Evaluation of an inhibitory effect of edible mushroom extracts against rotavirus infection

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Received 17 November 2017
Accepted 28 January 2018

Egyptian Pharmaceutical Journal
2018, 17:77–84

Background
According to recent statistics by the WHO, the annual mortality rate associated with diarrhea is 30 deaths per 100 000 among Egyptian children younger than 5 years. Nearly 3.9% of the reported deaths are because of rotavirus infection. It is necessary to look for alternative treatment against rotavirus.
Aim and methods
The aim of this study is to investigate the antiviral activity of aqueous (AqE) and ethanol (EtOHE) extracts of the fruiting bodies of *Agaricus* spp and *Pleurotus ostreatus* against rotavirus infection using cell culture-based MTT assay.
Results
The tested extracts demonstrated significant inhibition effects against rotavirus infection up to 96.7, 90.6, 86.3, and 83.1% at concentration of 1000 μg/ml of *P. ostreatus* EtOHE, *P. ostreatus* AqE, *Agaricus* spp EtOHE, and *Agaricus* spp AqE, respectively, when added at zero time of the infection. Nothing was observed when extracts were added after viral infection. The synergistic activity was observed when different extracts were combined. Our results exhibited an inhibitory effect against different phases of rotavirus infection.
Conclusion
The use of edible mushrooms as a potential antiviral substance might be an alternative treatment against rotavirus infection. Nonetheless, more investigations are requiring for studying the efficacy *in vivo* and for segregating their fractions, which might clarify the mechanism of the inhibitory effect.

Keywords:
antiviral activity, aqueous or ethanol extract, mushroom, rotavirus

Egypt Pharmaceut J 17:77–84

1687-4315

Introduction

Rotaviruses are a major cause of acute gastroenteritis in children worldwide [1]. To date, there are two rotavirus vaccines (Rotarix and RotaTeq). Their effectiveness requires more efforts to develop and improve [2]. However, rotavirus vaccines are costly and not recommended to use in immunocompromised children. There is urgent need to look for alternative treatment against rotavirus infections. Natural products are one of the best candidates for antiviral drugs as they are effective and inexpensive. Currently, many natural compounds have known to have antirotavirus effects in clinical studies [3–5], in animal experiments [6], and *in vitro* [7–10].

Edible mushrooms have been consumed for centuries as food and in a type of tea. It could be viewed as an extraordinary hotspot for proteins, carbohydrates, and bioactive metabolites for health promotion and disease prevention [11]. The previous studies suggested that mushrooms have been used as anticarcinogenic, antioxidant, or for stimulating the immune system. The therapeutic properties of mushrooms are mainly attributed to their content of polysaccharides and phenolic compounds, which exhibit anticancer, antiviral, antibacterial, and antioxidants activities [12–18].

The antiviral activity of edible mushroom has been demonstrated against HIV and western equine encephalitis virus (WEE), but there is nothing about its activity against rotavirus, particularly with respect to *Agaricus* spp. and *Pleurotus ostreatus*. For example, Wang and Ng [19] isolated a glycoprotein from fruiting body of *P. ostreatus* and demonstrated its antiviral activity against HIV. The previous report of Sorimachi *et al*. [20] showed that the aqueous extract of *Agaricus brasiliensis* has an inhibitory effect against WEE virus.

Generally, the antiviral activity of the compounds isolated from the consumable mushroom might be

attributed to direct effect against viral infection or indirectly by stimulate the immune system [14,21].

Materials and methods

Sample preparation

Both aqueous and ethanol extracts of *P. ostreatus* and *Agaricus* spp. were kindly provided by Dr Heba E. El-Henawy, Botany and Microbiology Department, Faculty of Science, Helwan University, Egypt. In summary, to obtain the extracts, two grams of the fruiting bodies of each mushroom were dissolved in 10 ml of water or absolute ethanol at ambient temperature under agitation for 1 h. Extracts were filtered using 0.2-μm syringe filter (Millipore Corp., Bedford, Massachusetts, USA).

