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Virucidal effect of *Moringa oleifera* against SARS-CoV-2 and Influenza A/H1N1

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In the last two decades, global human health problems and economic disasters have been brought on by many viruses, such as severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and middle east respiratory syndrome coronavirus (MERS-CoV), belonging to coronaviruses, and influenza viruses mainly H1N1. The lack of specific therapies and few available treatments for these viruses have made them more difficult to overcome. A significant barrier to antiviral therapy is the emergence of drug-resistant respiratory viruses; as a result of naturally occurring mutations, in addition to the misuse of already approved antiviral drugs. Therefore, this study will be on *Moringa oleifera* leaf extract as one of the most common medicinal plants. The ethanolic extract of the plant leaf powder was prepared and fractionated, later the crude and the fractions were tested for antiviral activity against H1N1 and SARS-CoV-2 as the causative agent for pandemic 2009 and 2019, respectively, after safety confirmation on Madin-Darby canine kidney (MDCK) and African green monkey kidney derived cells (Vero-E6) cell lines. The plaque reduction assay was performed for the crude ethanolic extract to determine the action of the antiviral medication with a low IC_{50}. The results showed high safety on the two cell types and the activity against both viruses with high percentage of direct inhibition (virucidal), indicating that *Moringa oleifera* can represent a promising antiviral agent against them.

***Keywords*:** SARS-CoV-2, H1N1, *Moringa oleifera*, virucidal effect, Vero-E6 cells.

INTRODUCTION

The respiratory system is a perfect candidate for many viruses to enter, either directly by inhaling the airborne virus particles produced by coughing and sneezing or indirectly by touching the mouth or nose after coming into contact with a surface that has been infected; hence, viral entry occurs successfully and easily in both cases (Dhand and Li, 2020). Respiratory diseases are a burden on society; according to the World Health Organization (WHO), influenza-related respiratory infections kill over 500,000 people each year, affecting infants, young children, and the elderly, who are more vulnerable, and costing billions of dollars (Sheridan *et al*., 2012).

Since it was first discovered in the sixteenth century, influenza has been a significant cause of mortality and morbidity throughout the world. The Orthomyxoviridae family, which includes influenza viruses, is huge and extremely diversified (Cui *et al.*, 2022), and typically has a single-stranded, enveloped and negative-sense RNA genome (Muraduzzaman *et al*., 2022).

The H1N1 Influenza virus strain caused a global pandemic in 2009 that affected more than 170 countries, and resulted in over 19,000 deaths (Vasileva & Badawi, 2019). On the other hand, COVID-19 is a respiratory disease caused by SARS-CoV-2 and it was characterized by fever, coughing, shortness of breath, and/or chest pain. Between the four genera of the Coronaviruses (CoVs); alpha, Beta, Gamma & Delta, two beta-CoVs and two alpha-CoVs have been linked to the common cold and self-limiting respiratory infections in humans. Two extremely pathogenic human beta-CoVs, SARS-CoV in 2003, the MERS-CoV in 2012 and SARS-CoV-2 in 2019 have recently been added to this list (Mostafa *et al.*, 2020).

Although there are vaccines and efforts are being made in the vaccine development against viruses, the need for alternative solutions was urgent due to the RNA viruses' proclivity for mutational alterations that render the approved vaccines no longer effective and the majority of clinically used drugs being used to relieve COVID-19 symptoms rather than their side effects (Ghaebi *et al*., 2020).

Plants are the most abundant source of both traditional and modern medicine, as well as food for humans and animals. Countless beneficial harvests obtained from plants, whether directly or indirectly, attest to their significance for both humans and other living. According to the WHO, 80% of the world's population uses herbal medicines for some aspect of primary healthcare (AL-Obaidi *et al*., 2021).

Moringa oleifera, or "the drumstick" or "miracle tree," as it is also known, is a member of the family Moringaceae and is native to the northern part of India, it is widely planted and used across the tropics due to its bioactive components known as phytochemicals (Saini *et al*., 2016).The plant's leaves are nutrient-rich and can be eaten, and their extract contains carotenoids, alkaloids, phenols, flavonoids, vitamins, isothiocyanates, saponins, tannins, steroids, and other compounds (Kashyap *et al*., 2022).

Nowadays, *Moringa oleifera* leaves can be used to make a variety of medications and dietary supplements, which iron, calcium, and potassium are all present in its leaves, along with vitamins A, C, and E. Vitamin A supports healthy immunity, eyesight, and embryonic development (Patil *et al*., 2022), and its antiviral activity was confirmed against many viruses (Xiong *et al*., 2021).

