ORIGINAL ARTICLE

Antiviral activity of Inonotusin A an active compound isolated from *Boletus bellinii* and *Boletus subtomentosus*

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Abstract

Mushrooms produce various classes of secondary metabolites that could be used as antivirals in the future. The aim of this study was to determine the antiviral activity of methanolic extracts obtained from two edible mushrooms, *Boletus bellinii* (*B. bellinii*) and *Boletus subtomentosus* (*B. subtomentosus*), collected from the north forests of Tunisia, against Herpes Simplex Virus type 2 and Coxsackie Virus B type 3. In vitro micro-inhibition assays and cytotoxicity screening were performed on Vero cells. The tested *Boletus* methanolic extracts were found to be non-cytotoxic at high doses (50% cytotoxic concentration – $CC_{50} > 1$ mg/mL) and exhibited relevant viral inhibition with 50% inhibitory concentration, i.e., IC_{50} of 3.60 ± 0.66 µg/mL and 35.70 ± 7.42 µg/mL for *B. bellinii*, and 5.67 ± 1.02 µg/mL and 56.88 ± 9.56 µg/mL for *B. subtomentosus*, against HSV-2 and CVB-3, respectively. Interestingly, *Boletus* methanolic extracts showed high selectivity index (SI) values against both viruses, with the highest values against HSV-2 (SI > 800). Both viral strains were inhibited when treated with extracts during the early stages of virus replication. Inonotusin A was isolated and identified as the compound responsible for these activities. The latter is a novel antiviral agent that may have clinical utility or serve as a lead compound for further development. This study is the first attempt to investigate the antiviral activity of inonotusin A, isolated from the genus *Boletus*. The information from the present work should be a valuable reference for future studies on the antiviral activity of inonotusin A.

Keywords Herpes simplex virus type 2 · Coxsackie Virus B Type 3 · *Boletus Bellinii* · *Boletus Subtomentosus* · Antiviral activity · Inonotusin A

Abbreviations

Boletus bellini	*B. bellinii*.
Boletus subtomentosus	*B. subtomentosus*.
CC_{50}	50% cytotoxic concentration.
IC_{50}	50% inhibitory concentration.
SI	Selectivity index.
HIV	Human immunodeficiency virus.
FDA	Food and Drug Administration.
HSV-2	Simplex Virus type 2.
CVB-3	Coxsackie Virus B type 3.
DMSO	Dimethyl sulfoxide.
DMEM	Dulbecco's Modified Eagle's medium.
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
PBS	Phosphate-buffered saline.
FBS	Fetal bovine serum.
TLC	Thin-layer chromatography.
MEA	Malt extract agar.
MEB	Malt extract Broth.
ITS	Internal transcribed spacer.
NCBI	National Center for Biotechnology Information.
PCR	Polymerase chain reaction.

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ECEs   Extracellular crude extracts.
TCID$_{50}$ Tissue culture infectious dose 50%.
Rf   Retention factor.
Rt   rotation time.
min   Minute.
h   Hours.
°C   Degree celsius.
μL   Micro litre.
μg   Microgram.
mL   Milliliter.
mm   Millimeter.
PFU   Plaque-forming unit.
w/v   Weight by volume.
v/v   Volume by volume.
OD   Optic density.
cm   centimeter.
nm   Nanometer.
UV   Ultraviolet.
AU   Arbitrary units.
SD   Standard deviation.

Introduction.

Viral infection is a global health problem affecting large populations all over the world. Virus elimination is complicated by the high prevalence of viral infections with no specific treatment and the constant appearance of new serotypes in virus groups (Pekosz and Glass 2008; Linnakoski et al. 2018). Currently, the best recognized examples of viral diseases are human immunodeficiency virus (HIV), herpes simplex virus, hepatitis, influenza and COVID-19 (Antonio et al. 2020). For thousands of years, natural products have served as traditional medicine and still provide the main source of biologically active molecules and they are potentially useful for drug development (Amzat and Razum 2018). One-third of all new molecular entities registered by the United States Food and Drug Administration (FDA) are based on natural products or their derivatives. Around one-half of these are derived from mammals, one-quarter from plants, and one-quarter from microbes (Patridge et al. 2015). Fungi represent a great source of bioactive molecules, which could potentially be used for novel drug discoveries (Linnakoski et al. 2018). Many studies have shown that a wide range of compounds with various biological activities isolated from mushrooms are of interest for medical applications due to their potential antitumor, immunostimulating, antibacterial, and antiviral effects (Lindequist et al. 2005; Santoyo et al. 2012). The bioactive mushroom products with antiviral properties are less extensively studied and characterized. However, some investigations have shown that bioactive compounds isolated from mushrooms offer a vast and unexplored diversity of chemical structures that can inhibit the virus infection by specifically targeting the viral enzymes, viral nucleic acid synthesis, or host cellular factors that the viruses use for their reproduction (Santoyo et al. 2012; Teplyakova and Kosogova 2015). On the other hand, direct antiviral effects have been mainly associated with the synthesis of phenolic secondary metabolites, whereas indirect antiviral effects have been linked to polysaccharides or other high-molecular weight molecules (Lindequist et al. 2005; Rincão et al. 2012).

