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EFFECT OF *MORCHELLA CONICA* AND *FOMES FOMENTARIUS* EXTRACTS AGAINST THE HUMAN RESPIRATORY SYNCYTIAL VIRUS *IN-VITRO*

Hasan Hüseyin Doğan *, Rüstem Duman and Pınar Tuncer

Department of Biology, Science Faculty, Campus, Selcuk University Konya, Turkey.

Keywords:

Morchella conica, *Fomes fomentarius*, Methanolic and aqueous extracts, Antiviral activity, Respiratory syncytial virus

Correspondence to Author:
Hasan Hüseyin Doğan

Department of Biology,
Science Faculty, Campus,
Selcuk University Konya, Turkey.

E-mail: hhuseyindogan@yahoo.com

ABSTRACT: Antiviral properties of methanol and aqueous extracts from *Morchella conica* and *Fomes fomentarius* were assessed by colorimetric XTT test against human respiratory syncytial virus (HRSV). HEp-2 cell line was used for the cytotoxic and antiviral effects of the extracts. Non-cytotoxic concentrations of methanol and aqueous extracts of *M. conica* were tested in 50000 to 195 µg/mL. In contrast, the cytotoxic *F. fomentarius* methanol and aqueous extracts and Ribavirin were tested at concentrations from 3347-13.1 µg/mL, 4906.5-19.2 µg/mL and 86.60-0.30 µg/mL, respectively. The results showed that aqueous extract of *F. fomentarius* had strong anti-HRSV activity (EC_{50} = 358.7 µg/mL, SI = 27.4) which could be compared with ribavirin (EC_{50} = 15.6 µg/mL, SI = 11.1) used as a positive control against HRSV, while other extracts (*M. conica* methanol extract EC_{50} = 28055 µg/mL, SI = >1.8; *F. fomentarius* methanol extract EC_{50} = 3000 µg/mL, SI = 2.2) were found to have weak antiviral activity. As a result, it can be said that aqueous extract of *F. fomentarius* is worthy of further study as an alternative drugs in order to develop anti RSV.

INTRODUCTION: Acute respiratory infections caused by viruses are a major reason of morbidity and mortality in children throughout the world. Human respiratory syncytial virus (HRSV) is the most important cause of pneumonia and bronchiolitis in infants, young children and even adults [1, 2]. The virus is also important and destructive on immunocompromised populations [3]. In addition, recurrent infections are common and naturally acquired immunity does not provide long-term protection [4]. Efforts to develop effective vaccines against RSV have so failed [3, 5, 6]. Moreover, even if one of this vaccine is accepted, it may not be suitable in RSV-sensitive populations, especially in neonates and immunosuppressed individuals [3].

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Immunoglobulins, including ribavirin and high-titer RSV-specific neutralizing antibodies, are now accepted antiviral agents for use in the treatment and prevention of RSV infections [7]. Nevertheless, both of these are not cheap and not easy to apply. Ribavirin has been reported to be myelotoxic when administrated intravenously and is therefore allowed to be used only in small aerosol particles [8, 9]. The application of medicines to illnesses, especially in infants and children is very difficult to control at home, therefore patients must go to hospitals for chemotherapy.

The absence of usable vaccine to prevent RSV and the presence of only one antiviral agent (RBV) which can be only used against severe infections is still constitutes a disruption in paediatric practice. Therefore, it is necessary to develop specific anti-RSV drugs that can be administered orally or parentally [10]. Mushrooms have been used for food sources and medicines for human beings since antiquity. Alternative medicine is growing popular as an aid to modern medicine because of its success.

