# In vitro anti-influenza virus (H1N1) activity of eleven species of Korean medicinal mushrooms

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# Abstract

**Background**: Oseltamivir is the most commonly used antiviral drug for the treatment and prevention of influenza. However, there are growing concerns about its use due to the risk of psychiatric side effects.

**Methods**: Eleven species of edible medicinal mushrooms (*Pleurotus ostreatus*, *Phallus rubrovolvata*, *Phallus luteus*, *Morchella esculenta*, *Grifola frondosa*, *Sarcodon imbricatus*, *Tricholoma bakamatsutake*, *Pachyma hoelen*, *Sparassis latifolia*, *Amanita caesareoides,* and *Marasmius siccus*) were collected from forests in Korea to evaluate their anti-influenza A properties. After collection, the identification of each mushroom type was verified with internal transcribed spacer (ITS) gene sequencing using fungal-specific primers. Extracts were prepared by heating dried mushroom powder at 100°C for 2 h. The cytotoxicity of the extracts was evaluated by MTT assay. The anti-influenza A properties of each extract were evaluated using the ASTM E1052-11 protocol, which is the international standardized approach.

**Results**: The efficacy of the mushroom extracts against influenza A was evaluated using hot mushroom extract solutions, each of which had a concentration of 10 g powdered mushroom per liter of hot water. This ratio was selected as all of the mushrooms had little cytotoxic effect at this concentration. The influenza virus reduction titer of *Pleurotus ostreatus* was 5.519, with a virus removal efficacy of 99.999%. This was the highest antiviral efficacy among the 11 mushroom species. The virus inhibition titers of *Phallus rubrovolvata* and *P. luteus* were 4.477 and 2.247, respectively. Their virus inhibition efficacies were 99.997% and 99.433%, respectively. The efficacy of *M. esculenta* was 90.303%. The antiviral effects of *Grifola*% that of *G. frondosa*was 78.788%, and that of *S. imbricatus*was 75.758%. The virus suppression efficacy of *Tricholoma bakamatsutake* and *Pachyma hoelen* were 66.667% and 63.636%, respectively. The extract solutions of the remaining three species (*Sparassis latifolia*, *Amanita caesareoides*, and *Marasmius siccus*) all showed a virus reduction efficacy of 60%.

**Conclusions**: This study demonstrates the potential of mushroom extracts for medicinal use as antiviral treatments for influenza A infections.

# Background

Swine influenza can be caused by several different subtypes of type A influenza viruses, including H1N1, H1N2, H2N1, H3N1, H3N2, and H2N3. It can lead to serious respiratory disease in both pigs and humans [1, 2]. After 30,000 cases of H1N1 infection were reported in over 70 countries around the world [3], the World Health Organization declared an influenza A (H1N1) pandemic on June 11, 2009 [4]. This influenza virus, which had previously affected only pigs, acquired the ability to infect humans through genetic rearrangement or mutation and quickly spread around the world [5]. Since that time, outbreaks among humans have continued to occur and H1N1 vaccines and treatments are needed to combat these [6].

The primary current treatment for H1N1 is oseltamivir, which targets neuraminidase [7]. However, reports of psychiatric side effects have led to growing concerns about oseltamivir [8]. Therefore, there is a need to discover and develop new antiviral agents [9].

Mushrooms are an established form of natural medicine and previous research has identified various bioactive metabolites in some species of mushroom that can effectively prevent and treat influenza viruses [10]. Substances derived from *Ganoderma pfeifferi*, *Phellinus baumii*, *Ph. linteus*, and *Pleurotus pulmonarius* have shown anti-influenza virus effects and are attracting attention as herbal medicines or health functional food ingredients that may reduce the risk of respiratory diseases [11–14].

Therefore, in this study, we tested the antiviral activity of influenza A virus using hot-water mushroom extracts of 11 species of edible medicinal mushrooms traditionally used to prevent colds. This study aims to present our antiviral efficacy findings for these mushroom extracts as a reference for the design of new antiviral drugs, we believe these extracts could be the basis for promising alternatives to current anti-influenza drugs.