Cells and viruses

Rotavirus SA-11 strain was obtained from Virology Department of National Institute for Cholera and Enteric Diseases, Kolkata, India, by Dr Mohamed Nasr, National Research Centre. For rotavirus activation, 10 μg/ml of treated trypsin was added to virus suspension followed by incubation time for 60 min at 37°C. *MA-104* (African green monkey kidney) cell line within the sight of 10 μg/ml of treated trypsin was used for rotavirus propagation. Use of treated trypsin was for increasing the infectivity of rotavirus [22,23]. The virus titer was estimated using the limit-dilution method and expressed as a 50% cell culture infective dose of 1×10^6 [24]. Virus stock solution was stored at −80°C until use.

Determination of cytotoxicity by morphological changes

Cytotoxicity of the prepared extracts was estimated on uninfected MA-104 cell monolayers, which was prepared in 24-well plates containing Dulbecco's modification of Eagle's medium with 10% fetal bovine serum (FBS, Gibco BRL) and 100 μg/ml of antibiotic mixture. After 24 h of incubation at 37°C in atmosphere 5% CO_2, the growth medium was discarded and 100 μl of various concentrations (1000, 500, 250, 125, and 0 μg/ml) of each extract were added to confluent MA-104 cell monolayers and incubated in fresh growth medium. After 24h and 48h of incubation, the morphological changes of examined cells were evaluated using inverted microscope.

Determination of cytotoxicity by MTT assay

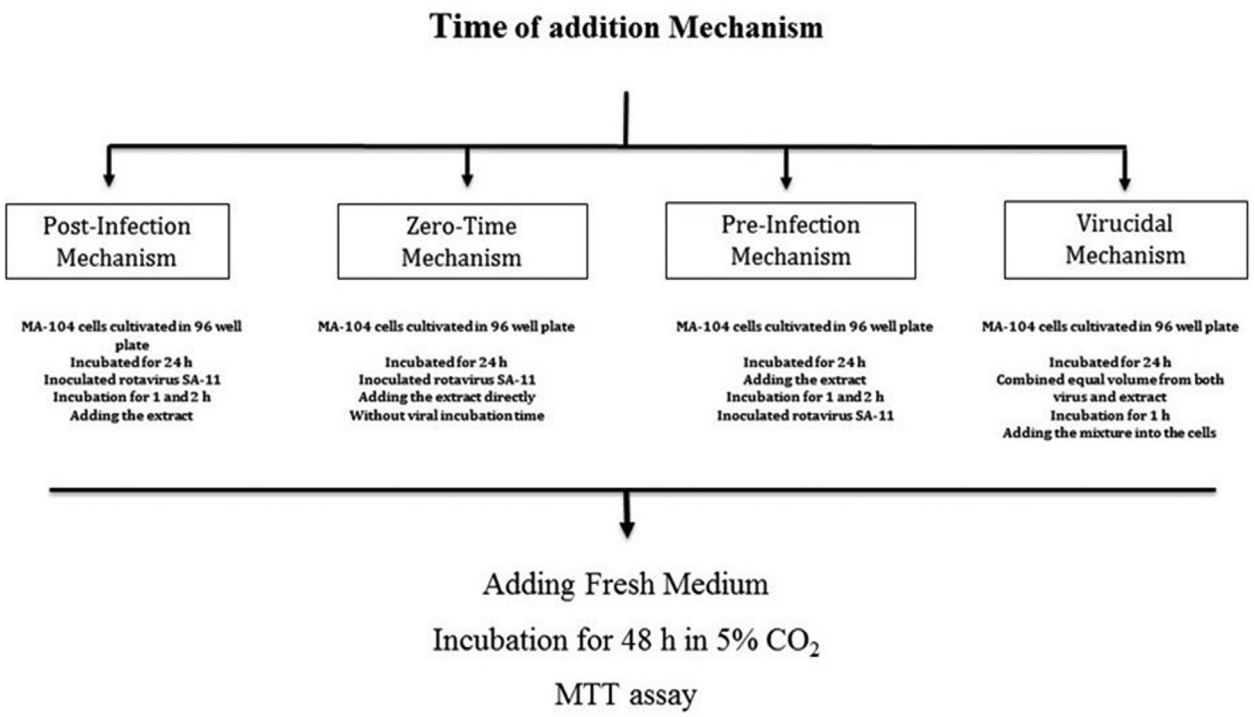
According to Miranda *et al*. [25], 100 μl of 2×10^4 uninfected cells/well was seeded into 96-well plates and incubated for 24 h. The growth medium was removed and inoculated by various concentrations (1000, 500, 250, 125, and 0 μg/ml) in fresh medium for additional 48 h of incubation time at 37°C under humidified atmosphere of 5% CO_2. The medium was replaced by 100 μl of MTT solution (5 mg/ml) and was incubated for further 4 h at 37°C. The MTT solution was replaced by 50 μl of acidified isopropanol and incubated for 30 min at 37°C. The optical density was identified using ELISA reader at 570 nm. The 50% cytotoxic concentration (CC_{50}) was calculated as $(A-B/A)\times100$, where A and B are equivalent to the mean of three OD_{570} of untreated and treated cells, respectively.