Mushrooms are one of the most important elements of this subject. Many fungal species are used in the production of bioactive compounds. *Morchella*, *Aleurodiscus*, *Coprinus*, *Clitocybe*, *Fomes*, *Fomitopsis*, *Ganoderma*, *Phellinus*, *Pleurotus*, *Poria*, *Polyporus* and *Tricholoma* genera are mostly used in researches. *β*-glucan, lectin, phenolic compounds, flavonoids, polysaccharides, triterpe-noids, lentinan, schizophyllan, lovastatin, pleuran, steroids, glycopeptides, terpenes, saponins, xanthones, cumarins, alkaloids, purines, purimidins, quinones, phenyl propanoids, calvasins, volvotoxin, fenugreys, flammutoxin, porisin, eryngeolsin, *etc*. were obtained from fungi and used in medical researches [11, 12, 13, 14, 15]. Fungi are rich in protein, fibre, vitamins B and C, calcium and other minerals. For nearly a thousand years, in Asia and Europe, fungi have been used not only as a public food, but also as a traditional medicine. Medicinally known fungi are used in relation to drug properties to promote heart health, increase immune function, reduce risk of cancer, fight virus and fungal diseases, fight against allergy, balance blood sugar level, and reduce inflammation. All of these functions can be useful in preventing and fighting ovarian cancer, in bacterial and viral infections, and in different diseases of the human body [11, 16]. Although, many fungal species in Basidiomycota and Ascomycota are able to form an important part of the fungi kingdom and provide an interesting source of new compounds with interesting structures, the biological activities of fungi, especially antiviral activities, have not been extensively investigated as in plants.

In addition, these few studies on the antiviral activities of different fungal species have also been conducted against certain virus types (herpes simplex virus type 1 and type 2, poliovirus, vesicular stomatitis virus, influenza virus type A) [17, 18, 19, 20, 21]. Nevertheless, only one study was conducted against the human respiratory syncytial virus, an important respiratory pathogenesis [22].

M. conica (Pers.) Boudier is well known species in Turkey as well as in the World. The trade of *M. conica* is very important and is a preferred species especially in European countries. This species is known as "kuzu göbeği" by the local villagers in Turkey. In spring, people collect and export this mushroom and make good profit.

Although, *F. fomentarius* (L.) Fr. is not an edible species, it has been used for medical purposes by the people for centuries due to the active ingredients it contains. It is known as "kav mantarı" in Turkey. This study was conducted in order to compare the antiviral effect of edible and inedible fungi. For this purpose *M. conica* and *F. fomentarius* were selected.

MATERIALS AND METHODS:

Collection of the Macrofungal Samples and Preparation of their Extracts: Two macrofungal species, *F. fomentarius* and *M. conica* were collected in various regions of Turkey (Konya, Karaman and surroundings) in 2015-2016. Macrofungal species were identified by the first author. Voucher specimens of the species (voucher numbers are HHD15453 and HHD7220 for *F. fomentarius* and *M. conica*, respectively) are kept at the Fungarium in Mushroom Application and Research Centre in Selcuk University.

For the preparation of methanolic extract (ME) and aqueous extract (AE), each mushroom samples was ground to get fine powder using a mill. Extraction processes of mushrooms have been conducted by using an ultrasonic homogenizator (Bandelin GM2070, Germany) in solvents (methanol and ultra-pure water). Mushroom powder sample (15 g) was extracted in each solvent (300 mL) by ultrasonicator for 60 min. The extract suspensions of samples were centrifuged and supernatant of the samples were obtained, this method were repeated several times until extracts completely dissolved. Then, extracts were filtered by millipore filter (Whatman no. 1). Filtered extract and solvent mixture were evaporated by using rotary evaporator (IKA RW10BT99, Germany) under low pressure and lyophilized (Labconco, USA).

Each lyophilized extract (10000 mg) was dissolved in 10 mL of EMEM (Eagle's Minimum Essential Medium) (without serum), and 1000 mg/mL stock solution were prepared and sterilized through 0.22 Millipore filter. They were stored at +4 °C until use. Extract dilutions used in cytotoxicity and antiviral activity assay were prepared from this stock.