# Methods

# Materials

Wild mushrooms collected from domestic forests were used to evaluate resistance to the influenza virus (Fig. 1). These mushrooms were identified by the internal transcribed spacer (ITS) region of the rDNA sequence using the fungus-specific primers ITS 1 and 4. Each mushroom was identified as the species with the highest homology score to its ITS sequence in the National Center for Biotechnology Information (NCBI) Genbank (https://www.ncbi.nlm.nih.gov). The analyzed nucleotide sequences were registered in the gene bank. Each hot-water extraction used dried powdered mushrooms in distilled water 10 times the dry weight of the powder. This was heated at 100°C for 2 h using and then filtered through a Millipore filter. Information on the materials used in this study is presented in Table 1.

Table 1
Materials used in this study

| Species | Collection locality | NCBI accession number | Extracted dry weight (grams) |
|---|---|---|---|
| Pleurotus ostreatus | Hongcheon | OR255977 | 10 |
| Phallus rubrovolvata | Jinju | OR255973 | 10 |
| Phallus luteus | Suwon | OR255975 | 10 |
| Morchella esculenta | Seoul | OR255969 | 50 |
| Grifola frondosa | Hongcheon | OR255971 | 20 |
| Sarcodon imbricatus | Hongcheon | OR255974 | 20 |
| Tricholoma bakamatsutake | Jeju | OR255970 | 50 |
| Pachyma hoelen | Hongcheon | OR255966 | 50 |
| Sparassis latifolia | Seoul | OR255972 | 20 |
| Amanita caesareoides | Jeju | OR255968 | 10 |
| Marasmius siccus | Seoul | OR255976 | 10 |
| NCBI, National Center for Biotechnology Information | | | |

# Cytotoxicity

For the cytotoxicity test, MDCK (Madin-Darby, canine kidney) (Korea Cell Line Bank [KCLB], Jongno-gu, Seoul, Korea) was used as the cell line, and Dulbecco's Modified Eagle Medium (DMEM) (BE12-604F, Lonza, Basel, Switzerland) as the medium for cell culture. Other reagents used for cell culture included HEPES buffer (90909C, Sigma-Aldrich, St. Louis, MO USA), fetal bovine serum (FBS) (10437028, Gibco, Thermo-Fisher Scientific, Waltham, MA, USA), gentamycin sulfate (G1914, Sigma-Aldrich), and trypsin-EDTA (T6689, Sigma-Aldrich). Cells were cultured in DMEM medium with 10−90% FBS. Cell viability was measured using a 3-(4,5-dimethythiazo-2-yl)-2,5-dipheny-tetrazoliumbrome (MTT) solution. The MDCK cells were seeded in a 96-well plate at a concentration of $1.5 \times 10^4$ cells/ well, and samples were added to each well at 80% incubation time. The MDCK cells were then cultured for 2 days in a carbon dioxide incubator. After removing the medium from the culture plate, 50 μl of 1 mg/mL MTT solution was added to each well and reacted for 4 h. Then, 10 μl of acid-isopropanol (0.04 N HCl in isopropanol) was added. The absorbance for each well was measured at a wavelength of 540 nm using a microplate reader (Microdigital, Korea). Cell viability[*] was calculated by comparison with a control group without mushroom extract.

* Cell viability (%) = ($OD_{540}$ of the treated samples/$OD_{540}$ of the untreated samples) × 100

# Mushroom antiviral activity against H1N1

An evaluation of antiviral activity was performed using the international standard method, ASTM E1052-11 (Standard Test Method to Assess the Activity of Microbicides Against Viruses in Suspension) [15]. The target virus for the evaluation was H1N1 and MDCK cells were used as the host. The virus suspension was diluted from $10^{-1}$ to $10^{-6}$ times, and 0.5 mL of each dilution was inoculated. After the cells had absorbed the diluted virus for 2 h, an overlay medium was added. To facilitate plaque counting, cell monolayers were stained with crystal violet vital dye. In both the control and the test group, the number of wells stained was counted to calculate the virus infection value (tissue culture infective dose 50% [TCID50]). The virus reduction concentration (log reduction [LR]) was calculated as follows:

## LR = LU − LT

LU and LT represent the titers of the virus control and test groups, respectively. The titer is expressed by taking the common log value ($\log_{10}\text{TCID}_{50}$/mL) of the virus infectivity titer.