Therefore, the current study was prepared to investigate and assess the possibility of using leaves extract of *Moringa oleifera* plant against two widely distributed respiratory RNA viruses (2009 H1N1 and SARS-CoV-2).

MATERIALS AND METHODS

Plant materials

The dried powdered leaves of *Moringa oleifera* were obtained in December 2020 from Moringa products at the National Research Centre, Giza, Egypt. It was identified by Prof. Dr. Abo-Elfotoh Mohamed, Egyptian Scientific Society of Moringa, National Research Centre, Giza, Egypt.

Preparation of *Moringa oleifera* extract and fractions

In a dark glass bottle, 1300 ml of 70% ethanol was added to 100 g of milled *Moringa oleifera* leaves. The extract was filtrated and evaporated under vacuum at 45°C using the rotary evaporator to obtain 16 g of the dry residue of leaves extract. The obtained residue was suspended in the least amount of distillated water and then fractionated via successive partitioning with petroleum ether, methylene chloride, and n-butanol. The obtained liquid fractions and the residual aqueous solution were dried via rotary evaporation, giving 0.13 g, 0.41 g, 7.5 g, and 8 g of the dry residues of petroleum ether, methylene chloride, n-butanol, and residual aqueous solution fractions, respectively.

The extraction and fractionation were done according to Mohamed *et al.* (2021).

Cells and viral strains:

Two types of cells were used; Vero-E6 cells for SARS-CoV-2 and MDCK cells for H1N1. The Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Verviers, Belgium) supplemented with 2% antibiotic antimycotic mixture (Lonza) and 10% fetus bovine serum (FBS) (Gibco, New York, NY, USA). Using 7.5% sodium bicarbonate solution, the media PH was adjusted to 7.2–7.4, and the cells were incubated in a carbon dioxide incubator at 37°C (Roshdy *et al.*, 2020). Infection medium was used for virus propagation supplemented with 2% bovine serum albumin (BSA) and 1% L-1-tosylamido-2-phenylethylchloromethylketone (TPCK)-treated trypsin (Kutkat *et al.*, 2022).

Viruses:

An hCoV-19/Egypt/NRC-3/2020 strain of the SARS-CoV-2 virus (GSAID Accession Number: EPI_ISL_430820) "NRC-03-nhCoV" virus and Influenza A virus A/California/04/2009 (H1N1pdm09), were obtained from the National Research Centre. The viruses were propagated in the appropriate cells until the appearance of distinct morphological changes which identified as cytopathic effect; consequently, the viral titre was calculated via plaque titration assay and preserved at -80°C.

Antiviral bioassay

Preparation of plant crude extract and fractions concentrations.

In 1 ml of 10% Dimethyl Sulfoxide, 0.1 g of the crude *Moringa oleifera* dry ethanolic extract and 0.01 g of its fractions were separately dissolved, and different concentrations were prepared by serial dilution of the plant extract and its fractions, giving ten concentrations starting with 10 µg/µl for the crude and 1 µg/µl for the fractions.

Cytotoxicity Concentration 50 (CC_{50}).

MDCK and Vero-E6 cells were cultured in 96-well plates and incubated at 37 °C in 5% humidified CO_2 until confluency, to test the extract or fractions toxicity on the cells. The cells were then treated with *Moringa oleifera* extracts concentrations and incubated for 3 days (the untreated wells contained only cells and were the cell control) (Kutkat *et al.*, 2022).

Inhibitory Concentration 50 (IC_{50}).

In 96-well microtiter plates, MDCK and Vero-E6 cells were cultured for 24 h before the infection and washed by 1x Phosphate buffer saline (PBS). Serially diluted concentrations were mixed with 0.5 ml/100 ml for H1N1 and 1 ml/100 ml of SARS-CoV-2 virus, and were incubated at 37°C for 1 h. The virus-extract mixtures were added to the cell monolayer sheet and incubated at 37°C for 72 h. The wells contained cells that had not been treated or infected, known as virus control.