Cells, Viruses and Reagents: As a host, HEp-2 cells (Human larynx epidermoid carcinoma cell

line, ATCC-CCL-23) which are sensitive to HRSV were used in all experiments. HEp-2 cells and HRSV Long strain (ATCC-VR-26) was obtained from the Faculty of Science, Virology Department, Selcuk University. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2, and were sub cultured twice a week.

The virus was propagated in HEp-2 cells and the titer of propagated viral stock was expressed as 50% tissue culture infective dose ($TCID_{50}$) per 0.1 mL by using Kaerber method [23]. The HRSV stock had a titer of $10^{4.75}$ $TCID_{50}$ 0.1 mL^{-1}. After titration, the viral stocks were dispensed in some sterile tubes and were stored at -80 °C.

EMEM (Eagle's Minimum Essential Medium), FBS (Fetal Bovine Serum), 0.25% trypsin-EDTA solution, antibiotic-antimycotic solution, 0.5% trypan blue dye solution, XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)- 2H- tetrazolium) and PMS (N-methyl dibenzopyrazine methyl sulfate) reagents were purchased from Biological Industries Ltd., Kibbutz Beit Haemek, Israel. Ribavirin (RBV, R9644-10 mg) used as a positive control for inhibition HRSV was purchased from Sigma (USA). A stock solution of RBV (2000 µg/mL) was prepared by using EMEM without FBS and stored at -80 °C until use.

Cytotoxicity Assay: XTT-based cell proliferation kit (Katalog no. 20-300-100, 1000 assays) developed by the company of Biological Industries (Kibbutz Beit Haemek, Israel) was used to determine cytotoxic effects of extracts and RBV. In accordance with the manufacturer's instructions, tests were done as follows; two-fold decreasing serial dilutions (50 to 0.10 mg/mL) were prepared from the stock solution of the extracts (1000 mg/mL) by using EMEM. Each 100 µL of the extracts were dispensed in to each 8 wells of microplate. Each 50 µL HEp-2 cell suspensions containing 1×10^5 cells per mL were placed onto extract dilutions. The same processes were applied for RBV using another microtiter plate (*i.e.* 1000-1.95 µg/mL serial dilutions were prepared). MC (Medium control) and CC (cells control) were also included on to Microplates.

Microplates were incubated with 5% CO_2 at 37 °C in a humidified incubator for 3 days. Then a mixture of 50 µL prepared from 5 mL XTT and 0.10 mL PMS activator was added onto each well. Microplates were lightly shaken for uniformly dispersed of the dye in the wells. Finally, microplates were incubated for 2 h to produce XTT-formazan. Absorbance were read at a wavelength of 540 nm in an ELISA reader (Multiskan EX, Labsystems), and average absorbance values obtained from the 8 wells were recorded.

Tests were performed in triplicate and the results were shown in % of the average cell cytotoxicity compared to controls. The following formula was used to determine the amount of cytotoxicity [24]:

$$\text{Cytotoxicity (\%)} = A - B \times 100 / A$$

A: the absorbance of cell control
B: the absorbance of cells treated extract (or RBV)

The calculated percentages of cytotoxic effects were converted to graphs against the respective concentrations of the tested samples (extracts and RBV). The concentration of sample with 50% survival of HEp-2 cells (CC_{50}) was analysed by the GraphPad Prism 5.03 software [25]. These determined CC_{50} values were used to assess the antiviral activity of the extracts (or ribavirin).

Antiviral Activity Assay: Antiviral assay was determined by the colorimetric XTT method. Non-cytotoxic *M. conica* extracts were tested in the concentrations of 500000, 250000, 125000, 62500, 31250, 15625, 7812.5, 3906.25, 1953.13 µg/mL. In contrast, the cytotoxic *F. fomentarius* methanol and aqueous extracts and RBV were tested at concentrations ranging from 3347 - 13.1 µg/mL, 4906.5 - 19.2 µg/mL and 86.60 - 0.30 µg/mL, respectively [26, 27].