## Results

## Cytotoxicity

The cytotoxicity of various mushroom hot-water extracts was evaluated by MTT assay using MDCK cells. Lactate dehydrogenase (LDH), a type of oxidoreductase, is secreted by cells when they die or their membranes are damaged. LDH reacts with water-soluble tetrazolium to form formazan, causing a color change. This enables the viability of the cells treated with mushroom extract to be measured using color differences as an indicator of the degree of cell viability. Cytotoxicity was found in the crude extract (100 g/L) of seven of the mushroom species, but not in *Tricholoma bakamatsutake*, *Sparassis latifolia*, *Amanita caesareoides*, and *Marasmius siccus* (Fig. 2). Noncytotoxicity was defined as cell viability greater than 80%. Influenza antiviral evaluations were conducted using tenfold dilutions of each species (10 g/L) as this had little toxicity in any of the variants.

## Antiviral efficacy

The virus removal efficacy of the mushroom hot-water extracts against H1N1 was evaluated and the results are summarized in Table 2. The titer (log $\text{TCID}_{50}$/mL) of the *Pleurotus ostreatus* control group was 7.519 and that of the test group was 2.000. The influenza virus reduction titer was calculated as 5.519. Accordingly, the virus removal efficiency was 99.999%. This antiviral efficacy was the highest among the 11 mushroom hot-water extracts. The control titers (log $\text{TCID}_{50}$/mL) of *Phallus rubrovolvata* and *P. luteus* were 8.477 and the titers of the test groups were 4.000 and 6.230, respectively. Their influenza virus inhibitory titers were measured as 4.477 and 2.247, respectively, and their virus inhibitory efficacies were 99.997% and 99.433%. The TCID50/mL value of *Morchella esculenta* was 6.505, 1.013 less than the control group value of 7.519. The virus reduction rate was 90.303%. The control titers (log $\text{TCID}_{50}$/mL) of

*Grifola frondosa* and *Sarcodon imbricatus* were 7.519, while the titers of their test groups were 6.845 and 6.903, showing similar antiviral effects of 78.788% and 75.758%, respectively. The infectivity titers of *Tricholoma bakamatsutake* and *Pachyma hoelen* were similar at 7.041 and 7.079, which were 0.478 and 0.44 lower than the control group titer. Their influenza virus inhibitory efficacies were 66.667% and 63.636%, respectively. The remaining three mushroom hot-water extracts showed virus reduction effects below 60%. The TCID50/mL values of *Sparassis latifolia*, *Amanita caesareoides*, and *Marasmius succus* were 7.146, 7.230, and 7.415, respectively, and their virus inhibition rates were 57.576%, 48.485%, and 21.212%.

Table 2
Antiviral activity of mushroom extract against H1N1

| Species | Infectivity titer of control (log) | Infectivity Titer of the test group (log) | Virus reduction of the infectivity titer (log) | Efficacy of virus reduction (%) |
|---|---|---|---|---|
| Pleurotus ostreatus | 7.519 | 2.000 | 5.519 | 99.999 |
| Phallus rubrovolvata | 8.477 | 4.000 | 4.477 | 99.997 |
| Phallus luteus | 8.477 | 6.230 | 2.247 | 99.433 |
| Morchella esculenta | 7.519 | 6.505 | 1.013 | 90.303 |
| Grifola frondosa | 7.519 | 6.845 | 0.673 | 78.788 |
| Sarcodon imbricatus | 7.519 | 6.903 | 0.615 | 75.758 |
| Tricholoma bakamatsutake | 7.519 | 7.041 | 0.477 | 66.667 |
| Pachyma hoelen | 7.519 | 7.079 | 0.439 | 63.636 |
| Sparassis latifolia | 7.519 | 7.146 | 0.372 | 57.576 |
| Amanita caesareoides | 7.519 | 7.230 | 0.288 | 48.485 |
| Marasmius siccus | 7.519 | 7.415 | 0.104 | 21.212 |

## Discussion

The ASTM E1052-11 (Standard Test Method to Assess the Activity of Microbicides Against Viruses in Suspension) used in this study is an internationally recognized standardized test for the antiviral activity

of test substances containing viruses in suspension [16]. It has been used to measure the activity of viruses including Ebola virus [17], human norovirus [18], severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [19], and human coronavirus-229E (HCoV-229E) [20].