The following steps are common for CC_{50} and IC_{50}:

The fixation step was done for 3 h using 100 µl of 10% formaldehyde solution at room temperature. After washing and drying the plates, staining with 50 µl crystal violet solution for 10 mins followed by washing carefully and drying the plates, to solubilize the dye 180-200 µl of absolute methanol was added to the completely air-dried plates and shaken for 20 minutes using a bench rocker (20 oscillations/minute) to measure the wells' average optical density (the absorbance) at λmax = 570 using the Anthos Zenyth 200 rt plate reader (Anthos Labtec Instruments, Heerhugowaard, Netherlands). The data were analyzed by Graph Pad Prism 5.01 through nonlinear regression, the results curves were drawn by plotting log inhibitor versus normalized response and (CC_{50}) and (IC_{50}) values were calculated by comparing the Optical density (OD) average results for cytotoxicity and antiviral wells to those of cell and virus control.

RESULTS

Cytotoxicity and antiviral activity of *Moringa oleifera* crude extract against H1N1

From CC_{50}, IC_{50}, and selectivity index (SI) (antiviral activity relative to cellular toxicity), results shown in Figure (1) Indicated that *Moringa oleifera* crude is extremely safe on MDCK cells with CC_{50} > 10.000 µg/ml, and it exhibits strong antiviral activity against H1N1 with IC_{50} = 10.61 µg/ml; hence SI value is larger than 10.000.

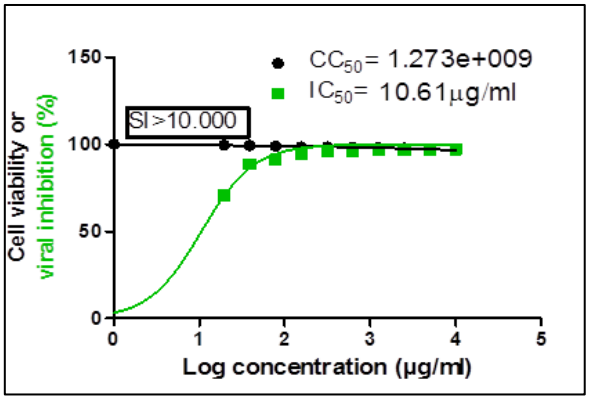

Fig.1. Cytotoxicity and antiviral assay curves of *Moringa oleifera* crude ethanolic extract against H1N1.

The assay was performed using the crystal violet method, and the curves were drawn using Graph Pad Prism 5.01. Using nonlinear regression, CC_{50} (cytotoxic concentration of 50% of cells) and IC_{50} (inhibitory concentration of 50% of cells) were calculated by plotting log inhibitor vs. normalized response (variable slope); hence their SI values (CC_{50} /IC_{50}).

The results in Figure (2) showed that all *Moringa oleifera* fractions are highly safe on MDCK cells in the following order: methylene chloride fraction, butanol fraction, residual aqueous fraction, and petroleum ether fraction.

The antiviral effect on Influenza A/H1N1 was observed for all fractions in the following order: n-butanol fraction, petroleum ether fraction, residual aqueous fraction, and methylene chloride fraction.

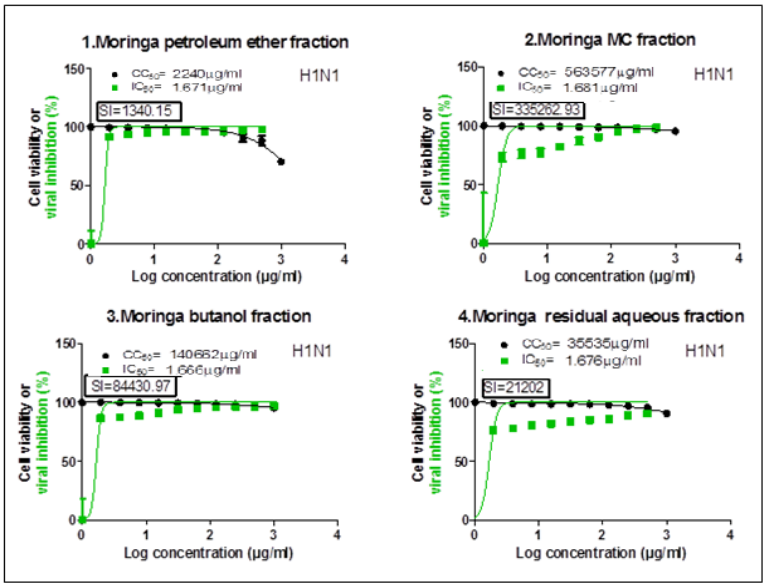

Fig.2. Cytotoxicity and antiviral activity curves of *Moringa oleifera* fractions against H1N1 on the order: Petroleum ether, methylene chloride, n-butanol and residual aqueous solution.

