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Inhibitory Activity of Extracts Isolated from *Parmelia* Lichen against Influenza A Virus (H1N1)

Tran Van Cuong[1*] and Duwoon Kim[1*]

[1]Department of Food Science and Technology and Foodborne Virus Research Center, Chonnam National University, Gwangju, 61186, Republic of Korea.

Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: In the present study, we aim to investigate the antiviral effect against influenza A (H1N1) virus of extracts of *Parmelia* lichen using the MDCK cell line.
Methodology: *Parmelia* lichens species were collected and extracted using acetone and methanol solvents, then chemically analyzed by HPLC. The antioxidant of the lichen extracts was measured using the DPPH method, meanwhile, cytotoxicity and antiviral activity were tested using the CCK-8 method. The cytopathic effect and apoptosis of infected-cells were observed under microscopy and using Muse Annexin V and Dead Cell Kit stains in the Muse Cell Analyzer. Finally, to confirm the antiviral effects of lichen against influenza A virus, the relative expression level of viral mRNA PA-gene in MDCK cells was measured using the qRT-PCR method.
Results: Based on the *in vitro* assays, we found that methanolic extract of *Parmelia* lichen shows extremely high activity against IAV, which is a significantly higher cell viability (more than 40%); however, a relatively low apoptosis and cell death compared to positive IAV-infected cells. Furthermore, the methanolic lichen extracts showed a significant reduction (by 1.9 log-fold) of influenza A viral replication with concentration of 100 µg/mL.
Conclusion: Our results show that methanolic extracts of natural *Parmelia* lichen highly inhibited influenza A virus replication *in vitro* assays. This knowledge has, in turn, allowed the researchers to further explore the specific molecules from natural lichens for pharmaceutical biomaterials of a new anti-influenza therapy.

**Corresponding author: E-mail: vcuong.edu.vn@gmail.com;*

Keywords: Influenza A virus; lichens; parmelia; pharmaceutical biomaterials; real-time RT-PCR; virus infection.

1. INTRODUCTION

Influenza A virus (IAV) is a group of negative-sense, single-stranded, enveloped RNA viruses belonging to the *Orthomyxoviridae* family is one of the most common infectious respiratory diseases [1,2]. There are three classes of influenza viruses include type A, B and C but only type A and B cause serious respiratory disease in humans. So those two viruses strains receive more research attention [3]. Up to date, there are two main methods for control and treatment of influenza viruses include vaccination and antiviral drugs [3,4]. Three active groups of anti-influenza drugs, which are M2 protein inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (oseltamivir, zanamivir, peramivir and laninamivir) and the third group was targeted to inhibit viral RNA polymerase (polymerase inhibitor), favipiravir (T-705) was recently approved in Japan (in 2011) [3]. However, due to the slow response to vaccines in dealing with epidemic outbreaks and the reducing the effectiveness of vaccination has been reported recently. Moreover, the resistance of influenza viruses to current anti-IVs drugs has been emerging, and seasonal influenza viruses continue to cause epidemics around the world each year. For example, according to the Centers for Disease Control and Prevention many strains of influenza, including the 2009 pandemic H1N1 influenza, are now resistant to amantadine and rimantadine. So, it has been extensive interest in developing a new antiviral treatment for this virus, and biomaterials from natural resources have been considered to be potential candidates for novel treatment against IVs infection. It is, therefore, an urgent requirement for an effective, more potent and risk-free therapy for the influenza infection.

Lichens are a well-recognized, self-supporting, mutualistic symbiosis between a dominant fungal partner (mycobiont) that provides shelter for one or several photosynthetic green algae and cyanobacteria (photobionts) forming a unique symbiotic structure [5]. The worldwide lichen flora is estimated to include approximately 18,500 species and covers about 8% of the earth's land surface [6]. Lichens are a promising source of the broad chemical diversity with around 1,050 lichen metabolites are known up to date and have been reported to display diverse biological activities [7,8].

