Robust Antiviral Activity of Santonica Flower Extract (Artemisia cina) against Avian and Human Influenza A Viruses: In Vitro and Chemoinformatic Studies

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ABSTRACT: The evolution of drug-resistant viral strains following natural acquisition of resistance mutations is a major obstacle to antiviral therapy. Besides the improper prescription of the currently licensed anti-influenza medications, M2-blockers and neuraminidase inhibitors, to control poultry outbreaks/infections potentiates the emergence of drug-resistant influenza variants. Therefore, there is always a necessity to find out new alternatives with potent activity and high safety. Plant extracts and plant-based chemicals represent a historical antiviral resource with remarkable safety in vitro and in vivo to control the emerging and remerging health threats caused by viral infections. Herein, a panel of purified plant extracts and subsequent plant-derived chemicals were evaluated for their anti-avian influenza activity against zoonotic highly pathogenic influenza A/H5N1 virus. Interestingly, santonica flower extract (Artemisia cina) showed the most promising anti-H5N1 activity with a highly safe half-maximal cytotoxic concentration 50 (CC50 > 10 mg/mL) and inhibitory concentration 50 (IC50 of 3.42 μg/mL). To confirm the anti-influenza activity, we assessed the anti-influenza activity of the selected plant extracts against seasonal human influenza A/H1N1 virus and we found that santonica flower extract showed a robust anti-influenza activity that was comparable to the activity against influenza A/H5N1. Furthermore, the mode of action for santonica flower extract with strong inhibitory activity on the abovementioned influenza strains was elucidated, showing a virucidal effect. To go deeper about the activity of the chemometric component of the extract, the major constituent, santonin, was further selected for in vitro screening against influenza A/H5N1 (IC50 = 1.701 μg/mL) and influenza A/H1N1 (IC50 = 2.91 μg/mL). The oxygen of carbonyl functionality in the cyclohexene ring succeeded to form a hydrogen bond with the neuraminidase active site. Despite the fact that santonin revealed similarity to both reference neuraminidase inhibitors in forming hydrogen bonds with essential amino acids, it illustrated shape alignment to oseltamivir more than zanamivir according to Tanimoto algorithms. This study highlights the applicability of santonica flower extract as a promising natural antiviral against low and highly pathogenic influenza A viruses.

1. INTRODUCTION

Influenza is an upper respiratory tract (URT) and/or lower respiratory tract (LRT) infection caused by a variety of influenza viruses (IVs), which are the major part of orthomyxoviridae family. It contains four genera of influenza viruses: influenza A, B, C, and D. Influenza A viruses (IAVs) are the major causative agents of the known seasonal flu epidemics. IAVs can be mainly classified according to their host range: avian influenza viruses (AIV) such as H5N1, H7N2, and H9N2; human influenza viruses such as H1N1 and H3N2; and bat-origin influenza-like viruses such as H18N11 and H17N10. The pathogenicity of AIVs can divide them into two main categories: low pathogenic AIVs (LPAIVs), which span a large set of AIVs (H1–H16), and highly pathogenic AIVs (HPAIVs), which is mainly restricted to H5 and H7 subtypes. The global burden of influenza disease is severe, leading annually to 290–650 thousand deaths every year, due to health implications related to influenza-like illnesses. Influenza vaccine effectiveness is reliant on the matching percent between...
Table 1. Plant Extracts Used in This Study

| Plant Extract | Major Active Constituents | Reported Biological Activities | Ref |
|---------------|--------------------------|--------------------------------|-----|
| cassia flower extract (C. fistula) | phytol and coumarin | antibacterial, antifungal, anti-inflammatory, and antioxidant | 8 |
| terminalia leaf extract (T. laxiflora) | ellagitannins, ellagic acid derivatives, and triterpenes | antibacterial | 9 |
| schinus extract (leaf, fruit, and stem) (S. terebinthifolius) | tetrahydrodoamantinolavone, apigenin, ellagic acid, masticadenionic acid, myricetin, gallic acid, and ursolic acid | antiviral | 11 |
| radish seed (R. sativus) extract | 5-vinyl-2-oxazolidinethione, 3-butenyl, 4-pentenyl, and phenethyl isothiocyanate | | |
| black mustard (B. nigra) seed extract | sinigrin | anti-cancer and antiviral (mustard seeds) | 12 |
| cinchona bark extract (Cinchona sp.) | quinine and quinidine and cinchonine and cinchonidine | anti-obesity, anticancer, antioxidant, anti-inflammatory, antimicrobial, and antiviral activities | 13 |
| tilia flower extract (T. cordata) | quercetin, rutin, kaempherol, caffeic acid, and chlorogenic acid | antibacterial and antioxidant activities | 14 |
| santonica flower extract (A. cina) | santonin, artemisinin, and achillin | antibacterial, antioxidant, anti-inflammatory, immunosuppressive, anti-roundworm, antimalarial (α-santonin) | 15 |
| black tea (C. sinensis) leaf extract | epigallocatechin gallate (EGCG), gallocteatin gallate (GCG), galacceatin (GC), catechin (C), and epicatechin (EC) | antifibrotic, antioxidant, and anti-adenovirus activity | 16 |
| coffee bean (C. arabica) extract | caffeine, trigonelline, and chlorogenic acids (5-CQA) | antiviral (pyridinium) antioxidant, antiviral, and antimicrobial activities | 17 |
| eucalyptus leaf extract (E. camaldulensis) | 1,8-cineol and α-pinene | antifibrotic, antioxidant, and antiviral activities | 18 |
| vinca (C. rosea) herb extract | vincristine, vinblastine, and raubasine | antitumor, anti-diabetic, and disinfectant | 19 |
| E. sativa fresh leaf extract | 4-hydroxygluconorhin, glucotropaeolin, glucosipidin, glucopherin, gluccosinarmin, glucosaein, glucosiercin, glucopherin | antiviral (glucosinolat derivatives) | 20 |