Tunisian forests are known for the variety of their soils and climatic conditions. This variability results in a wide range of wild mushroom production and provides interesting opportunities for the discovery of novel natural antiviral compounds. *Boletus* species are among the most widespread wild mushrooms in North Tunisia's forests (Ouali et al. 2018). The Boletales order include 300 species of *Boletus* whose spores are discharged from the surfaces of tubes beneath the mushroom cap (Money 2016). Carpophores of *Boletus* species have been frequently reported in conifer-dominated ecosystems. They are commonly found in pine forests, where they form mycorrhizal associations with a large variety of *Pinaceae* genera (Klofac 2013). Several species of the Boletales have been found to contain a diverse range of bioactive molecules with a high potential for therapeutic use, including phenolic secondary metabolites (Reis et al. 2011) and high-molecular-weight compounds (Zhang et al. 2018). However, few studies have been conducted on the antiviral activities of bioactive molecules isolated from *Boletus* species (Santoyo et al. 2012; Roy 2017; Linnakoski et al. 2018).

The appearance and dissemination of drug-resistant virus strains is a serious issue that strikes at the core of viral disease control. There is therefore an urgent necessity to develop new antiviral agents. The present study aimed to (i) assess the antiviral activity of two strains of *Boletus* against two viruses that are the causative agents of human pathologies, namely Herpes Simplex Virus type 2 (HSV-2) and Coxsackie Virus B type 3 (CVB-3) and (ii) identify the bioactive products and specify the mechanism of action of the characterized antiviral compound isolated from Boletus extracts within the different steps of the viral replication cycle.

The main novelties of this work are: (i) This is among the few works in the literature that investigate the antiviral activity of the genus *Boletus*; (ii) to the best of our knowledge, this is the first time that the antiviral compound identified as inonotusin A was isolated from Boletus species; (iii) this is the first study reports the antiviral activity of inonotusin A against CVB-3 and HSV-2.

Materials and methods

Chemical and reagents

Violet crystal, methylcellulose, methanol, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution were purchased from Sigma-Aldrich, ethanol and dimethyl sulfoxide (DMSO) from Scharlau, 96-well tissue culture plates and 35-mm dishes from Orange Scientific. Dulbecco's Modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), antibiotics-antimycotic (100 X), trypsine-EDTA (10 X), and fetal bovine serum (FBS) were from Gibco BRL; Malt extract media for mushroom culture from Bio-Rad. Thin-layer chromatography (TLC) silica gel 60 F254 was bought from Silicycle.

Isolation and mushroom culture

The fruiting bodies of the *B. bellinii* and *B. subtomentosus* strains were collected from the North-Western Tunisia. In order to isolate mycelium in pure culture, small pieces of context (about 10 mm^3) were aseptically drawn from the fruiting bodies and inoculated into malt extract agar (MEA) media with pH 6.0, in the dark at 25 °C for 15 days.

For the liquid culture, incubation was performed in 250 mL Erlenmeyer flasks containing 50 mL of 2% malt Extract Broth (MEB). Flasks with liquid medium were inoculated with three 8 mm diameter mycelial plugs cut from the Petri dishes using a sterile borer at the stage of actively growing mycelia. Mycelia were grown in static cultures for 14 days at 26 ± 2 °C.

Molecular identification of *Boletus* isolates

Ten days old mycelium grown in MEA was used for DNA extraction. Total genomic DNA from each sample was isolated using the FastDNA SPIN Kit (MP Biomedicals) following the manufacturer's instructions. Nanodrop 2000 UV (Thermo Fisher, USA) was used to determine concentration and purity of total extracted DNA. PCR amplification was carried out by using ITS1 and ITS4 primers to amplify the internal transcribed spacer (ITS) region of the rDNA. The nucleotide sequence of ITS1 was 5'-TCCGTAGGT-GAACCTGCGG-3' and of ITS4 was 5'CAGGAGACTTG-TACACGGTCCAG-3' as described by White et al. (1990). PCR products were sequenced by Applied Biosystems 3130 Genetic Analyzer. The resulting sequences were deposited in the GenBank database. Sequence analyses were carried out by BLASTN similarity search at the website (http://www.ncbi.nlm.nih.gov/BLAST) of the National Center for Biotechnology Information (NCBI).

Preparation of fungal extracts

Extracellular crude extracts (ECEs) were obtained from liquid cultures of *B. bellinii* and *B. subtomentosus*. The fermentation products were filtered using Whatman no. 4 paper and centrifuged at 4,500 x g for 10 min. The existing proteins were extracted using Sevag reagent (chloroform: n-butyl alcohol, 4:1). To precipitate the crude extracts, a four-fold volume of methanol was added for 24 h at 4 ˚C. Following 30 min of centrifugation at 4,500 x g, the residue was then extracted three times with methanol. After dialysis, the combined extracts were evaporated at 40 °C under reduced pressure (rotary evaporator) to remove methanol and freeze-dried using a vacuum freeze dryer with primary drying at 60 mT vacuum and a shelf temperature set at -100 ˚C for 20 h. The freeze-dried extracts were reconstituted for experimental use in distilled water. Insoluble material was removed by centrifugation.

