at 37ºC and 5% CO_2 were treated with varying concentrations of *A. blazei* extract (200, 100, 50, 40, 30, 20, 10 and 0 µg/mL).Cultures were observed for 7 days for alterations in normal cell morphology. The 50% cytotoxic concentration (CC_{50}) was determined by MTT assay kit (Sigma) according to the manufacturer's recommendation. HEp-2 cells were grown in 96-well microplates at 37ºC and in 5% CO_2. After the formation of a monolayer, the medium was replaced with fresh medium containing different concentrations of the extract (62.5 - 10000 µg/mL). Cells grown without extract was used as controls. The plates were incubated for three days at 37ºC and cell viability was then determined. The CC_{50} was calculated as the concentration of the extract capable of reducing the optical density of the MTT product by 50% in relation to the controls by regression analysis.

Antiviral activity

The antiviral activity was done using 2 treatment protocols:

- *Virucide activity:* For this activity, 3.5 x 10^6 plaque forming units (PFU) of HSV-1 and 4.6 x 10^6 PFU of BoHV-1 were incubated for one hour, at 37ºC, along with the extract of *A. blazei* at the concentrations of 50 and 100 µg/mL and then inoculated in HEp-2 cell culture, at final multiplicity of infection equal to 1 (MOI). The antiviral activity was monitored by plaque reduction and indirect immunofluorescence.

- *Therapeutic activity:* HEp-2 cell cultures were adsorbed with HSV-1 (MOI=1) and BoHV-1 (MOI=1) for 1h and after the removal of the inoculum cultures were washed with phosphate-buffered saline (PBS) treated with medium containing 50 and 100 µg/mL of the extract and 1.5% of agarose. The plaque assay was performed. The antiviral activity was assessed as before.

Plaque reduction assay

Plaque reduction assay was performed as previously described (17) with some modifications. HEp-2 cells, grown in 24-well plates and processed according to previous protocols were overlaid with nutrient agarose (DMEM containing 1.5% agarose). Cultures were incubated at 37ºC for 48 and 72 hours for BoHV-1 and HSV-1 respectively. Cultures were fixed with 10% formalin and stained with 0.5%violet crystal. The antiviral activity was defined as the percentage of plaque inhibition as follows. % Plaque inhibition = [1 – (Number of plaque in test/ Number of plaque in control) x 100].

Indirect immunofluorescence assay (IFA)

HEp-2 cell cultures, grown in Leighton tubes, on cover glasses, were infected and treated according to the protocols previously cited. After 14 hours, the cultures were collected, washed with PBS and fixed with cold acetone (-20ºC) for 20 minutes. Briefly, the cultures on coverglass were overlaid either with rabbit anti herpesvirus antibodies supplied by Virology Department - UFRJ, Rio de Janeiro, Brazil or bovine anti herpesvirus antibodies (LVA, DMVP - UEL, Londrina, Brazil), and incubated at 37ºC for 40 minutes. Three washings with PBS were done. After washings goat anti rabbit IgG or goat anti bovine IgG conjugated with FITC (Sigma Chem. Co. USA) was added, and maintained at 37ºC for 40 minutes (in dark). Cover glasses were mounted in slides with 50% buffered glycerol and cells were observed in a UV light microscope. Tests were done in duplicates and 100 cells were counted by cover glass.

RESULTS

Citotoxicity tests monitored by morphological changes of HEp-2 cells demonstrated that *A. blazei* Murill aqueous extract was innocuous at the concentrations varying from 10 to 200 µg/mL. The CC_{50}, determined by MTT, was 5000 µg/mL.

Fig. 1b shows that inhibition of 73.9 ± 0.38 % in the number of plaques was found formed when HSV-1 was treated with 100 µg/mL of the extract, and 78.4 ± 0.16% when treated with 50 ug/mL under virucide protocols. Such inhibition was confirmed by test of indirect immunofluorescence (Table 1), in which a reduction of 47% in the number of fluorescent cells was observed in both concentrations. In the therapeutic protocol, inhibitions of 47.3± 0.09% and 55.3 ± 0.02% were observed for concentrations of 100 µg/mL and 50 µg/mL, respectively. This inhibition was also verified by indirect immunofluorescence with a reduction of 44%, approximately, of fluorescent cells for both concentrations (Table 1).

