MORINGA OLEIFERA CRUDE EXTRACT AND THE REFERENCE M2-CHANNEL BLOCKER AMANTADINE AS ANTIVIRALS AGAINST THE EGYPTIAN H5N8 ISOLATE, SPECIFICALLY INFLUENZA A/CHICKEN/EGYPT/Q16684C/2019 (H5N8) VIRUS.

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Abstract

Despite that great effort is currently running in the direction of vaccine and drug development, limited number of therapeutics are currently approved against seasonal influenza virus. To limit the spread of the transmission of highly pathogenic influenza (HPAI) viruses including HPAI H5N8 to mammals, it is recommended to control them in their natural and intermediate reservoirs, namely domestic poultry. Herein we aim to test both *Moringa oleifera* extract against the reference M2-channel blocker amantadine as antivirals against the Egyptian H5N8 isolate, specifically influenza A/chicken/Egypt/Q16684C/2019 (H5N8) virus. Interestingly, beside its high safety on MDCK cells (CC_{50}= 15.42 mg/ml), the extract shows a promising and potent antiviral activity (IC_{50} = 0.665 µg/ml) against HPAI H5N8, while the 50% inhibitory concentration (IC_{50}) of the amantadine was 56.81 µM. *Moringa oleifera* extract has shown a very promising effect as an anti-influenza, which makes it worthy of further studies.

Key words: *Moringa oleifera,* Amantadine, H5N8, avian influenza, antiviral

1. Introduction

Emerging clade 2.3.4.4 HPAI H5N8 virus was first detected in live bird market in China in 2010 (Lee *et al*., 2014). In January 2014, H5N8 viruses caused different outbreaks in domestic poultry and wild birds in South Korea (Kang *et al.,* 2015). Several outbreaks were subsequently recorded either in wild or domesticated birds in many Eurasian and North American countries between 2014 and 2017. Since 2014, HPAI H5N8 virus has been detected in wild birds and domestic poultry in Egypt (Kandeil *et al.*, 2017; OIE, 2017; Yehia *et al.,* 2022).

Recently, much attention has been focused on the *Moringa* tree (*Moringa oleifera* Lam), also called the tree of life, as a source of active ingredients valuable for antimicrobial activities. Due to the presence of a broad spectrum of bioactive compounds, the plant has powerful antioxidant, antibacterial, toning, astringent, and anti-inflammatory properties (Nizioł-Łukaszewska *et al.,* 2020). Leaves of the *Moringa oleifera* tree have been found to contain flavonoids including myricetin, quercetin, kaempferol, isorhamnetin, rutin, as well as phenolic acids. Fresh leaves are a good source of carotenoids such as lutein, -carotene, and zeaxanthin. In addition, the *Moringa oleifera* tree is characterized by a high content of vitamins C and A. The active substances contained in the plant have shown to have beneficial *in vitro* antimicrobial activities suggesting it as natural antiviral supplementary against different animal viruses (Biswas *et al.,* 2020; Younus *et al.,* 2016).

In 1976 amantadine was approved for the treatment of influenza A by the US Food and Drug Administration (FDA). Although, it blocks many ion channels such as NMDA and M2, the influenza A shown resistance to it due to the several mutations it causes. Furthermore, many strains of influenza have become completely resistant to amantadine (CDC, 2008-2009).

Herein we aim to test both *Moringa oleifera* extract against the reference M2-channel blocker amantadine as antivirals against the Egyptian H5N8 isolate, specifically influenza A/chicken/Egypt/Q16684C/2019 (H5N8) virus.

2. Materials and methods

2.1. Virus and cell lines

MDCK cells were cultured in Dulbecco's modified Eagle's medium (Lonza) supplemented with 10% fetal bovine serum, and 1% antibiotic antimycotic mixture (Lonza). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. An influenza A/chicken/Egypt/Q16684C/2019 (H5N8) virus was propagated in MDCK cells. The virus was titrated using plaque titration assay as previously described *(Mostafa et al.*, 2020). Fresh *Moringa oleifera* extract was prepared from fresh leaves using 70% ethanol according to Ekpo and Etim, (2009). Amantadine hydrochloride was purchased from Sigma-Aldrich.