Determination of antiviral assay by MTT assay

One hundred microliter of *MA-104* cells was cultured in 96-well plate, with a total number of 2×10^4 cells/well, and incubated for 24 h. The culture medium was replaced by adding 100 μl of tested extract or virus, according to the next sections of distinctive methodologies. A 50% inhibitory concentration (IC_{50}) value was calculated as $[(A-B)/(C-B)\times100]$, where A, B, and C refer to the mean three absorbance of the tested extract with virus, virus positive control, and cell negative control, respectively, by using MTT assay. In addition, the therapeutic index has been calculated and characterized as the ratio of CC_{50}/IC_{50}.

The effect of addition time of each extract against CPE of rotavirus infection was determined according to Yang *et al*. [26]. Briefly, *MA-104* cells cultured in 96 microplates were submitted into each concentration of the extract before (−1 and −2 h), zero time, and after (1 and 2 h) viral infection (Fig. 1). After incubation, the antiviral activity was calculated using MTT assay, and each experiment was done in duplicates. (a) Postinfection assay was done as follows: 100 μl of MA-104 cells (2×10^4 cells/well) was cultivated in 96-well plate and incubated for 24 h. The culture medium was replaced by 100 μl of rotavirus SA-11 and incubated on the cells for 1 and 2 h at 37°C in 5% CO_2. After incubation time, 100 μl of extract was added, followed by fresh growth medium and incubated for further 48 h. (b) For the zero time assay, the same previous procedures were performed, except that the 100 μl of each extract was added directly after inoculation of rotavirus without any incubation time. (c) Preinfection assay was done according to Zhu *et al.* [27], where 100 μl of MA-104 cells was cultivated and incubated for 24 h. The medium was replaced by 100 μl of extract, followed by incubation time for 1 or 2 h at 37°C in 5% CO_2. Overall, 100 μl of rotavirus SA-11 was added to treated cells, followed by incubation time for 48 h. (d) For virucidal assay, 100 μl of both rotavirus and each concentration of extract was mixed together

Figure 1



Summary for time of addition mechanisms for evaluating the antiviral activity.

before inoculating into the cells, followed by 48 h of incubation.

Synergistic activity

Synergy effect, combined activity of two different extracts, was investigated. Separate concentrations (125 and 250 µg/ml), which was examined previously during zero time assay, were combined and tested in the half quantity used during zero time assay. Herein, 50 µl of extract at concentration of 125 or 250 µg/ml was combined with 50 µl of different extract at the same concentration. Overall, 100 µl of extract mixture was added directly after inoculation of rotavirus followed by 48 h of incubation.

Confirmation of rotavirus infection

Viral RNA was extracted from MA-104 cells by Qiagen Kit (Hilden, Germany), according to the manufacturer's instructions. Overall, 5 µl of extracted RNA was shock treated at 95°C for 5 min and chilled on ice for 5 min. A reverse transcription (RT)-PCR method based on amplification of a *VP6* fragment was used. Rota-A forward primer: 5-GGATGTCCTGTACTCCTTGTCAAAA-3, and Rota-A reverse primer: 5-TCCAGTTTGGAA-CTCATTTCCA-3, each at a concentration of 1 µmol/l, were used in an RT reaction. Five microliter of cDNA product has been used for a PCR program, which was as 10 min at 95°C and 30 cycles of 15 s at 95°C, 1 min at 60°C, and 1 min at 72°C, which amplify a 144-bp product [28].