In addition, it has been well known for novel biological activities of lichens, that such anti-cancer, anti-microbial, antioxidant, antiviral, anti-inflammatory activity as well as antiproliferative, antipyretic, allelopathic and UV protecting effects and healing, etc. [6,7,8,9]. Lichens have been used as ingredients in folk medicines for centuries, and many cultures have used lichens to treat a variety of ailments as part of their traditional medicines [10]. There was numerous related research about the biological functions of lichens have been reported in previously published papers. Amongst more than a thousand of identified secondary metabolites from natural lichens, usnic acid has been known as a novel compound [6,11,12]. Since its first isolation in 1844, usnic acid has become the most extensively studied lichen metabolite and one of the few that are commercially [6]. Recently, several studies have been reported that lichen acids showed potent antiviral activity against influenza viruses. Sokolov et al. reported usnic acids have highly antiviral effects on the pandemic influenza virus A(H1N1) in MDCK cells [11]. Shtro et al. and Shtro et al. also reported the activity of usnic acid (UA) derivatives against influenza virus *in vitro* and *in vivo* [12,13]. It, therefore, the characterization and utilization of new compounds from natural lichens are a promising source of new antiviral drugs. In the current study, we aim to investigate the antiviral effects of the extract from natural *Parmelia* lichen against influenza A (H1N1) virus *in vitro* assays using the MDCK cell line.

2. MATERIALS AND METHODS

2.1 Preparation and Extraction of Compounds from Lichen

Natural *Parmelia* lichen was collected from Sunchon Province, South Korea in 2017 and was authenticated by a taxonomist. Next, dried lichen sample was mortar ground to a fine powder. The lichen powder was weighed and extracted using methanol and acetone solvents at room temperature (approx. at 22^{o}C) in a Soxhlet apparatus. The extracts were filtered using syringe-driven filter (0.22μm, Merck Milipore Ltd.) and then concentrated under reduced pressure in a rotary evaporator. The dry extracts were stored at -20^{o}C before use. The phytochemical analysis of acetone extracts and methanol

extracts of the lichen was carried out using HPLC-UV (SHIMADZU, LC-20A) systems. The absorbance was read using the aforementioned UV-Vis spectrophotometer at a wavelength of 254 nm.

2.2 Analytical Methods

2.2.1 DPPH radical scavenging ability

In vitro antioxidant capacity of lichen extracts was measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma-Aldrich) radical scavenging activity assay. DPPH radical-scavenging activity was performed using the method described by Cuong and Chin (2016) with some modification [14]. The different concentrations (serial two-fold dilutions) of lichen by acetone (AcOH) and methanol (MeOH) were prepared. Each of 1000 µL sample extract was mixed with 500 µL of freshly prepared DPPH solution (0.2 mM) prepared in methanol. The mixture was shaken and incubated in the dark for 30 min at room temperature. The appropriate volume of the same solvent used for the sample was used instead of samples in the control group. The absorbance of triplicate was measured at 517 nm. DPPH radical-scavenging activity was calculated as follows:

DPPH radical-scavenging activity (%) = [(ODctl - ODspl) /ODctl] × 100,

where: ODctl is the absorbance value of the control group, and ODspl is the absorbance of the samples. The nonlinear concentration−inhibition response was plotted, and 50% inhibition concentration (IC_{50}) was calculated.

2.2.2 Cells and virus

Cells: Madin−Darby canine kidney (MDCK) cells were obtained from Korean Cell Line Bank (KCBL10034, Lot No. 30419). MDCK cells were cultured using standard methods at 37°C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM, Welgene, Daegu, Korea), supplemented with 10% heated fetal bovine serum (FBS), and antibiotics (streptomycin 100 mg/mL and penicillin 100 U/mL, Sigma-Aldrich, St. Louis, MO, USA).

Virus: The low pathogenic human IAV H1N1 strain A/PR/8/34 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and propagated by infecting MDCK cells at 37°C in 5% CO_2 atmosphere in virus infection media (DMEM supplemented with streptomycin 100 µg/mL, and penicillin 100 U/mL) containing trypsin treated with 2 µg/mL N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK, Sigma-Aldrich).