Although the development of antiviral influenza drugs has significantly reduced influenza mortality, this group of viruses remain a global threat to public health. Therefore, the need to discover and develop new antiviral agents has emerged [9]. There has been increased interest in natural remedies such as star anise, which have been used as antivirals in the past before the advent of modern medicine. The scientific examination of those herbal medicines and traditional remedies that show some degree of efficacy is potentially fruitful as a source of treatments in itself and as an investigative tool for the identification of new cellular and molecular approaches to the treatment of a given pathology [21]. The treatment and prevention of influenza viruses with extracts of various bioactive metabolites from fungi are among such natural remedies that have been found effective [10]. Therefore, in this study, we evaluated the efficacy of influenza A virus inhibition using hot-water extracts of edible medicinal mushrooms that have traditionally been consumed to prevent colds. Our results show that many variants of these mushrooms show considerable promise for use in antiviral treatments.

*Pleurotus ostreatus* is an edible mushroom cultivated worldwide [22]. This species showed the highest anti-influenza A virus efficacy (with a virus reduction rate of 99.999%) among the 11 mushrooms investigated. It is known to have various positive functional effects on health, having demonstrated antioxidant [23], anticholesterolemia [24], and anti-hyperlipidemia [25] effects in previous studies. It has also shown antiviral efficacy against human immunodeficiency virus (HIV) and hepatitis C virus (HCV) [26, 27]. The anti-influenza A virus efficacy of *Pleurotus ostreatus* was investigated in a previous study using NaCl-eluted mycelium extract, which showed a strong antiviral effect [28]. The mushroom extract using fruiting bodies, which was attempted for the first time in this study, also showed a significantly high antiviral effect.

Mushrooms of the *Phallus* genus, including the phylogenetically similar *P. rubrovolvata* and *P. indusiatus*, have been traditionally consumed in China since ancient times (618 AD) [29, 30]. *P. indusiatus* is known to have neuroprotective [31] and anti-inflammatory effects [32], while *P. impudicus* has antiviral effects. In particular, the hot-water extract of the fruiting body of *P. impudicus* has been found to inhibit intracellular influenza virus H5N1 replication by 5.20 (± 1.50) lg, and the cultured mycelium extract by 4.45 (± 1.60) lg [33]. *Phallus rubrovolvata* (Syn. *Dictyophora indusiata*) has recently attracted research attention as a medicinal mushroom due to the discovery of antioxidant and antiglycation properties in polysaccharides isolated from the pileus of the fruiting body [34]. However, this is the first study of the antiviral efficacy of this species, and it showed an antiviral efficacy of over 99.99%, further increasing its therapeutic value.

The pharmacological value of *P. luteus* came to light when a new sesquiterpene, phallac acid B(2), was isolated from the fruiting bodies of this mushroom [35]. An evaluation of its α-glucosidase inhibitory activity found that phallac acid B(2) has significantly greater inhibitory effects on α-glucosidase than

acarbose, an enzyme that breaks down carbohydrates in the small intestine. No other pharmacological research has been conducted, and the present study was the first to evaluate the antiviral properties of *P. luteus* against influenza. We found the antiviral efficacy of this mushroom to be more than 99%, indicating the need for further research on the antiviral components of *P. luteus*.

*Morchella esculenta* is a globally popular edible mushroom due to its unique taste and aroma and high nutrient content [36]. Polysaccharides of this species exhibit diverse bioactivities, including antioxidant, anti-inflammatory, immunomodulatory, hypoglycemic, atherosclerotic, and antitumor effects. In particular, its polyphenols, protein hydrolysates, and several crude extracts are known to exert strong effects [37]. Although this mushroom's bioactive substances have been widely studied, its antiviral efficacy has only been evaluated in relation to HIV [38]. Our study confirmed an influenza virus reduction rate of over 90%, adding new functional benefits to this mushroom's therapeutic repertoire.