Cells and viruses

Two virus strains were tested to evaluate the antiviral activity of *B. bellinii* and *B. subtomentosus*: Herpes Simplex Virus type 2 (HSV-2) and Coxsackie Virus B type 3 (CVB-3). These viruses were adapted to Vero (African green monkey kidney) cell line in DMEM supplemented with 2% FBS and containing 100 μg/mL streptomycin, 100 units/mL penicillin, and 0.25 μg/mL amphotericin B. The Vero cells were grown in the same medium with 5% FBS at 37 °C under humidified 5% CO_2. Vero cell line (ATCC® CCL-81™) and the HSV-2 clinical isolate were obtained from the Pasteur Institute of Tunis, Tunisia, and the CVB-3 clinical isolate was kindly provided by the Faculty of Pharmacy of Monastir, Tunisia.

Virus titration

HSV-2 and CVB-3 strains were titrated by the endpoint dilution method and the plaque assay method as described previously by Reed and Muench (1938) and Hankins and Heam (1970), respectively, with some modifications. The titles were expressed as plaque forming unit/mL (PFU/mL) for HSV-2 and 50 tissue culture infectious dose 50%/mL ($TCID_{50}$/mL) for CVB-3.

In vitro cytotoxicity assay

The cytotoxicity test was performed by the trypan blue dye exclusion method as described previously by Koyama and Miwa (1997) with some modifications. In brief, 100 μL of Vero cells (0.5×10^4) were seeded onto a 96-well plate (semi-confluent monolayer cells). The medium was

replaced after 24 h of incubation at 37 °C (5% CO2) with 100 μL of different concentrations of *B. bellinii* and *B. subtomentosus* ECEs. After 3 days of incubation at 37 °C (5% CO_2), monolayer cells were trypsinized and trypan blue solution (10% v/v) was added. Wells without ECEs were used as negative controls. The number of living cells was counted as compared to the cell control. The sample concentration that damages 50% of cell culture – which represents the 50% cytotoxic concentration (CC_{50}) – was calculated by linear regression analysis from the dose-response curve.

In vitro antiviral activity

The anti-HSV-2 and anti-CVB-3 activity assays were performed by the plaque assay method and the MTT method as previously described by Sasaki et al. (2016) and Guo et al. (2006), respectively, with slight modifications.

For the anti-HSV-2 activity, 2 mL of Vero cells (10^5) were seeded onto a 35-mm dish (confluent monolayer cells). After 24 h of incubation at 37 °C (5% CO_2), the medium was replaced by 2 mL of different concentrations of *B. bellinii* and *B. subtomentosus* ECEs from $CC_{50}/2$ with 150 PFU of HSV-2 for 1 h. Then, the cells were washed with PBS to remove unabsorbed viruses and overlaid with the same extract concentration in DMEM counting 1% of methylcellulose. After 2 days of incubation at 37 °C (5% CO_2), the medium was removed and the cells were stained with 0.3% crystal violet solution (0.3% w/v in 20% ethanol v/v), and then washed with PSB to remove the dye excess. The plaques were counted as compared to the virus control. The sample concentration that inhibits 50% of virus plaque – which represents the 50% inhibitory concentration (IC_{50}) – was calculated by linear regression analysis from the dose-response curve. The selectivity index (SI) value was determined as the ratio of CC_{50}/IC_{50}.

For the anti-CVB-3 activity, 100 μL of Vero cells (10^4) were seeded onto a 96-well plate (confluent monolayer cells). After 24 h of incubation at 37 °C (5% CO_2), the medium was replaced by 100 μL of different concentrations of *B. bellinii* and *B. subtomentosus* ECEs from $CC_{50}/2$ with 10 $TCID_{50}$ of CVB-3. Non-infected non-ECEs-treated cells and CVB-3-infected non-ECEs-treated cells were used as negative and positive controls, respectively. After 2 days of incubation at 37 °C (5% CO_2), the medium was removed and the cells were covered with 100 μL of MTT solution (1 mg/mL) for 3 h at 37 °C. The MTT reagent is reduced by the mitochondrial succinate dehydrogenase of active living cells to formazan, of which the quantity is proportional to the number of living cells. Then, the MTT solution was removed and 100 μL of DMSO was added to each well for to dissolve the formazan crystals product from the cells. After 15 min, the microplate was read with an ELISA plate

reader at 540 nm. The viral inhibition rate was determined following this formula: [(ODev – ODvc)/(ODcc-ODvc)] x 100, where ODev, ODvc and ODcc indicate the absorbance of the ECEs in presence of virus, the absorbance of virus control and the absorbance of cell control, respectively. The sample concentration that gave a viral inhibition rate of 50% corresponds to the IC_{50} and was calculated by linear regression analysis from the dose-response curve.

When SI ≥ 10 for HSV-2 (Dong et al. 2012) and ≥ 5 for CVB-3 (Abaza et al. 2019), the extract was considered as active.

