Virucidal Activity of Moringa a From Moringa Oleifera Seeds Against Influenza A Viruses by Regulating TFEB

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Research

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Abstract

Background

Influenza A viruses (IAVs) are highly contagious pathogens infecting human and numerous animals. The viruses cause millions of infection cases and thousands of deaths every year, thus making IAVs a continual threat to global health.

Methods

Moringa A was isolated from *Moringa oleifera* seeds and tested for antiviral activity against H1N1. The antiviral activity of *Moringa A* was tested by checking their effect on hemagglutination and PFU activities of the studied virus, and the cytopathic effect was observed too. Additionally, the different types of treatment experiments were performed to complement the analysis of the antiviral activity of *Moringa A*, and the contents of inflammatory cytokines and the expression of TFEB were detected.

Results

Moringa A inhibits virus replication in host cells, and it protects infected cells from cytopathic effect induced by IAVs. The EC₅₀ and EC₉₀ value of *Moringa A* for IAVs were 1.27 and 5.30 μ M, respectively. The different types of treatment experiments revealed that *Moringa A* has a significant inhibitory effect on the IAVs both before and after drug addition. What's more, *Moringa A* was observed to decrease the levels of inflammatory cytokines TNF- α , IL-6, IL-1 β and IFN- β in H1N1 infected RAW264.7 cells. Finally, *Moringa A* was found to inhibit the expression and nuclear transfer of the cellular protein transcription factor EB (TFEB), and weaken the autophagy in infected cells, which could be an important antiviral mechanism of *Moringa A*.

Conclusions

Moringa A has potent antiviral activity against IVAs, which could be due to the autophagy inhibition property.

Introduction

Influenza virus is the most threatening respiratory virus to human health, causing millions of people to suffer upper respiratory tract infections every year. In the past ten years, the large-scale outbreaks of SARS, MERs, H1N1, H7N9 and other viruses have brought serious harm to human life and health (Atkin-Smith G.K et al., 2018). The "cytokine storm" triggered by influenza virus infection can cause lung inflammation and acute respiratory distress syndrome, which is an important reason for mortality (Zhang Y et al., 2020; Xiaoyong C et al., 2018). So far, the prevention and control of influenza virus remains a worldwide problem. However, due to the high mutation rate of influenza viruses and the occurrence of recombination between viruses, it is difficult for existing anti-influenza drugs to cope with the changing flu viruses (Mazhar H et al., 2017). The M2 ion channel blockers and neuraminidase inhibitors, which are

main drugs for anti-IVAs, are facing drug resistance. Although the vaccine is a core drug for preventing and treating viral infections in humans, the current vaccines are not highly protective if antigenically different new strains emerge such as the outbreak of the 2019 pandemic SARS-COV2(Perez-Rubio A et al., 2018; Hye S.H et al., 2020). Therefore, it is highly desirable to develop new drugs that would have protective activity against the influenza viruses.

Moringa oleifera Lam., also known as the Drumstick tree, is a cultivated species from the genus *Moringa*, (family Moringaceae) and the order Brassicales. It is geographically distributed across South Asia, and in particular, India, Sri Lanka, Pakistan, Bangladesh, and Afghanistan. The *Moringa* seeds and leaves have broad uses in both the food industry and as therapeutics. Previous pharmacological investigations have shown *M. oleifera* has anti-inflammatory, anticancer, antioxidant, and anti-obesity properties, etc (Bhattacharya et al. 2018).

M. oleifera seeds are a promising resource for both food and non-food applications, as they have both edible and medicinal value. They have been shown to be rich in nutrients such as oils, proteins, and vitamins (Bhattacharya et al. 2018; Kc et al. 2020). The *M. oleifera* seeds also contain numerous bioactive compounds, such as flavonoids, phenolic acids, alkaloids, isothiocyanates, and thiocarbamate (Abd Rani et al. 2018), which have been shown to have good antimicrobial activity, as well as anti-tumour, anti-inflammatory, and anti-virus activities (Abd Rani et al. 2018). In terms of antiviral research, some reports have shown that extracts from *M. oleifera* leaves have good inhibitory effects on the herpes zoster virus, HIV, and hepatitis B virus (Abd Rani et al. 2018), but the key active ingredients are currently unclear.