Mode of action of *Moringa oleifera* against H1N1

To detect how the promising drug acts towards tested viruses, whether directly on virus (virucidal) or indirectly by hindering cell receptors (adsorption) or interfering with the replication cycle.

The data in Figure (3) revealed that *Moringa oleifera* crude extract has virucidal activity against H1N1, and the virus was directly inhibited with (39- 91%) in 625 -5000 µg/ml, and the *Moringa oleifera* effect on replication is very weak, with (0-21.4 %), while on adsorption was moderate (7.1-48.2 %).

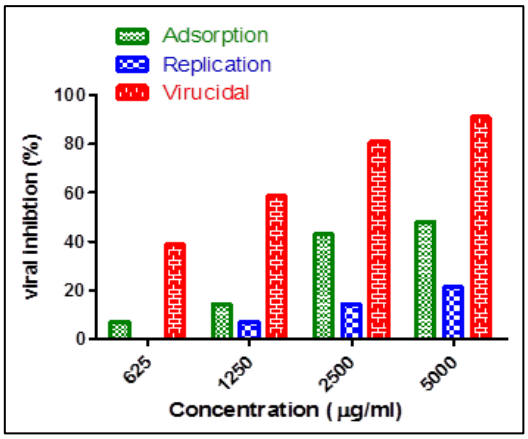

Fig.3. The mode of action of *Moringa oleifera* crude against H1N1 and the viral inhibition % calculation by comparing virus count in tested wells to those of virus control. Viral inhibition % = virus control count (untreated) – Viral count (post-treatment) /virus control count (untreated) x 100.

Cytotoxicity and antiviral activity of *Moringa oleifera* crude extract against SARS-CoV-2

From CC_{50}, IC_{50}, and selectivity index (SI); antiviral activity relative to cellular toxicity, results shown in Figure (4). *Moringa oleifera* crude extract is safe on vero-E6 cells till 7277 µg/ml and exhibits strong antiviral activity against SARS-CoV-2 with IC_{50}= 12.29 µg/ml, hence SI value of 564.

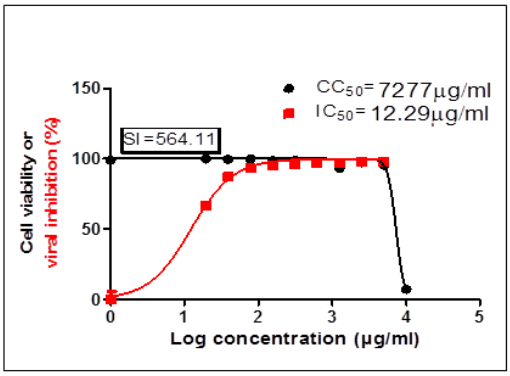

Fig. 4. Cytotoxicity and antiviral assay curves of *Moringa oleifera* crude ethanolic extract against SARS-CoV-2.

All the tested fractions are safe on Vero-E6 cells in the following order: residual aqueous fraction, petroleum ether, n-butanol, and methylene chloride. The highest antiviral activity was observed for

methylene chloride and petroleum ether fractions against SARS-CoV-2.

The butanol fraction and residual aqueous fraction don't show any detectable antiviral activity, and the CC_{50} was smaller than IC_{50} (Fig. 5).