2.2.3 Lichens treatment and cytotoxicity assay

The cytotoxicity of lichen extracts was evaluated in MDCK cells. Cell viability was measured using the Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan) method. MDCK cells were seeded in 96-well plates at a density of 2×10^4 cells/well and incubated for 24 hours in DMEM with the addition of 10% heated FBS. After the cell monolayer formation, cells were washed with PBS. The lichens extracted compounds were dissolved in DMSO to 10 mg/mL, and serial twofold dilutions with DMEM were performed to obtain the final concentration of 6.25,12.5, 25, 50, 100, 200, 400 and 800 µg/mL. The dilutions of lichens were used to treat the MDCK cells and incubated for 48 hours at 37°C with 5% CO_2. The CCK-8 kit reagent was added and after the incubation time (1 hour, 37°C, and 5% CO_2), and the absorbance was measured at 450 nm using a microplate reader (Synergy, Bio-Tek, VT, USA) and cytotoxicity was calculated as the method described by Thoa and Cuong (2018) [15]. Nonlinear concentration−response curves were plotted, and the half-maximal cytotoxic concentration (CC_{50}) was calculated.

2.2.4 Virus infection and inhibitory effect of the selected lichen compounds against IAV (H1N1)

For the evaluation of lichens treatment against IAV infection, MDCK cells were seeded in 96-well plates at a density of 10^5 cells/well and cultivated in DMEM with the addition of 10% heated FBS. After the cell monolayer formation, cells were washed with PBS. The compounds were dissolved in DMSO to 10 mg/mL, and the final concentration of 100 µg/mL lichen extracts was prepared in DMEM. The medium containing lichens of the above-mentioned concentration were used to treat the MDCK cells and incubated for 24 hours at 37°C, then influenza A virus (H1N1) was infected to the cells. IAV-infected MDCK cells were next incubated for 48 hours at the sample condition, and during the incubation, the virus-induced cytopathic effect was observed using an inverted microscope (Optinity KI 400,

Korea) attached with a digital microscope camera (DCM310). After 48 hpi (hours post infection), the CCK-8 kit reagent was added, and after the incubation time (1 hour, at 37°C, and 5% CO_2), the absorbance was measured at 450 nm using a microplate reader (Synergy, Bio-Tek, VT, USA) and the antiviral activity was calculated and the results were expressed as the percentage of inhibition. For IAV infection, the cells were overlaid with DMEM consist of 2 µg/mL tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK, Sigma)-treated trypsin (freshly make and add TPCK right before infection), while without the supplementation of FBS.

2.2.5 Apoptosis assay using the Muse cell analyzer

Cell apoptosis was analyzed using the Muse Cell Analyzer (Merck, KGaA, Darmstadt, Germany) according to the manufacturer's protocol. First, MDCK cells were treated with lichen extracts and infected with IAV for 48 hours as previously described for the antiviral protocol. The supernatant medium was discarded, and after twice washing using PBS, MDCK cells were separated using T-EDTA and collected for further analysis. Next, the cells were diluted in the same volume of PBS among the treatments and stained with Muse Annexin V and Dead Cell Kit (EMD Millipore Corporation, Hayward, CA 94545). After staining, the cells were incubated in the dark for 20 min, and were then quantitated using the Muse Cell Analyzer. The results were expressed as the percentages of the live cells, early apoptotic, and late apoptotic + dead.

2.2.6 RNA isolation, cDNA transcription and the expression pattern of IAV-PA gene

Total MDCK cells RNA was extracted using RNAiso plus reagent (Takara Bio, Shiga, Japan) according to the manufacturer's instruction. The total RNA concentration was measured using the Nanodrop 1000 Spectrophotometer (Thermo Scientific, USA), and equalized to all of the treatments. Aliquots of the RNA was reverse transcribed into cDNA using the Superscript First-Strand cDNA Synthesis Kit (Bioneer Corporation) in a TAKARA PCR Thermal Cycler Dice Real Time System at the following conditions: 63°C for 10 min, 37°C for 60 min and 95°C for 5 min. After the cDNA of each sample was synthesized, 2 µL of those was used as a template for real-time PCR. Real-time PCR was carried out in a TAKARA Thermal Cycler Dice Real Time System using SYBR Premix Ex Taq™ kit (Takara Bio). The PCR program run settings for detection of PA gene H1N1 virus were performed using the SYBR Green Kit (Takara Bio, Japan) as the following conditions: initial denaturation at 95°C for 30s followed by 45 cycles of denaturation at 95°C for 5s, at 55°C for 10s, 72°C for 20s and 1 cycle of dissociation at 95°C for 15s, 60°C for 30s and 95°C for 15s.