The test is summarized as follows:

For the experiment, HEp-2 cells were prepared at 1.43×10^5 cells/mL concentrations. These prepared cell suspensions were seeded in to 96-well culture plates [except for 8 wells in first column which was used as medium control (MC)] at a volume of 70µL per well (~10^4 cells / well) and incubated in 5% CO_2 at 37 °C for 6 h. Next the wells were drained and 70 µL maintenance medium (EMEM with 2% FBS) was added to all wells of the plate. Then, each 20 µL RSV suspensions which was diluted as

the ratio of 100 $TCID_{50}$ / 0.1 mL using maintenance medium were dispensed on to all columns of the plate [except 8 wells of first column used as medium control (MC) and 8 wells of second column used as cell control (CC)]. Eight wells in the third column of the microplate were used as virus control (VC). 20 µL maintenance medium were put on the each 8 wells in the first column used as MC and the second column used as CC and plates were incubated for two hours.

Concentrations of the fungal species to be used in dilutions were obtained from the stock solutions of the extracts (1000 mg / mL) using Maintenance medium. The final concentrations of *M. conica* for methanol and aqueous extract were arranged as 50000 - 195 µg/mL, the final concentration of *F. fomentarius* methanol extract was arranged as 3347 - 13.1 µg/mL and its aqueous extract was arranged as 4906.5 - 19.2 µg/mL. Final concentrations of Ribavirin were prepared as 86.60 to 0.30 µg / mL from RBV stock solution (2000 µg / mL). After 2 h of incubation, 10 µL of the prepared dilutions were added on to each 8 wells from 4^{th} to 12^{th} columns. In the wells of the microplates used as MC, CC and VC, 10 µL maintenance medium was added. They were incubated in 5% CO_2 at 37 °C for 3 days. After incubation, 50 µL XTT reagent were added to each wells. Plates were incubated for an additional 2 h to form the XTT formazan product. Optical densities were recorded by an ELISA reader (Multiscan EX, Labsystems) at a test wavelength of 490 nm and a reference wave length of 630 nm, and the average of the optical densities obtained from 8 wells were recorded. Percent virus protection rates of different extract (or RBV) concentrations were calculated by spectrophotometrically from the following formula [23]:

$$\text{Percent protection} = [(A-B) / (C-B) \times 100]$$

A = Mean optic density for each extract (or RBV) concentration in 8 wells.
B = Virus control optical density (average of optical density values at 8 wells).
C = Cell control optical density (average of optical density values at 8 wells).

The EC_{50} value, defined as the concentration of extract (or RBV) that provides protection in 50% of the infected cells was determined, taking advantage of the % protection against extract (or RBV) concentrations, by non-linear regression analysis using the GraphPad Prism Version 5.03 statistical program. The selectivity index (SI) of the samples was calculated from CC_{50} / EC_{50} ratio.

RESULTS AND DISCUSSION: It has been determined that there is no toxic effect on HEp-2 cells at the highest concentrations tested (50000 µg/mL) of methanol and aqueous extracts from an edible fungal species, *M. conica.* **Table 1** and this determined value (50000 µg/mL) was accepted as the CC_{50} value for the extracts [28].

This result demonstrates that more than 50000 µg/mL concentration can be used for an additional antiviral test. An additional test can be performed to find a toxic concentration above 50000 µg/mL. In the work of Vieira *et al*., [29] to determine the bioactive properties of the *M. conica* methanol extract, it was determined that methanol extract at 8000 µg / mL, the highest concentration tested, was to have no toxic effects which was parallel to the results obtained in this study, in primary pig liver cells. In a study to determine the antibacterial and cytotoxic activities of extracts from different solvents from *F. fomentarius*, the CC_{50} values of methanol and aqueous extracts against HeLa cells, a cancer cell line, were determined to be 20.10 and 8.31 µg/mL, respectively [30].