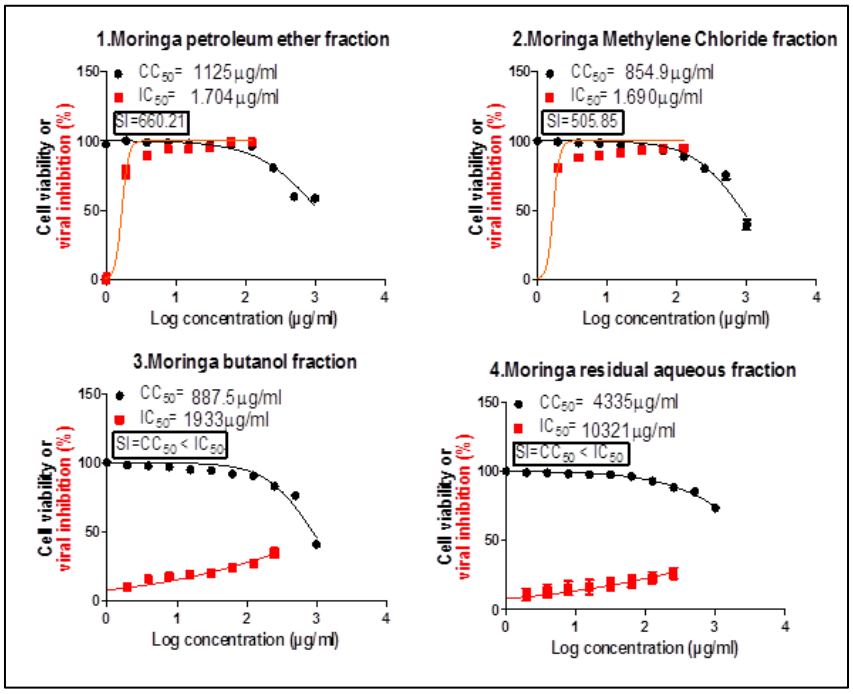

Fig.5. Cytotoxicity and antiviral assay curves of *Moringa oleifera* fractions against SARS-CoV-2.

Mode of action against SARS-CoV-2

The data in Figure (6) revealed that *Moringa oleifera* crude exerts a virucidal effect on SARS-CoV-2, inhibiting virus particles upon combination with it, and the viral particles were directly inhibited with 75.3–88.2% in 625–5000 µg/ml, and as well as having an effect on viral adsorption and replication with 62–75% and 17.6–74.7%, respectively.

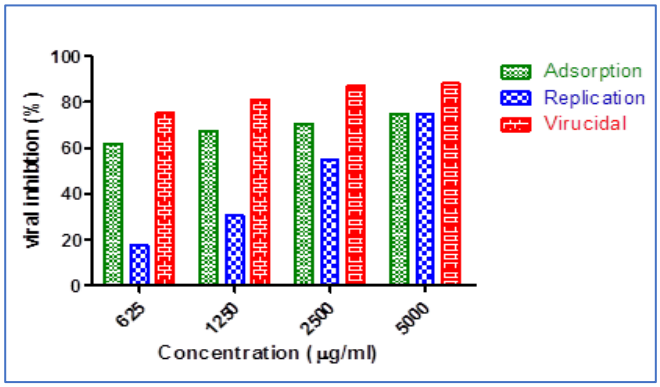

Fig.6. The mode of action of *Moringa oleifera* crude against SARS-CoV-2, as well as the viral inhibition percentage calculated by comparing the virus count in tested wells to those of the virus control.

DISCUSSION

A lot of work is currently being done to understand the biological activities of *Moringa oleifera* a in order to investigate its mechanism of action and how to benefit from it. With better understanding, it can be used effectively in a variety of health-improving products. The use of *Moringa oleifera* as a natural complement for drugs, may encourage the development of new drugs, and by combining it with synthetic drug, the negative effects could be reduced. However, thorough risk assessment studies must be conducted to guarantee the safety of the medications and items produced using *Moringa oleifera*.

Even after decades of monitoring with medical and non-medical measures, seasonal influenza viruses still spread epidemics every year. The main factor behind these repeated epidemics is how the viruses have evolved to evade the immunity brought on by previous infections or vaccinations. The timing and characteristics of the development of new virus strains are still largely unpredictable, despite the fact that scientists are starting to comprehend the mechanisms behind the evolutionary dynamics of influenza viruses. Since there is now no antiviral medication that has been clinically authorized for the management of H1N1 and SARS-CoV-2, the threat to public health has grown significantly.

Drug repurposing or repositioning, is an alternative approach of drug discovery that can support the identification of the new indications for already-approved medications (Rossler *et al*., 2021).

The antiviral potential of several *Moringa oleifera* extracts may be due to the bioactive phytoconstituents, which include flavonoids, and phenolic compounds, that having antiviral, antioxidant, anti-inflammatory, anti-diabetic, and anti-cancer properties (Senthilkumar *et al*., 2018). The current findings showed that, with SI value over 10,000, *Moringa oleifera* crude extract displays high safety and potent antiviral activity against H1N1 as given by Sofy *et al*. (2022), and against SARS-CoV-2 with SI value of 564.

The most secure *Moringa oleifera* fractions on MDCK was Methylene chloride fraction, while on Vero-E6 cells was residual aqueous fraction. The most potent *Moringa oleifera* fractions against H1N1 were butanol fraction, while only petroleum ether fraction and methylene chloride fraction are active against SARS-CoV-2.