In recent years, research into the *M. oleifera* seeds has focused on their active antiviral ingredients. Guided by antiviral activity, our team has obtained some new compounds from *Moringa* seed, of which the *Moringa* A(MA) has been proved to have good activity of anti-H1N1 virus (Yongai X et al. 2020). The chemical structure of *Moringa* A is shown in Fig. 1.

Research in this paper is a continuation of our previous work. In this paper, we aimed to study the antiviral properties of MA against IAVs by regulating transcription factor EB(TFEB), an important transcription regulator of autophagy-lysosomal pathway (Napolitano G et al., 2016). In recent years, the complex interplay between IAVs and host autophagy machinery has been extensively revealed, and many studies testify that the infection of IAV strains such as H1N1, H3N2, H9N2 can activate autophagy in host cells, the mechanism is reportedly involved in viral replication and immune escape(Wang Y et al., 2018; Choi Y et al., 2018). Mounting evidences have demonstrated that autophagy is an important approach for viral passage and virus particle production in host cells (Wang R F et al., 2019; Zhang J T et al., 2019). TFEB is a key transcription molecule that regulates autophagy at the transcriptional level. It can promote the transcription of multiple genes in the lysosome pathway, and regulate the production and efflux of autophagy lysosomes (Napolitano G et al., 2016). Therefore, inhibiting TFEB to block the production of lysosome may be an important strategy for host against viruses.

Materials And Methods

2.1. MA preparation

The MA with a purity of 99.8% was prepared according to our previous method (Yongai X et al. 2020). It's a highly polar compound and easy to be dissolved in water, therefore the cell culture medium was used to prepare the different concentrations of MA solution.

2.2. Chemicals

Fetal bovine serum (10099-141), DMEM Medium (C11995), 0.25% Trypsin- EDTA (25200-056), penicillin-streptomycin (15140-122), and pancreatin (H25200-056) were purchased from Gibco (ThermoFisher Scientific, USA). DMSO (D2650, 100 mL) was purchased from Duojindo (Japan). CCK-8 (CK04) was purchased from Duojindo

(Japan). TNF- α (K1051-100, 96T), IL-6 (K4144-100, 96T), IL-1 β (K4795-100, 96T) and IFN- β (K4795-100, 96T) Elisa kits were purchased from Biovision (USA). Anti-TFEB (ab270604) was purchased from Abcam (Cambridge, UK). Cavy red blood cells (1%, SBJ-RBC-GP001) was provided by Nanjing SenBeiJia Biological Technology Co., Ltd (Nanjing, China).

2.2. Cell lines and IAV strains

Raw264.7 and the MDCK cells were obtained from Mingzhou biotechnology co. LTD (Ningbo, China). Cells were cultured in DMEM containing 10% fetal bovine serum with 100 U/ml of penicillin and 100 μ g/ml of streptomycin at 37 °C with 5% CO₂. Influenza A/Weiss/43(PR8, mouse passaged H1N1) virus was provided by Wuhan institute of virology, Chinese academy of sciences (Wuhan, China). New viral stocks of IAV were passaged in 10 days old embryonic chicken eggs for 48 to 72 h. Allantoic fluid was collected and stored at -80 °C until required. Experiments were conducted in a physical containment level 3(PC3) laboratory.

2.3. Virus amplification

IAV was amplified as described before (Gao Li et al., 2017). Briefly, when MDCK cells grew to a density of 90% in cell culture flasks, 2 ml DMEM containing TPCK tyrosin (2 μ g/ml) were added. When the cells were suspended, 2 ml influenza virus stock (MOI = 10) were added into the cell culture flask. 4 hours later, 7 ml DMEM was added to the flask. Cytopathic changes were observed every 12 hours. 72 hours later, the mixture of virus and cells were collected and centrifuged, and the

supernatant was collected and stored in the refrigerator at 4 °C for later use.