For BoHV-1, under virucide protocol, reductions of 51 ± 0.33% and 46.8 ± 0.16% were observed for the concentrations of 100 µg/mL and 50 µg/mL, respectively (Fig. 1a). The number of fluorescent cells was reduced in 25.4% at the concentration of 100 µg/mL and 16% at the concentration of 50 µg/mL (Table 1). Under therapeutic protocol the values were 20.9 ± 0.31% and 23.8 ± 0.19% inhibition for concentrations of 100 and 50 µg/mL, respectively (Fig. 1a). By indirect immunofluorescence a reduction fluorescent cells of 19.6% and 3.5% was observed for 100 µg/mL and 50 µg/mL of the extract, respectively (Table 1).

The pre-incubation of HEp-2 cells with extract did not protect the cells from BoHV-1 or HSV-1 infection (data not shown).

DISCUSSION

A. blazei Murill has been studied for its antimutagenic effects (5,8,10) mainly related to the presence of polysaccharides and protein-polisaccharide complexes, but little is known on its antiviral activity.

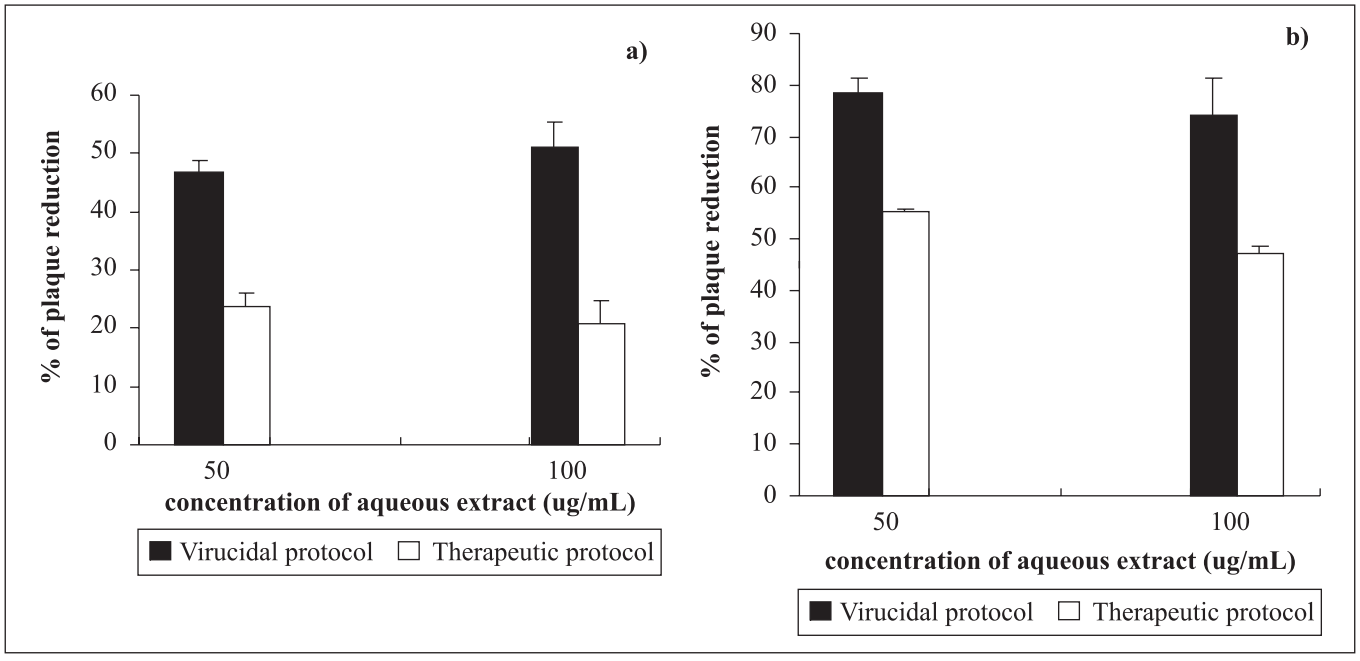

Figure 1. Percentage of reduction in plaque formation of **(a)** bovine herpesvirus type 1 (BoHV-1) and **(b)** herpes simplex virus (HSV-1) in HEp-2 cell cultures by the aqueous extract of *Agaricus blazei*, at concentrations of 50 and 100 µg/ml. Under virucide protocol, the viral suspension was pre-incubated with different concentrations of the extract before inoculation and, by the therapeutic protocol, different concentrations of the extract were maintained throughout viral replication. Data are expressed as mean ± SD. (n=3). (*p <0.05, **p < 0.01 by ANOVA).