To determine the potential drug's interactions with the tested viruses, according to the data in Figure (3), upon combination with H1N1, *Moringa oleifera* crude has virucidal effect. Viral particles were directly suppressed with 91% at 5000 µg/ml; and the effect decreases with dilution, with an effect on replication and adsorption with percentage less than 50% at the highest concentration; hence, the present research shows that *Moringa oleifera* has strong antiviral properties against H1N1 as one of influenza A viruses as mentioned by Xiong *et al.* (2021).

According to the data in Figure (6), *Moringa oleifera* crude also has virucidal effect on SARS-CoV2, with higher than 80% at 5000 µg/ml, and exerts an effect on viral adsorption and replication over 70% at the same concentration, which is similar to the results of (Rahayu and Timotius (2022); therefore, *Moringa oleifera* is regarded as a potent antiviral agent against H1N1 and SARS-CoV-2. These biological functions were suggested to be associated with phenolic compounds that have antioxidant activity (Kirindage *et al*., 2022) and aid in reducing the body's production of free radicals, which in turn causes oxidative stress and the onset of a number of chronic and acute diseases illness progression; and hence prevents DNA damage (Perrone *et al*., 2018).

CONCLUSION

The exploration of medicinal plants' therapeutic potential to treat human and animals is made possible by scientific study of these plants. *Moringa oleifera* crude ethanolic extract exerted inhibitory effects against H1N1 and SARS-CoV-2, with virucidal action on both viruses over 80%, furthermore, its effect on SARS-CoV-2 adsorption and replication over 70%. Separating out specific phyto-chemical components, however, will undoubtedly have positive outcomes, in which the antiviral activity of the fractions was better than those of the crude. The significant safety observed on MDCK and Vero-E6 Cells for the crude and fractions indicated the ability of *Moringa oleifera* ethanolic extract and its fractions to maintain cell viability upon co-incubation. High antiviral activity was obtained against both viruses, especially H1N1; hence, Moringa is considered as a promising antiviral agent against H1N1 and SARS-CoV-2, subsequently, it needs further studies to be formulated in suitable pharmaceutical form or involved in antiviral products.

In conclusion, this research contributes to the study of *Moringa oleifera* as virucidal agent on two RNA viruses (H1N1 and SARS-CoV-2).

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تأثير مبيد الفيروسات *للمورينجا أوليفيرا* ضد سارس كوف ٢ وانفلونزا Aاتش١ان١

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المستخلص

في العقدين الماضيين ، تسببت العديد من الفيروسات ، مثل سارس كوف ١و سارس كوف ٢ وميرس كوف ، التي تنتمي إلى فيروسات كورونا ، وفيروسات الأنفلونزا بشكل رئيسي اتش١ان١ ومع عدم وجود علاجات محددة وقلة العلاجات المتاحة لهذه الفيروسات أدى إلى زيادة صعوبة التغلب عليها .هناك عائق كبير أمام العلاج المضاد للفيروسات وهو ظهور فيروسات الجهاز التنفسي المقاومة للأدوية نتيجة للطفرات التي تحدث بشكل طبيعي ، بالإضافة إلى إساءة استخدام الأدوية المضادة للفيروسات المعتمدة بالفعل .لذلك تم تصميم هذه الدراسة لمعرفة قدرة المستخلص من مسحوق أوراق النبات *مورينجا أوليفيرا* كواحد من النباتات الطبية الأكثر شيوعا على معالجة بعض الفيروسات . تم تحضير مستخلص إيثانولي وتجزئته ، ومن ثم تم اختبار المستخلص الخام والكسور بحثا عن نشاط مضاد للفيروسات ضد اتش١ان١و سارس كوف ٢ كعامل مسبب للوباء 2009و 2019 ، على التوالي وذلك بعد تأكيد السلامة على خطوط خلايا الكلى مادين داربي للكلاب (MDCK) و الخلايا المشتقة من الكلى للقرد الأخضر الأفريقي (.Vero-E6). تم إجراء اختبار تقليل البلاك لمستخلص الإيثانول الخام لتحديد عمل الدواء المضاد للفيروسات الذي له IC_{50} منخفضة .أظهرت النتائج درجة الامان العالية للنوعين من الخلايا والنشاط ضد كل من الفيروسين مع نسبة عالية من التثبيط المباشر(مبيد للفيروسات)، مما يشير إلى امكانية أن تكون *المورينجا أوليفيرا* عامل مضاد للفيروسات واعد ضدهما.