*Grifola frondosa* has been consumed as an edible mushroom for health purposes for a long time in Asia. When modern science began to identify the various pharmacological effects and bioactive substances of this mushroom, its consumption increased and artificial cultivation began for the first time in Japan [39]. Compounds isolated from *G. frondosa* have been reported to have significant antiviral effects against hepatitis B virus (HBV), enterovirus 71 (EV71), herpes simplex virus type 1 (HSV-1), and other herpes simplex virus infections [40]. Based on previous research results, we had expected the influenza virus resistance of *G. frondosa* to be high, but it showed a relatively low efficacy of around 78%.

*Tricholoma matsutake* has been consumed in Japan for approximately 1250 years and is reported to have a unique taste and aroma. *T. bakamatsutake* is similar in appearance to *T. matsutake*, and its commercial value is recognized, making it popular enough to warrant study for potential artificial cultivation [41]. However, to date, there have been few studies on the pharmacological efficacy of *T. bakamatsutake*. Therefore, we evaluated the antiviral properties of this mushroom.

The polysaccharide isolated from *Sarcodon imbricatus* not only regulates immunity and has some anticancer efficacy but also significantly enhances the immune activity of the hematopoietic system [42, 43]. Although we expected to have high resistance to the influenza virus, its efficacy was relatively low.

*Pachyma hoelen* has excellent antitumor, immunomodulatory, anti-inflammatory, and antioxidant effects and has been used as a medicinal mushroom for approximately 2,000 years [44]. *Sparassis crispa*, a species phylogenetically similar to *S. latifolia*, is high in polysaccharides that strengthen immunity and have anticancer efficacy. It is widely consumed in Asia as a folk remedy, particularly in China, Korea, and Japan [45, 46]. Various bioactive substances have been identified in these mushrooms, but their extracts did not show satisfactory antiviral effects against influenza in the present study.

*Amanita caesareoides* mushrooms are eaten as a delicacy [47], and *Marasmius siccus* is an ecologically important mushroom as it facilitates the decomposition of fallen leaves [48]. Our antiviral evaluations to determine the pharmacological value of these two species did not obtain satisfactory results.

# Conclusions

Based on previous reports that have isolated various bioactive metabolites from mushrooms that are effective in preventing and treating influenza viruses we evaluated the activity of 11 species of mushrooms against influenza A. These mushrooms have traditionally been used to prevent colds. Analysis was performed according to an internationally standardized virus activity evaluation method using hot-water extracts of 11 mushrooms collected from Korean forests. The virus reduction titers calculated were indicative of We found high levels of virus resistance. This suggests that hydrothermal extracts of certain mushrooms are potential candidates for the development of new influenza A virus treatments. However, additional research is needed to isolate the virus-reducing substances and determine the mechanisms of action.

# Abbreviations

**ASTM-E1052-11** American Society for Testing and MaterialsStandard Test Method to Assess the Activity of Microbicides Against Viruses in Suspension

**DMEM** Dulbecco's Modified Eagle Medium

**EV71** Enterovirus 71

**FBS** Fetal bovine serum

**HBV** Hepatitis B virus

**HC1** Hydrochloric acid

**HCoV-229E** Human coronavirus-229E

**HCV** Hepatitis C virus

**HEPES** (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid

**HIV** Human immunodeficiency virus

**HSV-1** Herpes simplex virus type 1

**ITS** internal transcribed spacer

**LDH** Lactate dehydrogenase

**LT** Titers of the virus test group

**LU** Titers of the virus control group

**MDCK** Madin-Darby canine kidney (cell line)

**MTT** 3-(4,5-dimethythiazo-2-yl)-2,5-diphenyl-tetrazolium bromide

**NaCl** Sodium chloride

**SARS-CoV-2** Severe acute respiratory syndrome coronavirus 2

**Syn**. Synonym

**TCID50** Tissue culture infective dose 50%

**Trypsin-EDCA** Trypsin-ethylenediaminetetraacetic acid

# Declarations

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Data availability

All data generated or analyzed during this study have been included in this published article.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

RR: conceptualization, investigation, methodology, resources, original draft, visualization, HL: investigation, methodology, resources.