2.2. MTT cytotoxicity assay

To assess the half maximal cytotoxic concentration (CC_{50}), stock solutions of the dried ethanolic extract was prepared in 10 % DMSO in ddH_2O and diluted further to the working solutions with DMEM. The cytotoxic activity of the extract was tested in MDCK cells by using the 3-(4, 5-dimethylthiazol -2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method with minor modifications. Briefly, the cells were seeded in 96 well-plates (100 µl/well at a density of 3×10^5 cells/ml) and incubated for 24 h at 37°C in 5% CO_2. After 24 h, cells were treated with various concentrations of the tested samples in triplicates. 24 h later, the supernatant was discarded, and cell monolayers were washed with sterile 1x phosphate buffer saline (PBS) 3 times and MTT solution (20 µl of 5 mg/ml stock solution) was added to each well and incubated at 37°C for 4 h followed by medium aspiration. In each well, the formed formazan crystals were dissolved with 200 µl of acidified isopropanol (0.04 M HCl in absolute isopropanol = 0.073 ml HCL in 50 ml isopropanol). Absorbance of formazan solutions was measured at λ max 540 nm with 620 nm as a reference wavelength using a multi-well plate reader. The percentage of cytotoxicity compared to the untreated cells was determined with the following equation.

The plot of % cytotoxicity versus sample concentration was used to calculate the concentration which exhibited 50% cytotoxicity (CC_{50}) (Mosmann, 1983).

$$\%\ \text{cytotoxicity} = \frac{(\text{absorbance of cells without treatment} - \text{absorbance of cells with treatment})\ \text{X}\ 100}{\text{absorbance of cells without treatment}}$$

2.3. Inhibitory concentration 50 (IC_{50}) determination

The IC_{50} was determined as previously described (Mostafa *et al.,* 2020). In 96-well tissue culture plates, 2.4×10^4 MDCK cells were distributed in each well and incubated overnight at a humidified 37°C incubator under 5% CO_2 condition. The cell monolayers were then washed once with 1x PBS and subjected to virus adsorption for 1 h at room temperature (RT). The cell monolayers were further overlaid with 50 µl of DMEM containing varying concentrations of the tested samples. Following incubation at 37°C in 5% CO_2 incubator for 72 h, the cells were fixed with 100 µl of 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet in distilled water for 15 min at RT. The crystal violet dye was then dissolved using 100 µl absolute methanol per well and the optical density of the color is measured at 570 nm using Anthos Zenyth 200rt plate reader (Anthos Labtec Instruments, Heerhugowaard, Netherlands). The IC_{50} of the sample is that required to reduce the virus-induced cytopathic effect (CPE) by 50%, relative to the virus control.

3. Results and discussion

3.1. ***Cytotoxicity, anti-influenza activity and selectivity index of Moringa oleifera leaves extract*** **and amantadine**

The cytotoxicity of the *Moringa oleifera* extract and amantadine was evaluated in MDCK cell lines using MTT assay. Both samples were almost not toxic for MDCK cells, representing a high CC_{50} value of 15.42 mg/ml and 19.71 mg/ml for *Moringa oleifera* extract and amantadine respectively (Figure 1). The toxic effect of tested extract was a dose-dependent. Therefore, for further studies we selected the safe concentration of 1 mg/ml – 100 ng/ml for *Moringa oleifera* leaves extract for subsequent antiviral studies.

The antiviral activity of *Moringa oleifera* leaves extract against HPAIV H5N8 based on the dose response was determined as inhibitory concentration 50 (IC_{50}). The result showed that the 50% inhibitory concentration (IC_{50}) of the extract was 0.665 µg/ml, while, the 50% inhibitory concentration (IC_{50}) of the amantadine was 56.81 µM (Figure 1). In consistence with our results, the previous studies conducted by Abd Rani *et al*. (2018) was demonstrated that the *Moringa oleifera* extract contain various active components, such as alkaloids, phenolic acids, flavonoids, isothiocyanates, and thiocarbamate attributing their role as antivirals. In agreement with the current study, the findings by Yongai *et al*. (2020) reported that *Moringa oleifera* extract have a good activity of anti-influenza virus.

On the other hand, many previous studies were reported that amantadine exhibited an
inhibitory effect against the attachment and replication of influenza virus via several different mechanisms of action, whereas affecting neuraminidase, hemagglutinin activity and blocking M2 ion channel (Sarr *et al.*, 2021).