TABLE 1: THE PERCENTAGES OF CYTOTOXICITY OF *M. CONICA* AND *F. FOMENTARIUS* EXTRACTS CALCULATED AS THE RESULT OF THE XTT TEST APPLIED TO DETERMINE CC_{50} VALUES AGAINST HEP-2 CELLS

Concentration (µg/mL)	% Cytotoxicity			
	***M. conica* ME**	***M. conica* AE**	***F. fomentarius* ME**	***F. fomentarius* AE**
50000	0.0	0.0	83.3	76.9
25000	0.0	0.0	83.3	69.2
12500	0.0	0.0	70.2	55.8
6250	0.0	0.0	45.6	46.2
3125	0.0	0.0	32.5	26.9
1563	0.0	0.0	16.7	11.5
781	0.0	0.0	5.3	9.6
391	0.0	0.0	0.0	0.00
195	0.0	0.0	0.0	0.00
98	0.0	0.0	0.0	0.00

The CC_{50} values of *F. fomentarius* methanol and water extracts against HEp-2 cells, a cancer cell line, were also determined to be 6694 and 9813 µg/mL, respectively **Table 2**. However, it should be remembered that different cancer cell lines may have different sensitivities to the same extract [30].

TABLE 2: CYTOTOXIC AND ANTIVIRAL ACTIVITIES OF FUNGAL EXTRACTS IN HEP-2 CELLS DETERMINED BY XTT METHOD

Fungal species	Extract type	CC_{50} (µg/mL)	EC_{50} (µg/mL)	Selectivity index (SI)
M. conica	ME	>50000	28055	>1.8
	AE	>50000	Not detectable	Not detectable
F. fomentarius	ME	6694	3000	2.2
	AE	9813	358.7	27.4
Ribavirin (RBV)		173.2	15.6	11.1

As a result of the antiviral activity tests, it was determined that only *F. fomentarius* aqueous extract has significant antiviral activity at higher level than RBV (used as positive control). EC_{50} value of this extract was 358.7 µg/mL, SI value was 27.4, while EC_{50} value of ribavirin, used as a standard drug in the clinical treatment of HRSV infections, was 15.6 µg/mL and SI value was 11.1 **Table 2-3**.

TABLE 3: PERCENTAGE PROTECTION RATES OF FUNGAL SPECIES AGAINST HRSV

Concentrations (µg/mL)	Fungal species					
	Morchella conica		Concentration (µg/mL)	*F. fomentarius*	Concentration (µg/mL)	*F. fomentarius*
	ME Protection %	AE Protection %		ME Protection %		AE Protection %
50000	80.4	20.9	3347	58.4	4906.5	93.9
25000	46.3	18.2	1673.5	14.8	2453.3	84.5
12500	0.0	16.8	836.8	12.7	1226.6	75.8
6250	0.0	0.0	418.4	7.1	613.3	72.1
3125	0.0	0.0	209.2	3.9	306.7	50.9
1563	0.0	0.0	104.6	3.5	153.3	21.9
781	0.0	0.0	52.3	0.0	76.7	12.7
391	0.0	0.0	26.1	0.0	38.3	0.0
195	0.0	0.0	13.1	0.0	19.2	0.0

Chattopadhyay *et al.*, [31] reported that SI values greater than 3 and 3 should be considered as indicative of potentially reliable antiviral activity of test extracts. On the other hand, *F. fomentarius* methanol extract having CC_{50} value of 3000 µg/mL was found to have weak antiviral activity (SI: 2.2) **Table 2**. Antiviral activities of fungi are generally linked to the effects of aqueous extracts and are frequently associated with the presence of water-soluble polysaccharides and other compounds [21, 32, 33]. *Morchella conica* extracts, an edible fungal species, has been found to have negligible antiviral activity.

CONCLUSION: This research is the first study to determine anti-HRSV activities of *M. conica* and *F. fomentarius*. Comparisons and discussions could not be done in accordance with other studies. The aqueous extract of *F. fomentarius*, an antiviral agent, will contribute to the development of antiviral drugs by the future work of *in-vivo* studies and the discovery of active ingredients.

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CONFLICT OF INTEREST: There is no conflict of interest.

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