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# Figures

**Figure 1**

The Korean forest wild mushrooms used in this study. (A) *Pleurotus ostreatus*; (B) *Phallus rubrovolvata*; (C) *Phallus luteus*; (D) *Morchella esculenta*; (E) *Grifola frondosa*; (F) *Sarcodon imbricatus*; (G) *Tricholoma bakamatsutake*; (H) *Pachyma hoelen*; (I) *Sparassis latifolia*; (J) *Amanita caesareoides*; (K) *Marasmius siccus*.
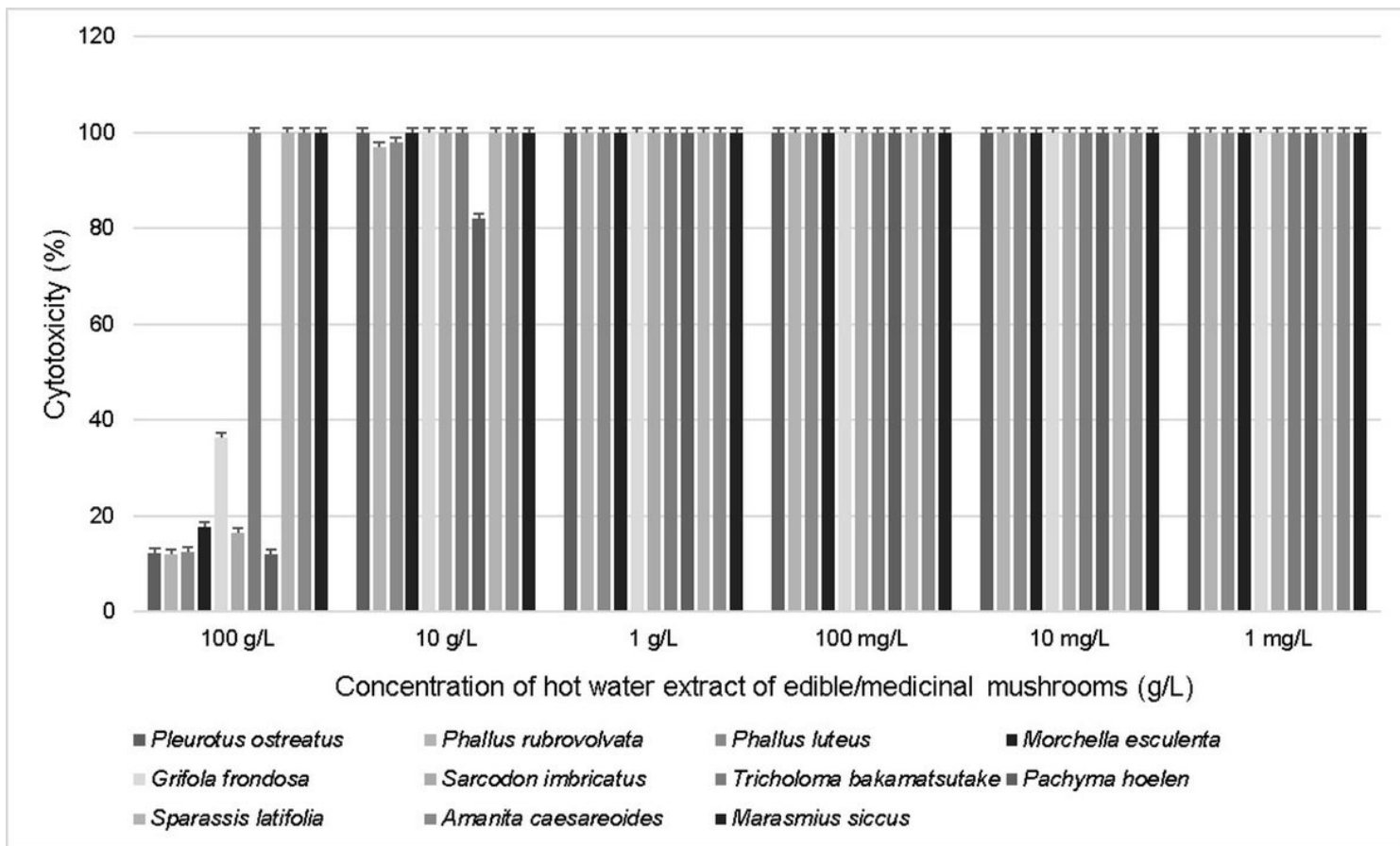
**Figure 2**

Cytotoxicity of medicinal mushroom hot-water extracts