Table 1. Effect of aqueous extract of *Agaricus blazei* in antigen expression of BoHV-1 and HSV-1. HEp-2 cells infected with the virus pre-treated with the extract (virucide protocol) or the extract maintained throughtout viral replication (therapeutic protocol) were submitted to indirect immunofluorescence assay. Test was performed in duplicates and 100 cells with specific fluorescence were counted, by cover glass. Data are expressed as mean ± SD. (n=4).

Aqueous extract (mg/mL)	Virucide Activity				Therapeutic Activity			
	number of fluorescentcells		%of inhibition		number of fluorescent cells		%of inhibition	
	BoHV-1	HSV-1	BoHV-1	HSV-1	BoHV-1	HSV-1	BoHV-1	HSV-1
0	37.5 ± 0.7	50± 1.4				43.5± 2.1	50.5±0.7	
50	31.5 ± 2.8	26.5± 2.1	16	47	42 ± 1.4	28 ± 3.0	3.5	44.6
100	28.0 ± 2.1	26.0± 3.1	25.4	47	35 ± 1.4	28.5 ± 3.2	19.6	43.6

In our work, we demonstrated that aqueous extract of *A. blazei* showed antiviral effect against HSV-1 and BoHV-1 in HEp-2 cell cultures. The aqueous extract was more effective regarding to virucide activity for both viruses, when compared to therapeutic action, shown by plaque reduction and the detection of viral proteins by IFA. A lower percentage of viral inhibition, in both treatment protocols (virucide and therapeutic), observed by IFA, is likely to be due to the fact that cells with fluorescent focus were counted as positive, independently on the intensity of the fluorescence emission, which might be understood as an inferior quantity of viral protein. This result was observed in cultures of infected and treated cells with two concentrations of the extract, because, besides, presenting a smaller number of fluorescent cells, they presented reduced fluorescence when compared to control cells (data not shown).

The highest viral inhibition, demonstrated in the virucide test, suggests a direct action of the extract over the viral particle, inhibiting the adsorption stage. The result found in the therapeutic test may indicate a lower efficacy in the inhibition of a posterior stage of virus replication in relation to adsorption one or even a direct action of the extract on the newly formed progeny, inhibiting the dissemination of the infection.

The virucide activity of substances derived from fungi and plants has been demonstrated for herpesvirus. Zhu *et al.* (20) demonstrated that a sulphated polysaccharide isolated from alga *Sargassum patens* inhibited up to 96% the formation of plaques in Vero cells (African green monkey kidney cells) infected by HSV- 2 (20). Sorimachi *et al.* (19) showed that aqueous extract and fractions obtained by alcoholic extraction of *A. blazei* Murill were able to inhibit the cytopathic effect of Western Equine Encephalitis (WEE) virus, poliovirus and HSV in cultures of Vero cells (19). The authors demonstrated a greater activity of the alcoholic fractions in comparison to the aqueous extract, however these effect were demonstrated in therapeutic protocol. The difference in susceptibility to antiviral agents between HSV-1 and BoHV-1 was observed to acyclovir, due to mutations in the genes of the thymidine kinase (1). In this study we observed that extract was more effective in inhibiting HSV-1 infection.

The use of fungus extract as possible drugs for the treatment of herpetic infections may be an alternative for the cases of strain resistant to the existing drugs.

This raw extract seems to act on the viral particle both before its adsorption to cell and during the replicative cycle of herpesvirus. However, studies with isolate fractions of this basidiomycete might clarify its mechanism of action.

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RESUMO

Inibição da replicação de herpes vírus humano e bovino pelo extrato de *Agaricus blazei* Murrill ss. Heinem em cultura de células

O extrato aquoso de *Agaricus blazei* Murill ss. Heinem, um basidiomiceto nativo do Brasil, usado na medicina popular, na forma de chá, foi avaliado quanto suas propriedades antivirais contra herpes simplex tipo 1 (HSV-1) e herpes bovino tipo 1 (BoHV-1) em cultura de células HEp-2. A inibição da replicação viral foi monitorada pelos ensaio de placa e reação de imunofluorescência. O extrato apresentou atividade virucida mais efetiva do que terapêutica para ambos os vírus, sendo mais efetivo portanto para HSV-1, inibindo em mais de 70% o número de plaques e em cerca de 47% o número de células apresentando fluorescência específica, nas concentrações de 50 e 100 µg/mL, nas duas técnicas utilizadas. Os resultados obtidos sugerem que o extrato aquoso de *A. blazei* deve agir principalmente sobre a partícula viral, embora a inibição durante o ciclo replicativo do vírus não deva ser excluída.

Palavras-chave: *Agaricus blazei*, BoHV-1, HSV-1, atividade antiviral

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