Virus direct inactivation assay

HSV-2 (15.000 PFU) and CVB-3 (1.000 TCID50) virus suspensions were pre-treated with an equal volume of *B. bellinii* and *B. subtomentosus* ECEs at two different concentrations (IC_{50} and 10 x IC_{50}). After 2 h of incubation, the mixture was diluted 100 fold to remove the effect of the extract on the virus replication and then added to confluent monolayer cells on 35-mm dishes / 96-well plates, respectively. After 2 days, the cells were subjected to plaque assay / MTT assay, as described in the antiviral activity assay. The percentages of viral infection were calculated as compared to the virus control.

Post-infection assay

Confluent monolayer cells on 35-mm dishes / 96-well plates were infected by 150 PFU of HSV-2 / 10 $TCID_{50}$ of CVB-3, respectively. After 1 h of incubation, the cells were washed twice by PBS to remove unabsorbed viruses and then overlaid by *B. bellinii* and *B. subtomentosus* extracts with two concentrations (IC_{50} and 10 x IC_{50}). After 6 h of incubation, the cells were washed twice with PBS to remove free compounds, and DMEM was added. After 2 days, the cells were subjected to a plaque assay / MTT assay, as described in the antiviral activity assay. The percentages of viral infection were calculated as compared to the virus control.

Viral adsorption and penetration assay

Confluent monolayer cells on 35-mm dishes / 96-well plates were incubated at 4 °C with 150 PFU of HSV-2 / 10 $TCID_{50}$ of CVB-3, respectively. After 1 h, the cells were washed twice with PBS to remove unabsorbed viruses and then overlaid by *B. bellinii* and *B. subtomentosus* ECEs with two concentrations (IC_{50} and 10 x IC_{50}) at 37 °C. After 2 h of incubation, the cells were washed twice with PBS to remove free compounds, and DMEM was added. After 2 days, the cells were subjected to a plaque assay / MTT assay, as described in the antiviral activity assay. The percentages of

viral infection were calculated as compared to the virus control by deducting the rates obtained in this assay from those obtained in the post-infection assay.

Cell pretreatment assay

Confluent monolayer cells on 35-mm dishes / 96-well plates were pre-treated at 37 °C with *B. bellinii* and *B. subtomentosus* extracts at two concentrations (IC_{50} and $10 \times IC_{50}$). After 2 h of incubation, the cells were washed twice with PBS to remove free compounds and then infected with 150 PFU of HSV-2 / 10 $TCID_{50}$ of CVB-3, respectively. After 2 days, the cells were subjected to a plaque assay / MTT assay, as described in the antiviral activity assay. The percentages of viral infection were calculated as compared to the virus control by deducting the rates obtained in this assay from those obtained in the virus adsorption and penetration assay.

Isolation of the active compound

The isolation of the active compound was performed by TLC. An amount of 242 mg of *B. bellinii* and *B. subtomentosus* ECEs were deposed on a pre-coated silica gel 60 F254 glass plate (20×20 cm; Glass Backed TLC Extra Hard Layer 60 Å) and eluted with methanol/acetonitrile/H_2O (3:3:0.5-v/v/v). After separation, the plate was air dried and observed under UV light at 254 nm. The visualized bands were scraped off and dissolved in the same mobile phase as used in TLC. After agitation for 15 min, the fractions were filtrated, dried, weighted, dissolved in 75% ethanol to a 10 mg/mL final concentration, and then evaluated for their cytotoxicity and antiviral activity.

Identification of the active compound

The analyses were performed using a Thermo Scientific LCQ FLEET system consisting of an LCQ FLEET ion trap mass spectrometer, a Surveyor MS Pump/Autosampler/PDA Detector (Thermo Fisher Scientific, Waltham, MA, USA) through an ESI source. The separation was obtained by using a Gemini® C18 110 A analytical column (150×2.00 mm i.d., 5 μm) and the pre-column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of aqueous formic acid at 0.1% and acetonitrile (solvent B) at 0.3 mL/min (the injection volume was 10 μL). A linear solvent gradient was used as follows: from 10% B to 95% B in 25 min with a final plateau of 3 min at 100% B. The ion trap operated in data-dependent, full scan (60–2000 *m/z*), and MS^n mode to obtain fragment ions *m/z* with a collision energy of 35% and an isolation width of 3 *m/z*. The negative and positive parameters of the ion mode ESI source have been optimized to an ionization voltage of 5.0 kV, a

capillary temperature of 320 °C, a capillary voltage of 32 V, a sheath gas flow rate of 25 arbitrary units (AU), and an auxiliary gas flow rate of 10 AU. Data was acquired using Thermo Excalibur 2.2 software (Thermo Fisher Scientific, MA, USA).

Statistical analysis

The experiments were carried out in triplicate and values are expressed as the mean ± standard deviation (SD).

Results

DNA sequencing analysis

A fragment of the DNA internal transcribed spacer sequences of mushroom samples was amplified and sequenced. The two *Boletus* strains could be identified up to species level from the available GenBank database. Molecular identification of the mushroom samples corresponds to morphological identification. The results were submitted to the GenBank database (Accession numbers: MN461459 and MN461458 for *B. bellinii* (synonym: *Suillus bellinii*) and *B. subtomentosus* (synonym: *Xerocomus subtomentosus*), respectively) with 100% similarity percentage.