The primer sequences for Influenza A virus PA gene were forward 5′-CGGTCCAAATTCCTGCTGA-3′ and reverse 5′-CATTGGGTTCCTTCCATCCA-3′. The 18S rRNA primer sequence used was forward 5′-TGTGCCGCTAGAGGTGAAATT-3′ and reverse 5′- TGGCAAATGCTTTCGCTTT-3′ for normalization. The IAV-PA gene expression was determined by the 2-ΔΔCt method; viral mRNA expression level was normalized against 18S mRNA expression for each sample and data was expressed relative to the mock control.

2.3 Statistical Analysis

All samples were performed in triplicate, and results were expressed as means ± SD. Statistical analysis was performed using IBM SPSS Statistics software (version 22.0; IBM Corp., Armonk, NY, USA). The means were compared by analysis of variance using Duncan test ($p<0.05$). Figure and IC_{50}/CC_{50} values were performed using Graph-Pad Prism software version 6.03 (Graph-Pad Software Inc., USA).

3. RESULTS AND DISCUSSION

3.1 HPLC Analysis and Antioxidant Capacity (DPPH radical scavenging) of *Parmelia* Lichen Extracts

The HPLC chromatogram of the acetone extract and methanol extract of *Parmelia* lichen is shown in Fig. 1. Lichen phytochemicals have been identified in *Parmelia* lichen species at 254 nm. HPLC analysis results revealed that the predominant lichen acids in acetone extract were norlobaridone and atranorin acid, whereas, in methanol extract was usnic acid. According to the study of Honda *at al*. (2016), showing that acetone extract of *Parmotrema screminiae* is a remarkable source of norlobaridone and atranorin acid [16]. In addition, depsidones/depsides from acetone extract (norlobaridone and atranorin acid) have been mentioned in several previous studies for their biological functions. For example, norlobaridone

acid from lichens was found to inhibit M-Phase Phosphoprotein 1 in a study by Talapatra et al. [17], and showed potent antibiotic activity against *S. aureus* and *E. faecalis* according to Honda at al. [16]; While as, atranorin showed neurotrophic activity in Neuro2A cells [18]. In addition, a previous study by Kosanić et al. reported the antioxidant, antimicrobial and cytotoxic activities of atranorin was extracted from several natural lichens [19].

The DPPH ability of acetone and methanol extracts from *Parmelia* lichen as the basis of percent inhibition is shown in Fig. 2. The DPPH ability (%) of both extracts was much lower than quercetin (as a positive control) at the same concentration. The DPPH values of AcOH and MeOH extract ranged from 1.73 to 30.9% and 2.97 to 23.6%, respectively (with different concentrations ranging from 6.25 to 800 µg/mL), whereas quercetin was highly inhibited DPPH radical scavenging with IC_{50} value at 9.56 µg/mL, which an excellent DPPH activity ranged from 40 to 99.8% in the range of 6.25–800 µg/mL. As based on the IC_{50} values, AcOH extract was higher antioxidant activity when compared with MeOH extract. This result suggests that the extracts with different solvents are varied in phenolic and other phytochemical compositions, which associated with radical scavenging activity. The present results are in the accordance to several previous authors, who have also reported that DPPH radical scavenging activity highly depended on the extraction solvents and lichen species [19,20,21,22]. On the other hand, the antioxidant activity *in vitro* and *in vivo* of lichen extracts and lichen substances have been studied and reported in several previous studies [21,23,24,25].