2.4. Cell viability assay

Cytotoxicity of MA was assessed by cck-8 assay. Briefly, RAW264.7 cells were seeded in 96-well plates at a density of 1×10^5 per well. At 24 h post-seeding, cells were treated with gradient concentrations of MA ranging from 10 to 320 μ M. At 48 h, 10 μ l cck-8 solution was added into every well and incubated for 2 h.

The OD values was estimated by measuring absorbance at 450 nm in a microplate reader (MK3, Thermo, USA).

2.5. Viral cytopathic effect (CPE) assay

RAW264.7 cells were seeded in a 96-well plate at a density of 1×10^5 per well. Experimental groups were set with the following conditions: normal control group (NC, saline + DMEM), virus control group (VC, H1N1 + DMEM), positive control group (H1N1 + DMEM supplemented with 10 μ M Oseltamivir (OST)), and treatment group (H1N1 + DMEM supplemented with 1, 5, and 10 μ M MA). Except the NC group, cells in other groups were infected with H1N1 (MOI = 1). After treatment for 24 h, the CPE in RAW264.7 cells was observed and photographed under inverted microscope (CKX53, OLYMPUS, Japan), and the cell viability was assessed by the CCK-8 assay.

2.6. Virucidal activity assay in vitro

The virucidal activity of MA in vitro was evaluated by hemagglutination assay and plaque assay, respectively. For hemagglutination assay, RAW264.7 cells were inoculated in 96-well plates at a cell density of 5×10^3 cells per well. 24 h later, 2-fold serial dilutions of H1N1 viruses were added to infect cells. After 24 h post-infection and treatment, the supernatant was collected and hemagglutination was detected using cavy red blood cell (RBC). Briefly, 50 μ L supernatant was added to each well of a U-bottomed 96-well plate. Then, 100 μ L 1% cavy RBCs were added to each well. Titers were detected after 1 h of incubation at room temperature and reported in HA units/50 μ L (HAU/50 μ L) (Jang Gi C et al., 2016). For plaque assays, RAW264.7 cells were seeded in 24-well plates at a cell density of 1.0×10^4 cells per well. 10-fold serial dilutions of H1N1 virus were added to infect cells. After 24 h post-infection and treatment, the supernatant was moved and an content of 1% ethyecellulose prepared in DMEM was added and plaques were counted after incubation for 12 hpi. Titers are presented as plaque forming unit(PFU) per ml (Wang M M., et al., 2017). In addition, the EC50 and EC90 of MA were also detected.

2.7. Transfection of siRNAs

2.0×10^4 RAW264.7 cells/well were plated in 24-well plates for 24 h. Then cells were transfected with 1.5 μ L of lipofectamine RNAi/Max (Thermo) and 10 nM of siTFEB (RNAi sequence: 5'-GGUCACAGUGUGCAUCAGCAT-3'(sense), 5'-GCUCAUGCAGACUCAGACGA T-3'(anti sense) and siRNA negative control (siNC, RNAi sequence: 5'-AGCUGACAGGUUCGC AGAATA-3', 5'-UUGUCGGAACGUGUGACGUAT-3'). When indicated, cells were infected by H1N1 after 48 h transfection.

2.8. Western blot

At the end of the experiment, cells were lysed in RIPA Lysis Buffer and proteins were extracted. The protein concentration was quantitated by using BCA kit (Sigma). An equal amount of protein was subjected to SDS-PAGE and transferred onto a PVDF membrane. After blocking with 5% skim milk, the membrane was incubated with primary antibody diluent (The antibody against TFEB 1:1000; The antibody for β -actin 1:5000) and secondary antibodies (HRP-conjugated antibodies and anti-mouse). The

membrane blots were developed with the enhanced chemiluminescence method and visualized by using the ChemiDoc XRS camera system (Bio-rad, USA).