Cytotoxic effect of the extracts on viability of Vero cells

Cell viability against different concentrations of *B. bellinii* and *B. subtomentosus* ECEs in Vero cells for 72 h of incubation was screened. In vitro cytotoxicity assays showed that the concentrations associated with 50% cytotoxicity concentration (CC_{50}) of *B. bellinii* and *B. subtomentosus* ECEs on Vero cells were 2677 and 3414 μg/mL, respectively (Table 1). The CC_{50} values indicated that these extracts had no significant cytotoxic effect against Vero cells up to a concentration of 2 mg/mL.

The antiviral activity of extracts against HSV-2 and CVB-3

The *Boletus* ECEs were subjected to an in vitro plaque reduction assay and an MTT assay to evaluate the antiviral activity against HSV-2 and CVB-3, respectively. The 50% inhibitory concentration (IC_{50}) of *B. bellinii* and *B. subtomentosus* ECEs exhibited a high inhibitory effect both against HSV-2 and CVB-3 (Table 1). *B. bellinii* ECE displayed relatively strong inhibitory activity as compared to *B. subtomentosus* ECE. The relevant values of the selectivity index ($SI = CC_{50}/IC_{50}$) of *B. bellinii* and *B. subtomentosus*

Table 1 Cytotoxicity and antiviral activity of *Boletus bellinii* and *Boletus subtomentosus* extracts

Extracts	CC_{50} (µg/mL)	IC_{50} (µg/mL)		SI = CC_{50}/IC_{50}	
		HSV-2	CVB-3	HSV-2	CVB-3
Boletus bellinii	2677 ± 189	3.60 ± 0.66	35.70 ± 7.42	744	75
Boletus subtomentosus	3414 ± 312	5.67 ± 1.02	56.88 ± 9.56	602	60
Active compound	722 ± 96	0.81 ± 0.11	19.04 ± 5.29	891	38

CC_{50}: Cytotoxic Concentration 50%. CVB-3: Coxsackie Virus B type 3. HSV-2: Herpes Simplex Virus type 2. IC_{50}: Inhibitory Concentration 50%. SI: Selectivity Index. CC_{50} and IC_{50} values are expressed as the mean values of three independent experiments

Table 2 Mode of antiviral action of *Boletus bellinii* and *Boletus subtomentosus* extracts

Extracts	Virus	Antiviral activity	Virucidal effect	Before virus penetration	During virus penetration	After virus penetration
Boletus bellinii	HSV-2	100	100	NA	92	NA
	CVB-3	100	NA	NA	76	NA
Boletus subtomentosus	HSV-2	100	100	NA	85	NA
	CVB-3	100	NA	NA	64	NA

CVB-3: Coxsackie virus B type 3. HSV-2: Herpes Simplex Virus type 2. Concentrations of *Boletus bellinii* and *Boletus subtomentosus* extracts = 10 x IC_{50}. Data represent the percentage of viral inhibition as compared to virus control and are expressed as the mean values of three independent experiments. The viral infection percentages of virus adsorption and penetration assay (during virus penetration) were calculated by deducting the rates obtained in this assay from those obtained in post-infection assay (after virus penetration). The viral infection percentages of cell pretreatment (before virus penetration) assay were calculated by deducting the rates obtained in this assay from those obtained in virus adsorption and penetration assay (during virus penetration). NA: no activity
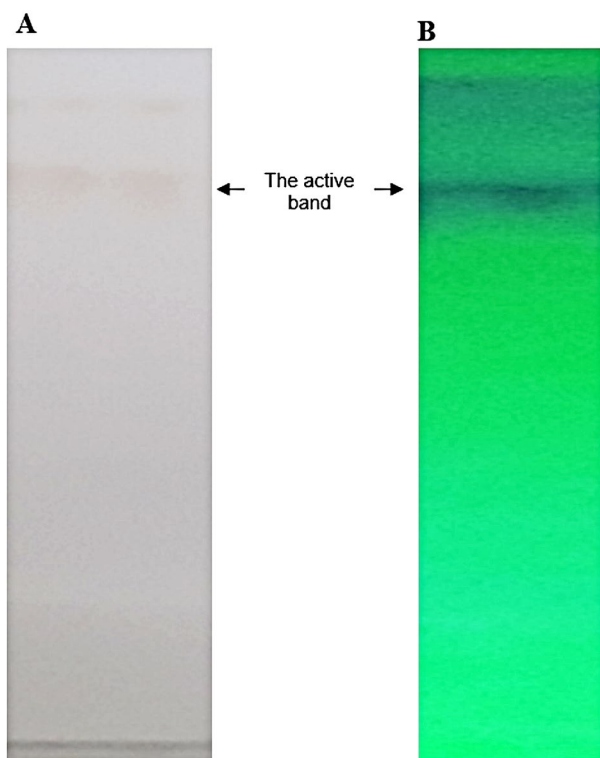

Fig. 1 TLC profile of *Boletus bellinii* extracellular crude extract under white light (A) and $UV_{254 \text{ nm}}$ light (B). The mobile phase was methanol/acetonitrile/H_2O (3:3:0.5-v/v/v)

ECEs obtained against HSV-2 were higher than those obtained against CVB-3 (SI > 600 for HSV-2 vs. < 100 for CVB-3).