3.2 Cytotoxicity of *Parmelia* Lichen Extracts

As a result is shown in Fig. 3 that *Parmelia* lichen extracts effectively changed the viability of MDCK cells at the concentrations in the range of 6.25–800 µg/mL. Interestingly, the results showed that methanol extract had no cytotoxic effect on MDCK cells at the concentration tested, whereas acetone extract was toxic at the concentration higher than 100 µg/mL. The CC_{50} value of methanol (CC_{50}>800 µg/mL) was much higher than acetone extract (176.7 µg/mL), which indicates that methanol extract from *Parmelia* has lower cytotoxicity than acetone extract and had the less cytotoxic effect to the MDCK cells. The difference in the cytotoxic dose of lichen extracts may in part be due to the specific

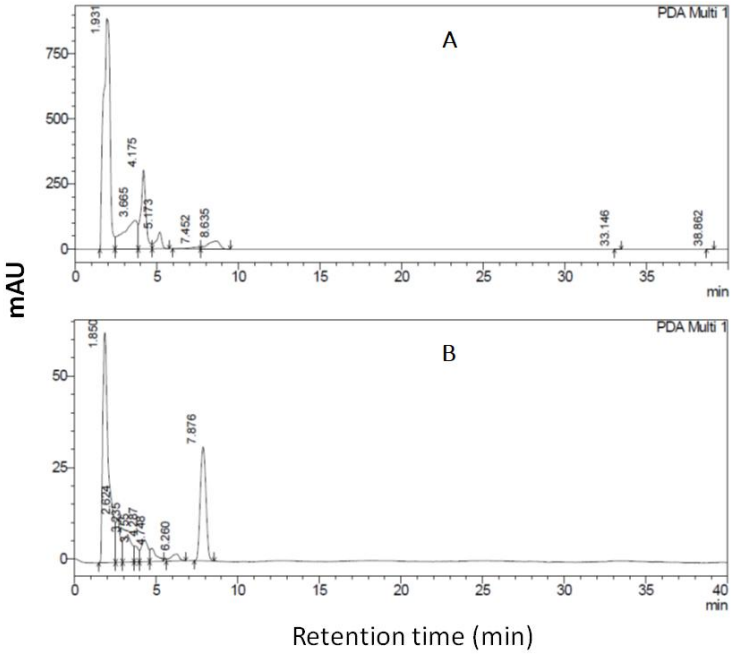

Fig. 1. HPLC spectrum of acetone extract (A) and methanol extract (B) of *Parmelia* lichen at 254 nm, together with the chemical structures of their predominant constituents.

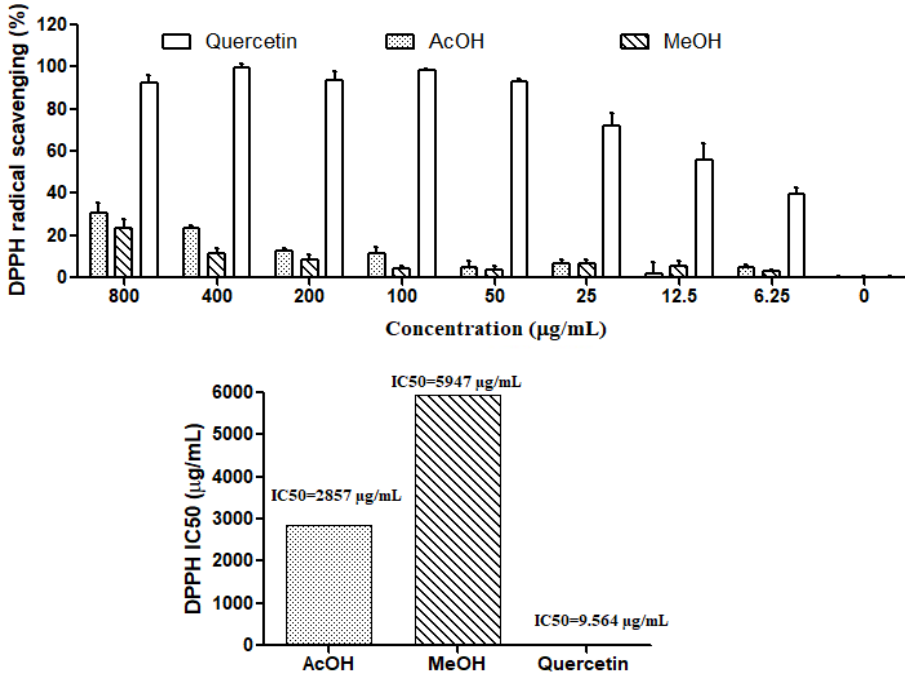

Fig. 2. DPPH radical scavenging activity of acetonic and methanolic extracts from *Parmelia*.

compound of phytochemical characteristics of different solvents, which showed in HPLC results. In a previous study reported a similar result that acetone extracts of *Cladonia* lichens induced a significant cytotoxic effect on several cell lines (human melanoma and human colon carcinoma) [19] and Raji cells [26]. In addition, different results showing different CC_{50} values for different lichens extracts and their constituents on different cell lines have been published [18,19,20,25].