Results

Cytotoxicity assay

In this work, the tested extracts of edible mushrooms (*Agaricus* spp. and *P. ostreatus*) have been investigated to study the inhibitory activity against rotavirus infection. The cytotoxicity was identified by using (a) optical microscopy for examination of the morphological changes of the treated cells and contrasted with untreated cells as a control. (b) Cytotoxicity was evaluated using *MTT* assay to calculate the CC_{50}. No concentration of both extracts was poisonous at 1000 µg/ml. The CC_{50} for the AqE and EtOHE of *Agaricus* spp. was 2902 and 2525 µg/ml, respectively, whereas the CC_{50} was 6447 and 3550 µg/ml for the AqE and EtOHE of *P. ostreatus*, respectively (Table 1).

Effects of time of addition

Our outcomes demonstrated that the strongest percentages of viral inhibition were 96.7, 90.6, 86.3, and 83.1% at concentration of 1000 µg/ml of EtOHE of *P. ostreatus*, AqE of *P. ostreatus*, EtOHE of *Agaricus* spp., and AqE of *Agaricus* spp., respectively, when added at zero time. During virucidal mechanism of an infection, the tested extract (1000 µg/ml) showed moderate direct effect of viral inhibitions, which were

71, 56.5, 50.3, and 22% for EtOHE of *P. ostreatus*, AqE of *P. ostreatus*, EtOHE of *Agaricus* spp., and AqE of *Agaricus* spp., respectively, whereas in the preinfection period, the tested extracts showed little effect of viral inhibitions when added before viral infection, which were 27.9, 26.2, 41.0, and 20.0% for EtOHE of *P. ostreatus*, AqE of *P. ostreatus*, EtOHE of *Agaricus* spp., and AqE of *Agaricus* spp., respectively (Figs 2–5). Finally, no inhibitory effect was observed when extracts were added after viral infection. The IC_{50} and the therapeutic index were identified depending on the outcomes of time of addition obtained at zero time (Table 1).

Table 1 An inhibitory concentration and therapeutic index of aqueous and ethanol extracts of *Agaricus* spp. and *Pleurotus ostreatus* against rotavirus on MA-104 cells

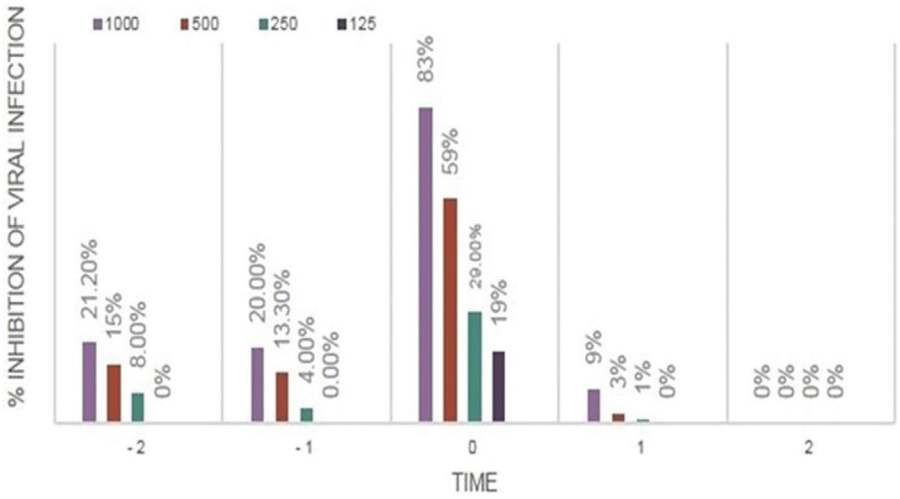
Extracts	CC_{50}	IC_{50}	Therapeutic index[a]
AqE extract of *Agaricus* spp.	2902	502.0	5.78
EtOHE of *Agaricus* spp.	2525	426.5	5.92
AqE extract of *Pleurotus ostretus*	6447	511.5	12.6
EtOHE of *Pleurotus ostretus*	3550	210.0	16.90

AqE, aqueous; CC_{50}, 50% cytotoxic concentration; EtOHE, ethanol; IC_{50}, 50% inhibitory concentration. [a]Therapeutic index=CC_{50}/IC_{50}.