2.9. Immunofluorescence assay

3×10^4 RAW264.7 cells per well were plated in 24-well plates on 13-mm coverslips, transfected/infected/treated as designated in the scheme and incubated for 48 h. The cells were fixed with 4% paraformaldehyde in PBS and followed by permeabilisation in 0.2% Triton X-100 in PBS for 10 min at room temperature with agitation. Coverslips were then blocked in 5% skim milk-TBS-T blocking solution. Primary antibody anti-TFEB was diluted (1:400) into 4% BSA in PBS and added to coverslips. After incubation for 20 min, the secondary antibody goat anti-rabbit (1:1000) was added (1:500) in a solution of 4% BSA in PBS. DAPI was added to the secondary antibody solution to stain nuclei (1:10000 in PBS). The confocal images were acquired by using a OLYMPUS-HGLGPS fluorescence microscope and accompanying Cellsens standard software (OLYMPUS, Japan) (Rusmini P et al., 2017).

2.10. Autophagic flux assay

The autophagic flux in RAW264.7 cells was detected by cell autophagy staining kit (MDC methods) (Dai S et al., 2017). Raw264.7 cells were infected with IAV at MOI 1 and treated with MA. After 48 hrs, autophagic vacuoles were labelled with MDC by incubating cells with 0.05 mM MDC in DMEM at 37 °C for 10 min. After incubation, cells were washed three times with wash buffer and immediately photographed and analysed by using a fluorescence microscopy (Nikon Eclipse TE 300, Japan) and accompanying excitation/emission (380/420 nm) filters. Images were captured with a CCD camera and the mean fluorescence intensity was quantified by the Image Pro Plus 6.0.

2.11. Cytokines assay

Raw264.7 cells were seeded in 96-well plates at a cell density of 5.0×10^3 cells per well. The siTFEB or siNC was transfected at 24 hpi, then cells were infected with IAV of MOI 10 at 48 hpi. The MA was added at 72 hpi. After treating and incubating, culture supernatant was harvested. The inhibition of TNF- α , IL-6, IL-1 β and IFN- β production was detected according to the manufacturer's instruction of Elisa kits, respectively.

2.12. Statistical analysis

All data were analyzed in OriginPro 2017 software. Data are presented as mean $\pm$ SEM from five samples. The significance of the statistical difference between the means was determined using ANOVA (*p < 0.05, **p < 0.01). The level of significance was determined using Duncan's multiple-range test (*p < 0.05, **p < 0.01).

Results

Cytotoxicity of MA was evaluated against RAW264.7 cells by the cck-8 assay. The result showed that the MA was largely non-toxic in Raw264.7 cells (Fig. 2A). The CC_{50} value was 72.44 μ M. In the following experiment, 1, 5 and 10 μ M concentrations were chosen to investigate the anti-H1N1 effect of MA in Raw 264.7 cells.

When virus proliferates in host cells greatly, it usually leads to a cytopathic effect (CPE) in host cells, which can cause injury and death of infected cells (Laghlali G et al., 2020). Therefore, the CPE can reflect the amount and interaction of virus in host cells. To investigate the protection effect of MA on RAW264.7 cells infected by H1N1, we observed and photographed the CPE of RAW264.7 cells under inverted microscope, and detected the viability of infected cell by cck-8 assay. As shown in Fig. 2B, compared to virus control group (Fig. 2C(b)), MA could markedly increase the survival rate of H1N1-infected cells, and there were no visible CPE in MA treatment groups (Fig. 2C (d, e, f)). The results indicated that MA was able to protect infected cells from viral apoptosis and increase cells viability.

To attest the anti-influenza virus activity of MA, we performed the plaque and hemagglutination assay to evaluate the viral load of RAW264.7 cells after MA treatment, furtherly. The hemagglutination assay was performed with the supernatant of 2-fold serial dilutions of H1N1 and treated with MA in different concentrations (1–10 μ M). As shown in Fig. 3A, the HA was significantly reduced when the cells were treated with gradient concentrations (1, 5 and 10 μ M) of MA for 24 h. The Fig. 3B showed that RBCs sedimented to the bottom of U-bottomed 96-well plate and exhibited obvious agglutination in the NC group without virus, whereas VC group had an epinephelos appearance and exhibited hemolysis with no sedimentation, since the IAVs can induce hemagglutination of RBCs. From Fig. 3B we can see the obvious agglutination in RBCs, which indicates that the viral particles in the supernatant of MA groups have been significantly reduced, particularly at the concentrations of 5 and 10 μ M.