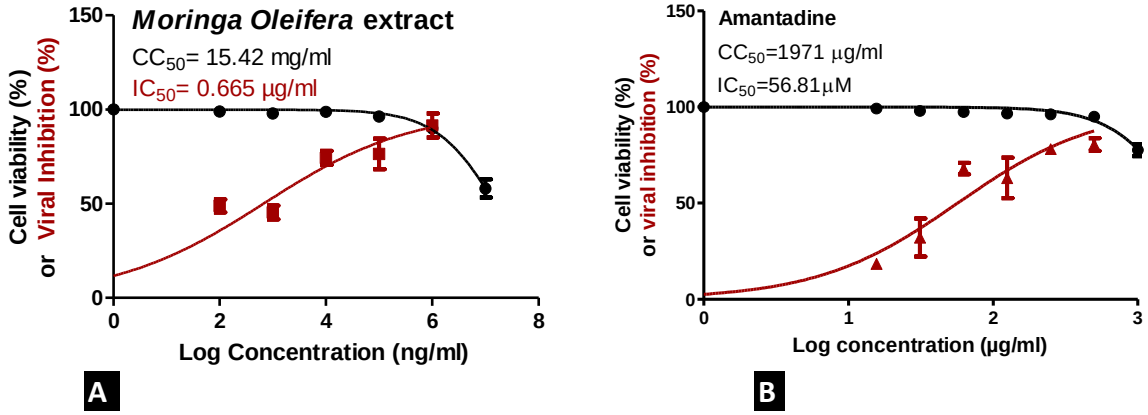

Figure (1). Half maximal inhibitory concentration "IC_{50}" of the tested *Moringa oleifera* leaves extract (A), and amantadine (B), against HPAIV H5N8 virus in MDCK cells. The IC_{50} values were calculated using nonlinear regression analysis of GraphPad Prism software (version 5.01) by plotting log inhibitor versus normalized response (variable slope).

3.2. Selectivity index

These data demonstrated that *Moringa oleifera* leaves extract is effective as anti-influenza with high selectivity index ($SI=CC_{50}/IC_{50}=23187$) (Table 1). Previous studies have recommended a selectivity index that is higher than or equal 10 (≥ 10) to be candidate for further investigation as antiviral (McGaw *et al.*, 2014).

This is consistent with documentation of previous reports, representing that the plant *Moringa oleifera* extract showed inhibitory activity principally against a few infectious disease causing viruses including Human Immunodeficiency Virus (HIV), causative agent for Acquired Immune Deficiency Syndrome (AIDS) in human; Herpes Simplex Virus (HSV) causes fever blisters or cold sores around the mouth and lips and genital infection in human body; Hepatitis B Virus (HBV) which affects human liver and causes inflammation, cirrhosis and liver cancer; Epstein-Barr Virus (EBV) responsible for various non-malignant, premalignant and malignant lymphoproliferative diseases in human; Foot-and-Mouth Disease Virus (FMDV) causes blisters in mouth and feet of human and some hoofed animals; and Newcastle Disease Virus (NDV) affecting avian species and transmissible to human through poultry birds (Biswas *et al.*, 2020).

Table (1). The CC_{50}, IC_{50} and selectivity index of *Moringa oleifera* leaves extract and amantadine

Extract	CC_{50} (ng/ml)	IC_{50}	SI
Moringa oleifera leavesextract	15420	0.665 Mg/ml	23187
Amantadine	19710	56.81 µM	346.945

Abbreviations: "CC_{50}" half maximal cytotoxic concentration; "IC_{50}" half maximal inhibitory concentration; "SI" Safety index

4. Conclusion

Ethanolic extract of *Moringa oleifera* leaves extract exhibited stronger anti-H5N8 virus activity at a nontoxic Micromolar concentration in MDCK cell lines. The results recommend that the plant extract may be helpful to elucidate effective antiviral activity against H5N8 virus.

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مستخلص المورينجا أوليفيرا والمانع المرجعى لقناة M2 الأمانتادين كمضاد للفيروسات ضد العزلة المصرية H5N8 وتحديدا الإنفلونزا /A / الدجاج / مصر / 2019/ Q16684C / فيروس (H5N8)

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على الرغم من الجهود الكبيرة المبذولة حديثا في اتجاه تطوير اللقاحات والأدوية، تمت الموافقة حاليا على عدد محدود من العلاجات ضد فيروس الإنفلونزا الموسمية. للحد من إنتشار فيروسات الإنفلونزا شديدة الإمراض (HPAI) (HPAI H5N8) بما في ذلك الثدييات، يوصـــى بمكافحتها في عوائلها الطبيعية والوســيطه، وهى الدواجن المنزلية. في هذا البحث تم إختبار كل من مستخلص المورينجا أوليفيرا مقابل مقفلات قنوات ال M2 وهو الامانتادين (amantadine) ضـــد العزلة المصـــرية H5N8 وتحديدا انفلونزا/ الدجاج/ 2019/ Q16684C / مصـــر (H5N8) . من المثير للاهتمام، أنه إلى جانب الأمان الكبير على خلايا MDCK حيث كانت (CC_{50}= 15.42 mg/ml) أظهر المستخلص النباتى نشاطا واعدا وقويا كمضاد للفيروسات ضد HPAI H5N8 (IC_{50}= 0.665 µg/ml) بينما كان 50% من التركيز المثبط من الامانتادين (amantadine) هو 56.81 µM. أظهر مستخلص المورينجا أوليفيرا تأثيرا واعدا كمضاد للإنفلونزا مما يجعله يستحق الكثير من الدراسات.

الكليمات المفتاحية : المورينجا أوليفيرا , الامانتادين , H5N8 , انفلونزا الطيور , مضادات الفيروسات