3.3 The Inhibitory and Apoptotic Effect of Lichen Extracts on IAV-infected MDCK Cells

As the results are shown in Fig. 4, IAV infection induced the cytopathic effect in MDCK cells after 48 hours with a multiplicity of infection (MOI) of 1. The effect of acetone (AcOH) and methanol (MeOH) extracts from *Parmelia* lichen on the cell viability of IAV-infected was assessed using a CCK-8 assay. In fact, the relative cell viability of IAV-infected cells was reduced to 48% as compared to the mock cells. However, both extracts AcOH and MeOH treatment (with the concentration of 100 µg/mL) significantly increased the relative cell viability of the IAV-infected cells (88.2% and 91.3% of the mock cells, respectively).

Furthermore, to test the apoptotic effect of lichen extracts on IAV-infected MDCK cell, the total apoptotic cells were quantified and expressed as the percentage of live cells and apoptotic cells by annexin V and dead cell kit staining. The present results demonstrated that IAV infection induced apoptosis in MDCK cells, while lichen treatment protected cells from death. As the results are shown in Fig. 5, the percentage of live cells in the IAV-infected sample (45.3%) was significantly lower than in AcOH extract (65.7%) and MeOH extract (77.8%), and those values by lichen treated samples was not a significant difference from the mock sample (75.5%). Whereas, the percentage of early apoptotic, and late apoptotic/dead cells in the IAV-infected samples (27.6 and 20.7%, respectively) was significantly higher than in AcOH extract treated sample (20.4 and 11.8%, respectively), and MeOH extract treated samples (10.4 and 8.7%, respectively). This result suggests that lichen extract treatment produced a significantly lower apoptosis in the IAV-infected cells as compared to IAV-infected cells. In addition, the methanol extract of *Parmelia* lichen showed a relatively high protection effect of IAV-infected cells from apoptosis than acetone extract.

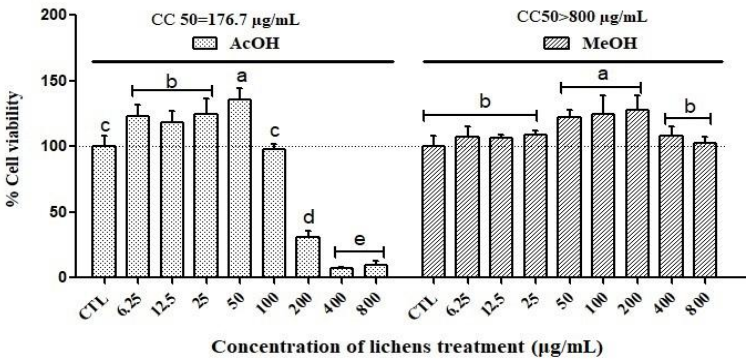

Fig. 3. Cytotoxicity of acetonic and methanolic extracts from *Parmelia* lichen on MDCK cells. MDCK cells were treated with different concentration of lichen extracts for 48 hours, and CCK-8 kit was used to measure cells viability. Cell viability was expressed as a percentage of the viability of Mock. Each bar represents the mean ± SD. Different letters above bars represent means that are significantly different at *p<0.05.*