Mode of antiviral action of *B. bellinii* and *B. subtomentosus* extracts

At a concentration of 10 x IC50, *B. bellinii* and *B. subtomentosus* ECEs completely inactivate HSV-2 by direct contact, inducing 100% of viral inhibition (Table 2). On the other hand, the ECE of both species revealed no virucidal effect against the non-enveloped virus CVB-3. Moreover, *B. bellinii* and *B. subtomentosus* ECEs demonstrated antiviral activity against both HSV-2 and CVB-3 during the early steps of virus replication, including virus-cell attachment, viral adsorption and penetration. However, after virus inoculation, the Boletus ECEs did not show any significant antiviral activity.

Isolation and identification of the active compound of *B. bellinii* extract

The thin layer chromatographic profiles of *B. bellinii* and *B. subtomentosus* ECEs were similar, characterized by the presence of two individualized bands (Fig. 1). Only the band with a retention factor (Rf) value of 0.88 exhibited antiviral activity against HSV-2 and CVB-3. This active band had the following characteristics: brown color under white light and blue color under UV light. The subjection of this fraction to a second TLC using the same protocol (with some variations in the proportion of the mobile phase) did not reveal other bands. Since the two extracts showed the same active band, only the one isolated from the most active extract (*B. bellinii* ECE) was scraped and subjected to HPLC-DAD-ESI-MS/
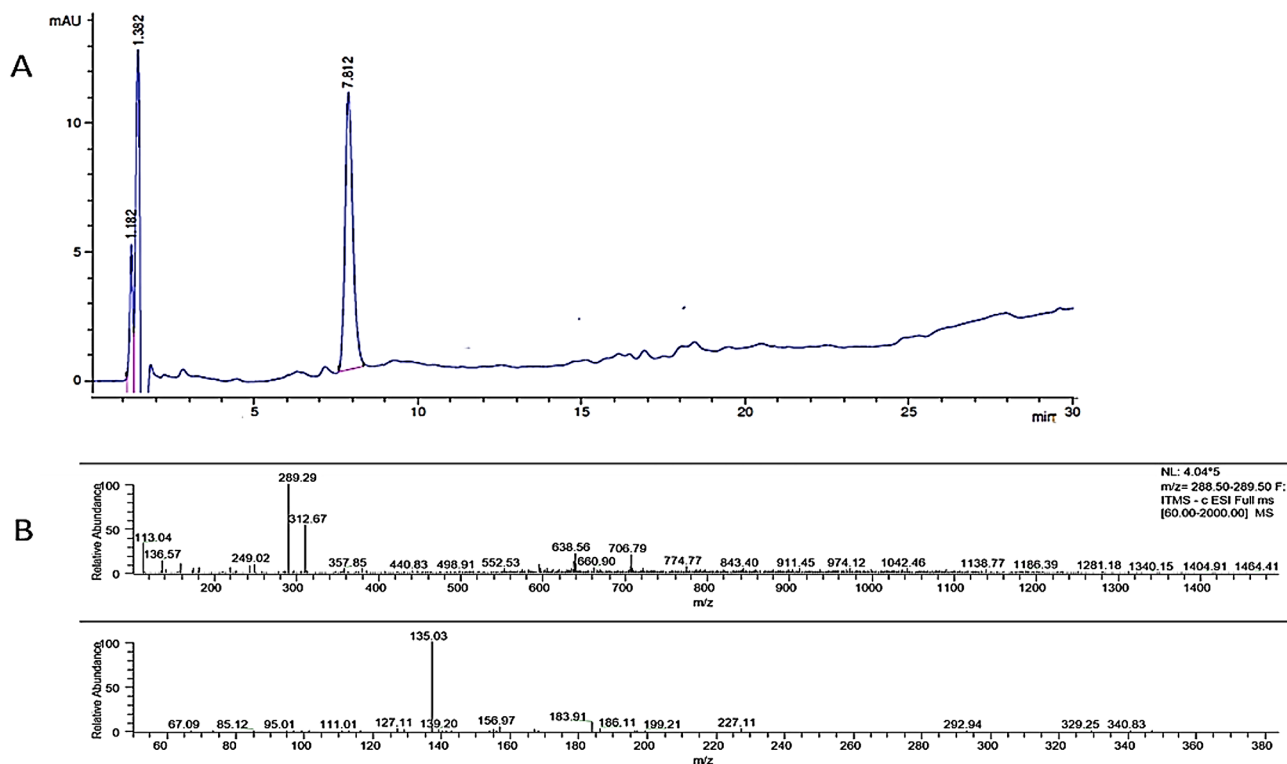
Fig. 2 UV profile registered at 315 nm (A) and MS and MS/MS spectra of the *Boletus bellinii* active fraction registered in negative ionization mode (B)
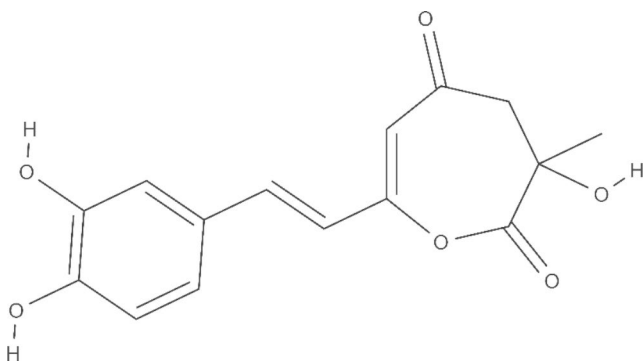