The plaque assay results verified the anti-influenza virus activity of MA, furtherly. The plaque assay was performed with the supernatant of 10-fold serial dilutions of H1N1 and treated with MA in different concentrations (1–10 μ M). As shown in Fig. 3C, H1N1 of MOI 1 induced an average 4.60 ± 0.43 Log progeny infectious particles per ml in virus control group. In the presence of 1, 5 and 10 μ M MA, the viral particle production was reduced by 1.16 ± 0.18 , 0.83 ± 0.35 and 0.29 ± 0.22 Log when compared to VC group, respectively. Correspondingly, the lysis plates inhibition of three concentrations was 74.8%, 82% and 93.7%, respectively.

The EC_{50} of MA against H1N1 in vitro were calculated and presented in Fig. 3E. When the RAW264.7 cells were infected by H1N1 of MOI 1, the EC_{50} and EC_{90} values were 1.27 and 5.30 μ M MA, respectively, and when the RAW264.7 cells were infected by H1N1 of MOI 10, the EC_{50} and EC_{90} values were 2.39 and 8.38 μ M, respectively.

The different types of treatment experiments were performed to complement the analysis of the antiviral activity of MA (Mayra D.T et al., 2019). Here, Raw264.7 cells were infected with H1N1 at MOI 1, and MA at a concentration of 5 μ M was added at different phase of infection (Fig. 4A). In the Pre- experiments, MA

was added 1 or 3 h before infection. In the during- experiments, both the virus and the MA were added simultaneously during the infection for 3 h. The post-infection means the MA was added at 3, 6, 9, 12, 24 hpi after H1N1 infection, respectively. At 24 hpi cells were harvested and subjected to PFU detection. The results showed that MA exhibited an inhibition to H1N1 when added pre, during and up to 24 hpi (Fig. 4B). These findings also suggested that the anti-H1N1 activity of MA is likely due to its activity against both the stages of intracellular replication of H1N1 and early stages of its replication cycle such as virus attachment or entry. In addition, We noticed a minimum production of virus particle was decreased by of 65% in MA pretreated cells when compared to the control. When the MA was added to cells at 3, 6, 9, 12, 24 hpi, maximum reduction of virus particle can reach almost 95%. Obviously, MA post-treatment is more effective than pre-treatment after infection, which suggest that MA might act on the late stages of H1N1 infection.

TFEB is a member of the MiTF/TFE (microphthalmia-transcription factor E) family of transcription factors of the leucine zipper bHLH-LZ, which is involved in the regulation of many important cellular physiological processes, and now it has been demonstrated that it is the master regulators of macroautophagy/autophagy and lysosome function raises the possibility that it may be of central importance in linking autophagy and lysosome (Settembre C et al., 2019). However, the autophagy/lysosome pathway is important for viruses to replicate and escape in infected cells (Wang Y et al., 2018), so the TFEB may have important effects on the replication and spread of viruses. Thus, MA may clear H1N1 virus by inhibiting TFEB. To verify this, TFEB protein expression in H1N1 infected (MOI = 10) RAW 264.7 cells was detected. The result in Fig. 5A displayed that H1N1 increased TFEB levels by 1.5, 2.0 and 3.1-fold at 12, 24 and 36 hpi when compared to uninfected cells, respectively. Yet, After MA intervention, the TFEB levels of infected cells were reduced up to 70% (Fig. 5B). To reveal the affect of TFEB on H1N1 replication, the expression of TFEB was silenced with siTFEB, meanwhile the blank siRNA was transfected as the negative control (siNC). Finally, the PFU was determined. As shown in Fig. 5C, compared to the siNC transfection cells, the TFEB levels was reduced by 85% after 48 h siTFEB transfection. Base on this, H1N1 of MOI 1 was added into transfection or MA treatment cells. At 24 hpi, the PFU was determined. As shown in Fig. 5D, compared to the virus control group and the siNC group, the viral particle production was reduced by 4-fold in siTFEB-transfected group. While after MA treatment, We can also see 3.2-fold decrease of virus particle production. These results indicated the expression of TFEB protein is positively correlated with the increase of virus particle production. Yet, when the TFEB levels was decreased by MA in infected cells, the virus particle production was prominently reduced.