Synergistic effect between two mushroom extracts

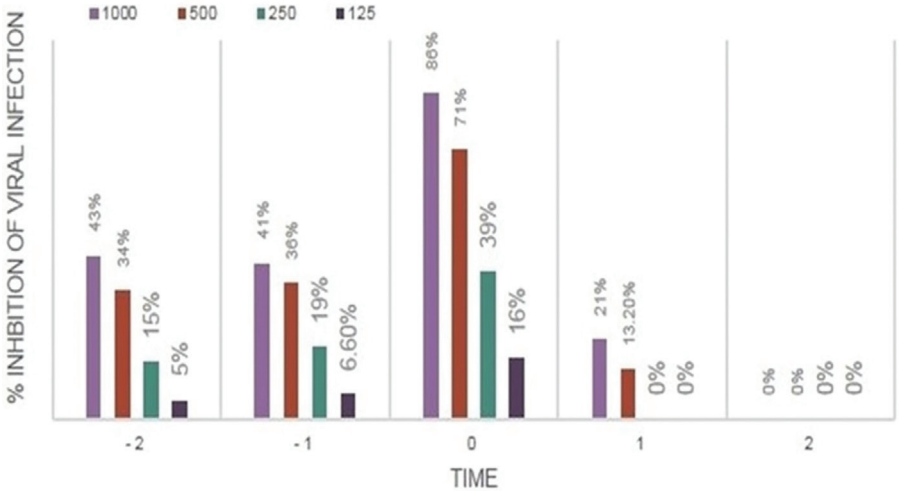
Two mixed mushroom extracts demonstrated synergistic activity in this study. The AqE of *Agaricus* spp. (125 μg/ml) and EtOHE of *P. ostreatus* (125 μg/ml) inhibited viral infection with 19.0 and 51.1%, respectively, whereas a combination of both

Figure 2



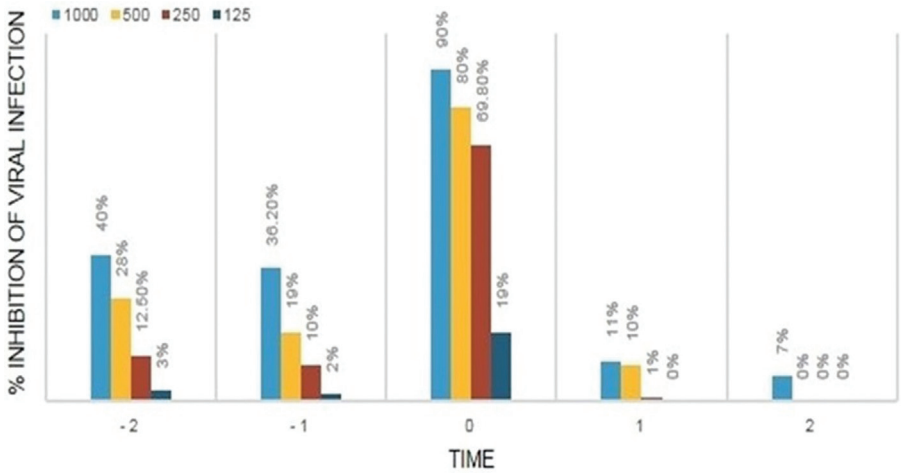
Inhibitory effects of aqueous extract of *Agaricus* spp. against rotavirus, according to time addition. Data represents the mean±SD of duplicate samples with similar results.

Figure 3



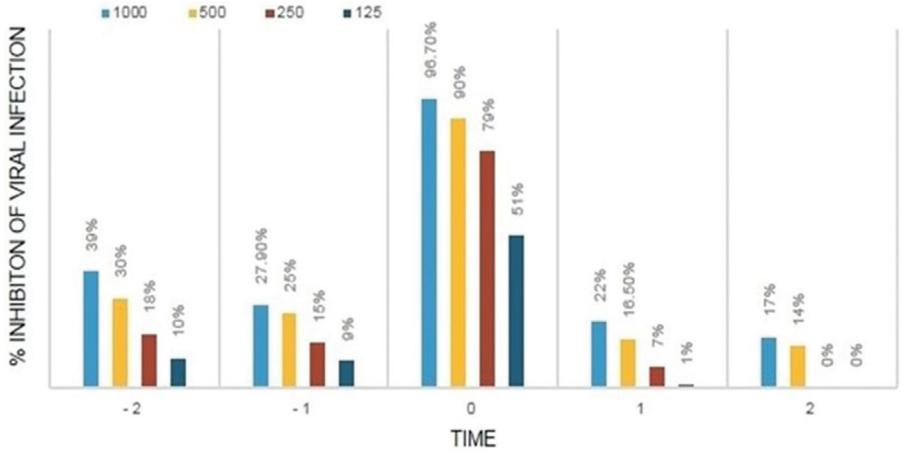
Inhibitory effects of ethanol extract of *Agaricus* spp. against rotavirus, according to time addition. Data represents the mean±SD of duplicate samples with similar results.