Fig. 3 Chemical structure of inonotusin A

MS analysis. The amount of the recovered active compound was 5.2 mg.

Figure 2 reports the UV chromatograms at 315 nm for *B. bellinii* active fraction. The HPLC profile showed a peak eluting at rotation time (Rt) 7.81, which gave a pseudomolecular ion at m/z 289 [M-H]$^-$ and 291 [M+H]$^+$ in negative and positive ionization modes, respectively. An adduct with sodium was also revealed in positive ionization mode at m/z 313. The fragmentation in the negative ionization mode led to a base peak at m/z 135 due to the cinnamic acid residue. The MS/MS fragmentation pattern led to the putative identification of inonotusin A (Fig. 3) by comparison of its

spectral data with those reported in the literature (Zan et al. 2011).

However, further analyses such as NMR spectroscopy will be conducted for the determination of the relative stereochemistry of the active compound.

Cytotoxicity, antiviral activity and mode of action of the active compound of *B. bellinii* extract

The purified active compound was evaluated for its cytotoxicity and antiviral activity. It showed a more cytotoxic effect than that of the native active extract (722 µg/mL versus 2677 µg/mL) and also a higher antiviral activity against both HSV-2 and CVB-3 (0.81 and 19.04 µg/mL versus 3.60 and 35.70 µg/mL, respectively) (Table 1). Therefore, the SI of the purified active compound increased compared to the native extract against HSV-2 (891 versus 744); however, the selectivity seems to decrease against CVB-3 since an augmented cytotoxicity was recorded.

The test of the mode of antiviral action performed on this compound showed similar results to those of the active extract. Better still, this compound showed a total inhibition of the virus (100%) during the virus penetration step against both HSV-2 and CVB-3 when it was tested at a concentration of 10 x IC50.

Table 3 Summary of data from some studies reporting the antiviral activity of Basidiomycota species, the target virus, their active extracts or compounds and their mode of action

Basidiomycota specie	Target virus	Active extract / compound	Mode of antiviral action	Reference
Agaricus brasiliensis	PV	Aqueous and ethanol extracts & isolated polysaccharide	Inhibition at the initial stage of the viral replication	Faccin et al. 2007
Agaricus brasiliensis	HSV	Sulfated derivative polysaccharide	Inhibition of virus attachment, penetration, cell-to-cell spread & protein expression	Cardozo et al. 2011
Auricularia uricula-judae	NDV	Sulfated Polysaccharides	Before, during and after virus infection	Nguyen et al. 2012
Ganoderma lucidum	HSV	Proteoglycan	Inhibition of virus adsorption and penetration	Liu et al. 2004
Ganoderma lucidum	EV71	Triterpenoids (Lanosta-7,9(11),24-trien-3-one,15;26-dihydroxy and Ganoderic acid)	Virucidal effect	Zhang et al. 2014
Grifola frondosa	EV71	Heteropolysaccharide	Inhibition during the viral replication (RNA synthesis, protein expression) & induction apoptosis by suppressing virus induced caspase cleavage	Zhao et al. 2016
Inonotus hipidus	IFV	Phenolic compounds (hispolon and hispidin)	Not done	Lv et al. 2017
Inonotus tamaricis	HIV	Hispidin and other molecules	Inhibition of HIV-1 integrase	Singh et al. 2003
Lentinula edodes	PV	Aqueous and ethanol extracts & isolated polysaccharide	Inhibition at the initial stage of the viral replication	Rincão et al. 2012
Lentinula edodes	HCV	mycelia solid culture extract & lignin	Inhibition of virus penetration & subgenome replication	Matsuhisa et al. 2015
Phellinus baumii	IFV	Polyphenols (hispidin, hypholomine B, inoscavin A, davallialactone, and phelligridin D)	Inhibition of neuraminidase activity	Tu and Tawata 2014
Phellinus ignarius	IFV	Sesquiterpen (the most active) and other compounds	Interaction with neuraminidase enzyme & binding site	Song et al. 2014
Pleurotus tuber-regium	HSV	Beta-glucans and sulfated derivatives	Virucidal effect	Zhang et al. 2004