TFEB overexpression promotes the expression of multiple lysosomal genes, thereby regulating the production and efflux function of autolysosomes and increasing autophagic flux. TFEB subcellular localization and its transcriptional activity are strictly regulated by many protein molecules, of which the mTORC1 is the foremost regulator. It mainly affects the nuclear entry and transcriptional activity of TFEB by means of phosphorylation (Noda T. 2017). Therefore, the nuclear transfer of TFEB can reflect the autophagy status. To reveal the efficacy of MA on the nuclear transfer of TFEB and autophagy, we detected the nuclear transfer rate of TFEB and autophagy flux in H1N1 infected cells (MOI = 10) using immunofluorescence. As shown in Fig. 6A, after MA treatment, the nuclear transfer rate of TFEB is

statistically significant compared to virus control group, and we observed a concentration-dependent decrease in the levels of TFEB in the nucleus of MA treatment groups (Fig. 6B). To determine whether MA inhibited autophagy induced by H1N1, the autophagic flux of infected cells was determined. The results showed that MA observably suppressed the autophagy in H1N1- infected cells, since the autophagic vesicles stained by MDC, a lysosomotropic compound used to label lysosomes and autophagosomes, were dramatically reduced after MA treatment (Fig. 6C). The autophagic vacuole formation was observed by fluorescence microscope (Fig. 6D).

Influenza virus infections usually cause severe interstitial pneumonia and induce an uncontrolled host-immune response, leading to a life-threatening condition called cytokine release syndrome (CRS) (Liu D et al., 2018). CRS represents an emergency scenario of a frequent challenge, which highlights the complex and interwoven link between infections and autoimmunity, and it has been believed to be a primary cause of death in IAV H1N1-infected patients (Downey J et al., 2018; Peteranderl C et al., 2016). Of note, autophagy has been revealed to be critical for the generation of cytokines from innate immune cells (Deretic V., et al., 2018), which provides possible targets for immunotherapy to combat IAV infection via inhibiting autophagy. Base on the suppressive effect of MA on autophagy of RAW264.7 cells infected by H1N1 by modulating TFEB, we investigated the anti-inflammatory effect of MA. Raw264.7 cells were infected with IAV at MOI 10, and MA was added. Meanwhile, cells transfected with the siTFEB or siNC was seted as the contrast. At 48 h post-transfection or treatment, MA could inordinately decrease the levels of TNF- α , IL-6, IL-1 β and IFN- β in the H1N1 infected RAW264.7 cells,as is shown in Fig. 7A, Fig. 7B, Fig. 7C and Fig. 7D, respectively. These four cytokines were also inordinately decreased after the silence of TFEB. This experiment suggested that MA might decrease the levels of inflammatory cytokines induced by H1N1-infection by inhibiting TFEB.

Discussion

M. oleifera seeds have become a focus of research in recent years, as they have extensive pharmacological activities and abundant levels of valuable chemical components. However, there have been relatively few antiviral studies on the chemical compositions of *M. oleifera* seeds. Our team has obtained many compounds from the *M. oleifera* seeds, the MA is one of them. In this paper, we reported that MA has good antiviral activity against H1N1 in RAW264.7 cells, as it can inhibit RBC hemolysis and plaque in H1N1-infected cells. The EC50 and EC90 values in RAW264.7 cells relate to 1.27 and 5.30 μ M MA when infected by H1N1 of MOI 1, respectively. When cells were infected by H1N1 of MOI 10, the EC50 and EC90 values were 2.39 and 8.38 μ M, respectively. In addition, the MA protects cells from cytopathic effect induced by H1N1 and increases cell survival. The different types of treatment experiments showed that the main anti-H1N1 activity of MA is likely due to its activity against the late stages of infection.