Figure 4



Inhibitory effects of aqueous extract of *Pleurotus* spp. against rotavirus, according to time addition. Data represents the mean±SD of duplicate samples with similar results.

Figure 5



Inhibitory effects of ethanol extract of *Pleurotus* spp. against rotavirus, according to time addition. Data represents the mean±SD of duplicate samples with similar results.

extracts of AqE of *Agaricus* spp. and EtOHE of *P. ostreatus* demonstrated an inhibition of up to 68.7%. In addition, the same mechanism of the synergistic effect was observed when EtOHE of *Agaricus* spp. and EtOHE of *P. ostreatus* have been combined, with an inhibition activity up to 77.0%. However, each single extract showed antiviral activity up to 16.4% with EtOHE of *Agaricus* spp. (125 µg/ml) and 51.1% with EtOHE of *P. ostreatus* (125 µg/ml) (Figs 6 and 7).

Analysis

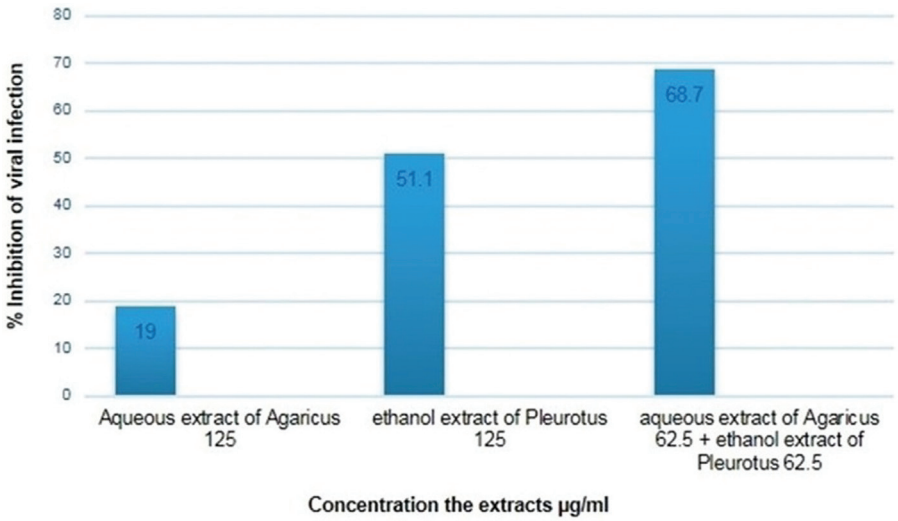
The measurement of 50% cell culture infective dose of simian rotavirus SA-11 strain was computed according to the Reed and Muench method [24]. In addition, the IC_{50} values were identified by statistically validated nonlinear regression curve and calculated from the mathematical regression curve formula.

Discussion

Recently, there is an evolving eagerness for associating pharmacology with nourishment science. Pharmaceuticals are created to cure sickness, and the essential objective of nourishment is to keep up or enhance well-being. This does not propose that there is no part for nourishment in anticipating or curing affliction [29]. The extracts of consumable mushrooms have been investigated previously for their antioxidants, antibacterial, or antiviral effects, but nothing was reported about their antiviral activity against rotavirus [12,13,30,31]. In this work, the antiviral activity of aqueous (AqE) and ethanol (EtOHE) extracts of *Agaricus* spp. and *P. ostreatus* has been investigated against CPE of rotavirus infection.