Discussion

The development of novel antiviral agents is required to prevent the continuous appearance of resistant viral strains and to control the propagation of new viral infections (Pekosz and Glass 2008). Earlier research suggested that mushrooms could be a potential natural source for the development of new antiviral drugs (Linnakoski et al. 2018). It has been reported that basidiomycete fruiting body and mycelia extracts are active against various viruses, including human immunodeficiency virus, enterovirus 71, herpes simplex virus, poliovirus, and influenza viruses (Table 3). Basidiomycetes produce a variety of secondary metabolites like polysaccharides and phenolic compounds with the potential to inhibit viral replication through diverse modes of action such as virucidal effect (Zhang et al. 2004), inhibition of integrase (Singh et al. 2003), inhibition of neuraminidase activity and binding site (Song et al. 2014), inhibition of virus adsorption and penetration (Faccin et al. 2007) and inhibition during viral replication as RNA synthesis and

protein expression (Zhao et al. 2016). *Boletus* is a cosmopolitan mushroom genus of basidiomycetes with over 100 species. They are well known for their potential therapeutic and antimicrobial benefits (Garcia et al. 2022). Nevertheless, this is the first study to evaluate the antiviral activity of two edible *Boletus* species, *B. bellinii* and *B. subtomentosus*, against an enveloped DNA virus HSV-2 belonging to the Alphaherpesvirinae and a non-enveloped RNA virus CVB-3 belonging to the Picornaviridae.

The most crucial requirement for an antiviral agent is safety, and when searching for new drugs, it is important to look into potential side effects (Saxena et al. 2010). According to our research, *B. bellinii* and *B. subtomentosus* ECEs showed no significant cytotoxicity on the Vero cell line, with a CC_{50} value higher than 2000 µg/mL. The lower toxic effects imply that *B. bellinii* and *B. subtomentosus* ECEs are safe for use even at high doses (Ellan et al. 2019). On the other hand, our results provide evidence of the antiviral effect of *Boletus* extracts against HSV-2 and CVB-3, as indicated by their SI values (SI > 50 for CVB-3 and > 600

for HSV-2) (Dong et al. 2012; Abaza et al. 2019). In comparison to other natural extracts tested against HSV-2 and CVB-3 viruses, these findings suggest that *Boletus* extracts could be a very promising source for the development of new and less toxic antiviral compounds (Miaoxian et al. 2006; Hassan et al. 2015).

Phytochemical screening revealed that the ECEs of *B. bellinii* and *B. subtomentosus* contain two major fractions, of which only one showed significant anti-HSV2 and anti-CVB3 activities. Chromatographic separation and spectral analysis revealed that the active fraction contains a hispidin derivative named inonotusin A, which was first isolated from the methalonic extract of the fruiting bodies of *Inonotus hispidus* (Zan et al. 2011). Numerous researches have reported immunomodulatory (Gründemann et al. 2016), anti-allergic (Tamrakar et al. 2019), antioxidant (Zan et al. 2011), cytotoxic (Lee and Yun 2011), anti-inflammatory (Shao et al. 2015), anti-cancer (Lv et al. 2017), and anti-obesity (Tu and Tawata 2014) activities of mushroom hispidin. Additionally, it has been shown that hispidin demonstrated antiviral activities against diverse enveloped viruses, including influenza virus (IFV) types A and B (Awadh Ali et al. 2003; Hwang et al. 2015) and human immunodeficiency virus (HIV-1) (Singh et al. 2003). However, the antiviral activity of inonotusin A against HSV-2 and CVB-3 was demonstrated for the first time in this report. Furthermore, the presence of this molecule in Boletus was not described previously, even though hispidin and derivatives were mainly derived from the *Hymenochaetaceae* family, including *Phellinus* and the *Inonotus* genus (Ma et al. 2018). In addition, this compound has been rarely studied and the only report have described a potential antioxidant activity (Zan et al. 2011), as well as a moderate cytotoxic activity ($IC_{50} = 19$ μM) against a human breast carcinoma cell line (MCF-7) (Angelini et al. 2019).

In order to determine the mode of antiviral action of inonotusin A, the *B. bellinii* and *B. subtomentosus* ECEs were added at different stages during the viral infection cycle. The results indicated that both Boletus extracts exhibited anti-HSV-2 and anti-CVB-3 activities with two modes of action. Against CVB-3, these extracts acted at early steps of infection by inhibiting viral attachment and/or penetration through the cell membrane, while against HSV-2, these extracts acted directly on virus (virucidal effect), which could disturb the interaction of their envelope glycoproteins with target cell receptors, preventing them from binding to host cells.

To the best of our knowledge, this is the first study reporting the antiviral activity of *B. bellinii* and *B. subtomentosus* ECEs against CVB-3 and HSV-2. Indeed, this work reported for the first time the great antiviral potential of inonotusin A. These findings suggest a novel approach to using inonotusin A to control viral infections, with two distinct modes of action depending on the nature of the virus. In addition, our experiments demonstrated that this molecule directly inactivated HSV-2 particles and inhibited the CVB-3 viruses in the cell entry process. Further investigations are ongoing to verify the underlying antiviral mechanisms involved in these inhibitions, such as the nature of the virus attachment glycoproteins and the cellular receptors involved in this inhibition. Our results suggest that inonotusin A could be considered as a promising compound for the development of a novel and efficient naturally occurring antiviral agent with broad-spectrum.

Ethics declarations

Statements and declarations The authors did not receive support from any organization for the submitted work.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

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