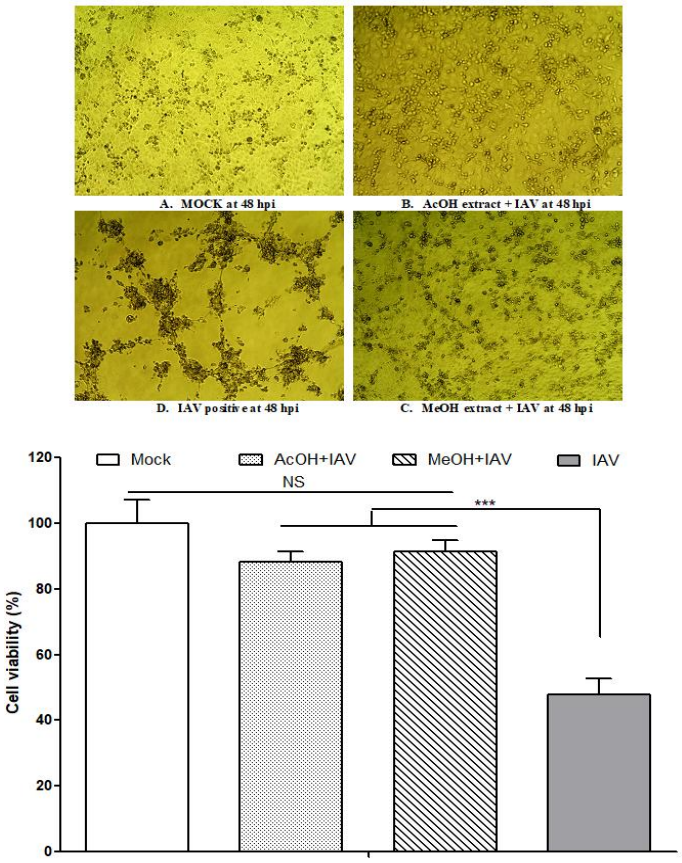

Fig. 4. Effect of acetonic and methanolic extracts from *Parmelia* lichen on cell viability and cytopathic effect in IAV-infected MDCK cells. A: Mock, MDCK cells without virus infection; B: 100 µg/mL treatment of acetone extract in MDCK cells and IAV infection; C: 100 µg/mL treatment of methanol extract in MDCK cells and IAV infection; D: IAV-infected cells without lichen treatment. Each bar represents the mean ± SD. Asterisks (*) represents means that are significantly different (NS, not significance and ****p<0.005*).

3.4 Relative mRNA Expression of IAV-PA Gene

The influenza A virus genome contains eight single-stranded RNA molecules, which include three viral polymerase subunits and multiple copies of the viral nucleoprotein [27]. The IAV PA gene is one of the three components of the viral RNA polymerase complex (PB2, PB1, and PA) [1]. Together with the viral nucleoprotein (NP), the viral RNA polymerase complex plays a crucial role in replication of influenza A virus [1]. To confirm the inhibitory potential of lichen treatment, we measured the relative expression level of viral mRNA PA-gene in IAV-infected MDCK cells treated with lichen extracts with concentration of 100 µg/mL. After 48 hpi, the virus-induced cytopathic effect was observed, the supernatant of infected MDCK cells was harvested, then RNA and cDNA were isolated and synthesized, respectively. The relative mRNA expression of IAV PA-gene was performed using qRT-PCR. Fig. 6 shows the inhibitory effects of lichens during influenza A virus infection in MDCK cells. Our results showed that both extracts (AcOH and MeOH) were highly active to control influenza A virus replication in the MDCK cells compared to IAV-infected sample. Lichen extracts highly reduced the expression of IAV PA gene at 48 hours after IAV infection ($p<0.005$). However, the inhibitory effect of AcOH extract was significantly lower than methanol extract. In fact, MeOH extract inhibited virus propagation approximate three-fold when compared with IAV positive control, and the viral mRNA expression level was not significant from the mock sample. The result is suggesting that the difference in the effect of these two lichen extracts can most likely be attributed to differences in the chemical compositions since the antioxidant capacity and cytotoxicity also differed from one other. There are several previous studies both *in vitro* and *in vivo* have been reported the anti-influenza effect of various phytochemical components from natural sources, which showed different results due to differences in the chemical characteristics. For example, according to the previous study, *Rubus coreanus* seed extract and its gallic acid had potent and broad antiviral activity against influenza A and B type viruses in MDCK cells and mouse [2]. Earlier, Lee et al. also reported that green tea by-products possessed strong anti-influenza activity *in vitro* and against influenza virus infection in chickens [28]. Furthermore, He et al. also evaluated the antiviral activity of extracts from dandelion against influenza A virus infection in MDCK cells [29]. These inhibitory activities to inhibit virus propagation of phytochemicals from plant extracts have been mentioned in several previously published papers, which might associate with various steps in the life cycle of influenza viruses. The mechanisms of those inhibitory activities which include the prevention of virus entry into host cells by disrupting or binding to the viral particles [27,30,31], the decreasing the NP viral RNA level and inhibition of viral polymerase activity [29,32] and the inhibition of transcription and release of the virus [28].

