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 – CC50 > 1 mg/mL) and exhibited relevant viral inhibition with 50% inhibitory concentration, i.e., IC50 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 bellinii B. bellinii.
Boletus subtomentosus B. subtomentosus.
CC50 50% cytotoxic concentration.
IC50 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.
shown that bioactive compounds isolated from mushrooms
ied and characterized. However, some investigations have
products with antiviral properties are less extensively stud
al. Many studies have shown that the few works in the litera
can inhibit the virus infection by specifically targeting
the viral enzymes, viral nucleic acid synthesis, or host cel-
lar factors that the viruses use for their reproduction (San-
toyo et al. 2012; Teplyakova and Kosogova 2015). On the
other hand, direct antiviral effects have been mainly asso-
ciated with the synthesis of phenolic secondary metabo-
lites, 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 wide-
spread wild mushrooms in North Tunisia’s forests (Ouali
et al. 2018). The Boletales order include 300 species of Bole-
tus 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 spe-
cies of the Boletales have been found to contain a diverse
range of bioactive molecules with a high potential for therap-
etic 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 Bole-
tus 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 knowl-
dge, this is the first time that the antiviral compound identi-
fied as inonotusin A was isolated from Boletus species; (iii)
this is the first study reports the antiviral activity of inonoto-
sin A against CVB-3 and HSV-2.
Materials and methods

Chemical and reagents

Violet crystal, methylecellulose, 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), trypsin-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 mm3) 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-GAACTGCGG-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% CO2. 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 (TCID50/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 x 104) were seeded onto a 96-well plate (semi-confluent monolayer cells). The medium was
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% CO₂), 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 containing 1% of methylcellulose. After 2 days of incubation at 37 °C (5% CO₂), 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 IC_{50}/CC_{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% CO₂), 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% CO₂), 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: \{[(ODv – ODc)/(ODv-ODc)] x 100, where ODv, ODc and ODc 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 x 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\(_2\)O (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 filtered, 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\(^2\) 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*
Mode of antiviral action of *B. bellinii* and *B. subtomentosus* extracts

At a concentration of 10 x IC<sub>50</sub>, *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/
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.

**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
## 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

| 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 | Cardoso 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 hispidus* | 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 mycelia solid culture extract & lignin | Inhibition at the initial stage of the viral replication | Rinção et al. 2012 |
| *Lentinula edodes* | HCV | Polyphenols | Inhibition of virus penetration & subgenome replication | Matsuhasha et al. 2015 |
| *Phellinus baumii* | IFV | Polyphenols (hispidin, hypholomine B, inoscavin A, davallialactone, and phelligrinin 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 |

### 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.
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 methanolic 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 (IC50 = 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 (viralcidal 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.

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Author contributions Conceptualization and Investigation: [Soumaya Boudagga]; Formal Analysis: [Lamjed Bouslama] and [Adele Papetti]; Validation: [Raffaella Colombo]; Data curation: [Fatma Arous]; Supervision: [Atef Jaouani]; Writing: [Soumaya Boudagga] and [Lamjed Bouslama].

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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