Autophagy has been confirmed to be a important mechanism of RNA viruses immune evasion, replication and release from infected cells. Double-membrane compartments formed during autophagy can provide a physical platform for the viral replication machinery, locally concentrate essential intermediates and protect viral RNAs from detection by innate immune sensors and degradation (Arrey F et al., 2009; Zhou Z

et al., 2009). IAV also triggers the accumulation of autophagosomes for viral replication. Our study showed that the autophagic flux of RAW264.7 cells was markedly enhanced after H1N1 infection, meanwhile the virions increased dramatically too. Hence it was suggested that the induction of autophagosome during IAV infections might promote virus replication within infected cells. The MA could repress H1N1-induced autophagic flux, therefore it blocked the important pathway of virus amplification and restricted viruses replication and virulence. The suppressive effect of MA on autophagy of RAW264.7 cells infected by H1N1 was positive related to the reduction of TFEB protein, which is the master regulator for transcription of genes involved in autophagy and lysosome biogenesis.

In this paper, it was proved that cellular factor TFEB may contribute to the replication of H1N1 virus. We revealed the expression of TFEB was upregulated gradually with the extension of H1N1 infection time, so the H1N1 virus could promote the TFEB expression in infected cells, and it was a possible target for H1N1 virus triggered autophagy. In fact, the production of infectious virus particles were reduced by 80% after the silence of TFEB. What's most important, the nuclear transfer rate of TFEB in H1N1-infected cells was markedly increased. The nuclear transfer of TFEB can promote the transcription of multiple genes in the lysosomal pathway, and regulate the production and efflux function of autophagy lysosomes (Napolitano G et al., 2016). Therefore, the nuclear transfer promotion of TFEB was another evidence for H1N1 virus inducing autophagy in infected cells. It may be an important mechanism for H1N1 to complete its life cycle by utilizing autophagy.

MA was found to inhibit TFEB expression in H1N1-infected cells, and it could also decrease the nuclear transfer rate of TFEB. Accordingly, MA could repress autophagic activity caused by H1N1 infection. This inhibition was shown to be positively correlated with the antiviral activity of MA. However, whether MA acted directly on TFEB or regulated other proteins in relevant pathway needs further study.

What's more frightening than the virus is the cytokine storm, which is an uncontrolled excessive immune response and an important pathogenesis of viral pneumonia. The virus usually hijacks the immune system, leading to the loss of negative feedback in immune regulation, abnormally increasing a variety of cytokines, impairing the alveolar diffusion function and causing multiple organ dysfunction (Gerlach T et al., 2019; Gounder A. P et al., 2019). In addition to investigating the antiviral activities of MA, we also focused on its anti-inflammatory activity. The result showed that MA could reduce the levels of inflammatory cytokines TNF- α , IL-6, IL-1 β and IFN- β in H1N1-infected cells. These four cytokines have been confirmed to play important roles in inflammation and apoptosis, which are responsible for organ dysfunction. The protection of MA on infected cells might be related to the reduction of inflammatory cytokine levels.

Mounting evidence have confirmed that many inflammatory diseases may fundamentally be caused by autophagy-lysosome pathway dysfunction (Ahmad L et al., 2019; Racanelli A C et al., 2018). As the master regulator of macroautophagy /autophagy and lysosome function, the TFEB may be a bridge in linking autophagy and lysosome dysfunction with inflammatory disorders. Our study also demonstrated the TFEB has a close link with cell inflammatory induced by H1N1. In the H1N1 infected cells with high

TFEB expression, we found that higher levels of inflammatory cytokines compared to normal or siTFEB transfected cells. Yet, following the TFEB expression was down-regulated by MA, the inflammatory cytokines of infected cells was dramatically decreased.

Conclusion

This study indicated MA is a new compound from *M. oleifera* seeds which exhibit antiviral activity towards H1N1. The possible mechanism of MA anti-H1N1 virus was described in Fig. 8. The observed EC50 and cytotoxicity together with the different types of treatment data are hopeful and indicate that MA is a potential compound for the prophylaxis and treatment of influenza virus infection.