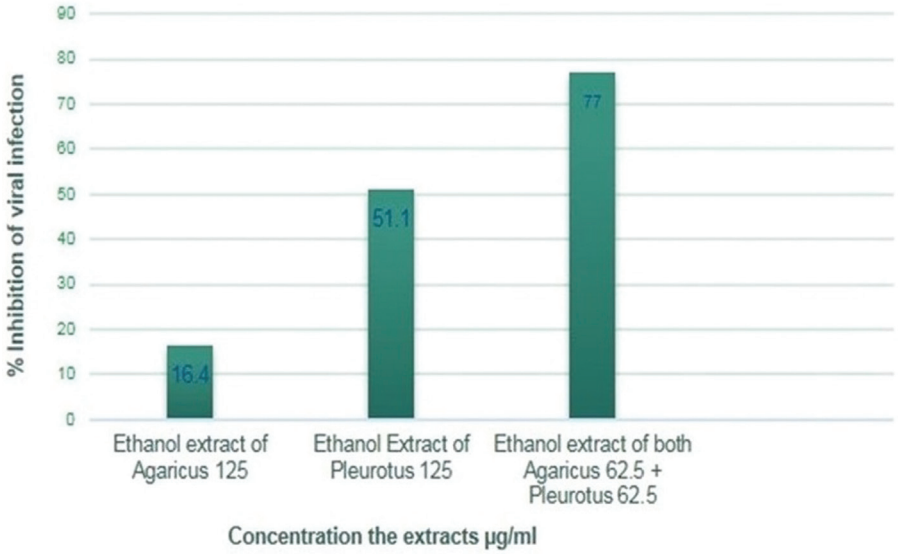
These extracts demonstrated a capability to inhibit the cytopathic effect of rotavirus infection depending

Figure 6



Synergistic effect of antiviral activity of mixed aqueous extract of *Agaricus* spp. and ethanol extract of *Pleurotus* spp. Data represents the mean±SD of duplicate samples with similar results.

Figure 7



Synergistic effect of antiviral activity of mixed ethanol extract for *Agaricus* spp. and *Pleurotus* spp. Data represents the mean±SD of duplicate samples with similar results.

on the concentration of the extract and the contact time. Both the AqE and the EtOHE extracts have solid inhibitory actions when added at zero time of viral infection, and moderate to little effects during virucidal and preinfection stages at concentration of 1000 µg/ml. Our findings are similar with the previous reports of Zhang *et al.* [32] and Faccin *et al.* [33]. They demonstrated that the fractions obtained from B-glycan isolated from *P. tuberregium* were more effective when added simultaneously with herpes simplex type 1 and 2 at the time of viral infection. In addition, the findings of virucidal and preinfection stages are similar with the results of Sorimachi *et al.* [20] and McClure *et al.* [34] They demonstrated that the fractions isolated from *A. brasiliensis* and *Ganoderma lucidum* mycelia slightly inhibited the early steps of viral replication.

Our results recommend a direct action of the extracts on viral replication and viral molecule itself, which prompted to repress the replication and adsorption stages. However, the outcomes of the present study showed no efficacy during postinfection phase in contrast with other phases of the viral infection. These results are similar with the previous reports of Liu *et al.* [21] and Lopes *et al.* [35]. They observed a remarkable antiviral activity during pretreated and

treated herpes virus infection more than the treatment of postinfection.

The present study demonstrated that the effect of ethanol extracts was more valuable than aqueous extracts against rotavirus. These results were in agreement with the report of Sorimachi *et al.* [20], who studied the antiviral activity of ethanolic extract of *Agaricus blazei* against WEE virus, poliovirus, and herpes simplex virus. He found higher antiviral activity for ethanol extract more than aqueous extract.

The findings of this study showed that each extract alone have a potent inhibitory activity against rotavirus infection, but low concentrations have a weak effect. The combination between EtOHE of *Agaricus* spp. and EtOHE of *P. ostreatus* greatly inhibited rotavirus, which tended to be further decreased when compared with that of EtOHE of *Agaricus* spp. or EtOHE of *P. ostreatus* alone. These results suggested that EtOHE of *Agaricus* spp. and EtOHE of *P. ostreatus* have synergistic effect. This kind of combined treatment will offer an excellent method for treating severe diseases like those caused by viruses, because this kind of treatment would be benefit to inhibit the viral infection and decrease the toxicity [36].

Conclusion

The utilization of mushroom extracts as a possible element for the prevention or cure rotavirus infection might be an alternative treatment. These extracts might act on the viral particle and the replication cycle of rotavirus. However, more studies are required for studying the isolated fractions of these extracts that might clarify the mechanism of the antiviral activity.

Financial support and sponsorship

Nil.

Conflicts of interest

There is no conflict of interest.

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