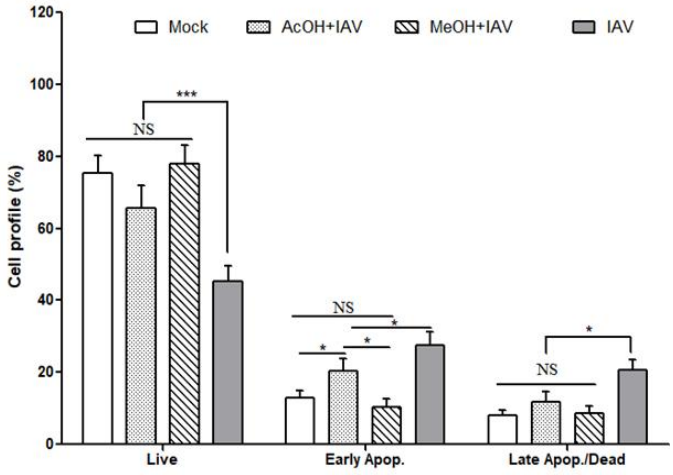

Fig. 5. Effect of acetonic and methanolic extracts from *Parmelia* lichen on apoptosis in IAV-infected MDCK cells. Each bar represents the mean ± SD. Asterisks (*) represents means that are significantly different (NS, not significance * *p<0.05* and ****p<0.005*).

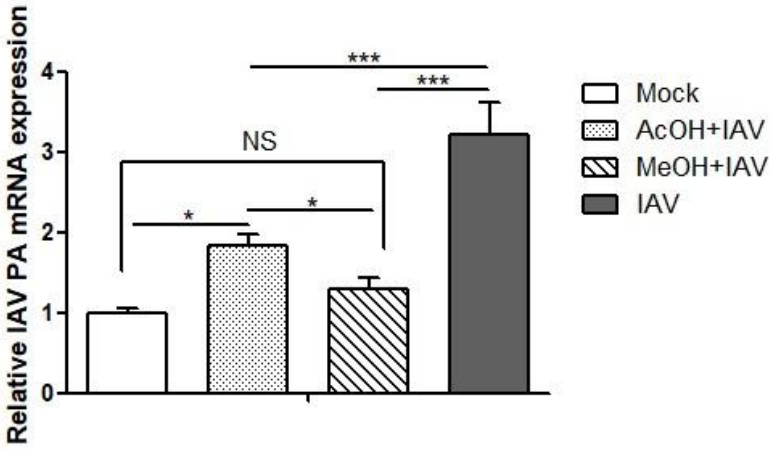

Fig. 6. Antiviral effect of acetonic and methanolic extracts from *Parmelia* lichen on IAV replication. Each bar represents the mean ± SD. Asterisks (*) represents means that are significantly different (NS, not significance, *$p<0.05$ and ***$p<0.005$).

4. CONCLUSION

The present study showed that the methanolic extract from *Parmelia* lichen highly inhibits influenza virus A, which enhance cell viability and reduce virus replication after influenza virus A/PR/8/34 infections in MDCK cells. Our finding is the first report of an excellent effect against influenza A virus infection without any cytotoxicity of the methanolic crude extract from *Parmelia* lichens, which have not been reported in any previous publications. Furthermore, lichens produce a broad spectrum of unique secondary compounds; thus, the bioactive compounds from *Parmelia* may be a potential target for the development of drugs to treat IAV. However, more detailed *in vitro* as well as *in vivo* studies are necessary to understand the inhibitory mechanisms and figure out the potential antiviral activity against IAV of specific molecules from natural *Parmelia* lichens.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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