List Of Abbreviations

PFU: plaque forming unit

HA: Hemagglutination

TFEB: transcription factor EB

MA: *Moringa A*

CPE: Viral cytopathic effect

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publication

Not applicable.

Availability of data and material

Data and materials are available upon request by the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceived and designed the experiments: Zhendan He. Performed the experiments: Yongai Xiong, Mengxun Zhang. Analyzed the data: Ning Liang. Drafted the manuscript: Muhammad Shahid Riaz Rajoka. All authors read and approved the manuscript.

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Figures

Figure 1

The chemical structure of Moringa A

Figure 2

MA alleviates the viral cytopathic effect (CPE) in RAW264.7 cells. A. Dose response curve showing the cytotoxicity of MA in normal RAW264.7 cells determined by cck-8 assay (n=3). B. Cytopathic effect (CPE) in RAW264.7 cells treated with MA and Oseltamivir 48h after the H1N1 infection. C. CPE pictures: (a) Normal control: saline + DMEM. (b) Virus control: H1N1 + DMEM. (c) Positive control: H1N1 + Oseltamivir (10 μM). (d) H1N1 + MA (1 μM). (e) H1N1 + MA (5 μM). (f) H1N1 + MA (10 μM).

Figure 3

Anti-viral activities of MA on the H1N1 virus in vitro. (A) The agglutination in RBCs showed a dose-dependent behavior when compared to virus control group. (B) Viruses were detected via the hemagglutination assay. (C) The PFU showed a dose-dependent behavior when compared to VC group. (D) Plaques quantification of the infected cells. (E) Dose response curve analysis of MA against H1N1.

Results were statistically significant (**p < 0.01) when analyzed using one-way ANOVA and Tukey's post-test.

Figure 4

Anti-viral activity of MA on the H1N1 when added up to 24 hpi. (A) Schedule of different phase of infection and treatment. (B) H1N1 virus particle production was detected by the plate-forming method. In all experiments, cells were infected at MOI 1 and 5 μ M MA was added. Data were obtained from five independent experiments and analyzed using one-way ANOVA and Tukey's post-test, with *p < 0.05, **p < 0.01.

Figure 5

Anti-viral activities of MA is independent of TFEB. (A) TFEB expression increased with the extension of infection time (MOI = 10). (B) The levels of TFEB protein in H1N1-infected cells (MOI = 10) after MA intervention. (C) The levels of TFEB protein in siTFEB-transfected and MA (5 μ M) intervention cells. (D) RAW264.7 cells were infected with H1N1 (MOI = 1) and transfected with siNC and siTFEB. Data were obtained from five independent experiments and analyzed using one-way ANOVA and Tukey's post-test, with *p < 0.05, **p < 0.01.

Figure 6

Suppressive effect of MA on autophagy of RAW264.7 cells infected by H1N1 by modulating TFEB. (A) Quantification of mean percentage of TFEB localized in the nucleus. (B) Immunofluorescence images displaying TFEB translocation after MA treatment. (C) The mean fluorescence intensity was measured by Image Pro Plus software. (D) Autophagic vacuole formation was observed by fluorescence microscope: (a) Normal control: untreated and uninfected cells. (b) Virus control: infected and untreated cells. (c)

siTFEB: infected and si TFEB transfected. (d) MA treated (1μM) and infected cells. (e)MA treated (5μM) and infected cells. (f) MA treated (10μM) and infected cells. Data are presented as mean ± SEM from five independent experiments.

Figure 7

MA decreased the levels of inflammatory cytokines induced by H1N1-infection. (A) Quantification of TNF-α content in normal and H1N1-infected cells. (B) Quantification of IL-6 content in normal and H1N1-infected cells. (C) Quantification of IL-1β content in normal and H1N1-infected cells. (D) Quantification of IFN-β content in normal and H1N1-infected cells. Data are presented as mean ± SEM from five independent experiments.

Figure 8

Anti-H1N1 and anti-inflammatory activity of Moringa A by regulating TFEB