2. MATERIALS AND METHODS

2.1. Cell Lines and Viruses. Madin Darby Canine Kidney (MDCK) cells obtained from the Center of Scientific Excellence for Influenza Viruses, National Research Centre, Egypt, propagated in MDCK and embryonated eggs, respectively, as previously described.7

2.2. Studied Plant Extracts and Phytochemicals. The tested plant extracts that were used in this study are listed in Table 1. Briefly, Cassia fistula, Eucalyptus camaldulensis, and Catharanthus roseus plant materials were collected from the botanical garden at the Faculty of Pharmacy, Zagazig University, Egypt. Schinus terebinthifolius was collected from 1st district, 10th of Ramadan City, Egypt. Terminalia laxiflora leaves were collected from 1st district, 10th of Ramadan City, Egypt. The tested plant extracts and phytochemicals are listed in Table 1. Cassia fistula, Eucalyptus camaldulensis, and Catharanthus roseus plant materials were collected from the botanical garden at the Faculty of Pharmacy, Zagazig University, Egypt. Schinus terebinthifolius was collected from 1st district, 10th of Ramadan City, Egypt. Terminalia laxiflora leaves were collected from El-Orman Garden, Giza, Egypt. Raphanus sativus, Cinchona sp., Brassica nigra, Tilia cordata, Artemisia cina (santonica), Camellia sinensis, Coffee arabica, and Eruca sativa leaves were purchased from a local market, while santonin was purchased from Sigma Aldrich. These plant extracts were selected based on previously reported biological activities, making them good candidates to be tested as anti-influenza agents.

Five grams from each of the studied plants was extracted with 100 mL of methanol, and the resulting extracts were concentrated to afford the different extracts. As to E. sativa, the leaves were either extracted with methanol or squeezed to give Eruca extract or juice, respectively. The process was repeated twice to obtain two sets from each product; one set was heated in a water bath at 80 °C for 10 min to kill the myrosinase enzyme and to keep the integrity of glucosinolates, while the other set was kept at room temperature overnight (16 h) to obtain glucosinolate hydrolysis products. Concerning black mustard and radish seeds, the methanolic extracts were heated in a water bath at 80 °C for 10 min to protect their glucosinolates content from the myrosinase hydrolyzing effect. All the extracts were kept in a refrigerator till investigation.

2.3. Tissue Culture Infective Dose 50 (TCID50). To determine the virus titer, a tissue culture infective dose 50% assay was used where serial decimal dilutions of the virus were incubated with cells in triplicates at 37 °C/5% CO2 in a humified incubator for 72 h. Then, the highest dilution of the virus showing a 50% cytopathic effect (CPE) was used to calculate the.
amount of virus in the stock samples by a Reed–Muench method.\textsuperscript{21}

2.4. Plaque Titration Assay. To define the viral titers as plaque-forming units (PFU)/mL, a plaque infectivity assay was done with some modifications.\textsuperscript{22} Serial decimal dilutions for both viruses were prepared in DMEM (supplemented with 4% bovine serum albumin (BSA) (Gibco-BRL, New York, USA), 2% pen/strep mixture, and 1 μg/mL L-1-tosyl-aminom-2-phenyl-ethyl chloromethyl ketone (TPCK)-treated trypsin), and after washing with 1X phosphate buffer saline (PBS), the 80% confluent MDCK cell monolayers (previously cultured in six-well plates for 24 h at 37 °C/5% CO\textsubscript{2}) were infected with 100 μL/well (except for one well to work as the cell control, which is covered with 400 μL of viral infection medium) and 300 μL/well of viral infection medium to keep the cells in a moisturized environment. Under humidified conditions with 5% CO\textsubscript{2} and 37 °C, the plates were incubated for 1 h to allow for viral adsorption. During the incubation period at 15 min intervals, the plates were shaken gently to guarantee the equal distribution of the virus on the cells and to keep the cells moisturized. After incubation, the inoculum was aspirated, 3 mL of 2% agarose overlay in 2X DMEM supplemented with 10% FBS and 1 μg/mL TPCK was added, and the plates were allowed to set; then, the plates were incubated at 37 °C/5% CO\textsubscript{2} in a humified incubator for 72 h. The plates were fixed with 10% formaldehyde for 2 h to inactivate the virus activity and to fix the cells into the plates, and then, the overlay was removed and the plates were stained with 0.1% crystal violet in distilled water for 10 min to visualize the plaques. The virus titer was calculated for each virus using the following formula:

\[
\text{PFU/mL} = \frac{\text{number of plaques} \times \text{reciprocal of virus dilution}}{\text{dilution factor (to 1 mL)}}
\]

2.5. Cytotoxic Concentration 50 (CC\textsubscript{50}) and Inhibitory Concentration 50 (IC\textsubscript{50}). To assess the half-maximal cytotoxic and inhibitory concentrations 50 for each extracts and/or compounds, a crystal violet assay was used as previously described.\textsuperscript{23} Briefly, MDCK cells with a density of 1×10\textsuperscript{5} per well were cultured in 96-well plates and incubated for 24 h at 37 °C/5% CO\textsubscript{2} in a humified incubator. The next day, serial decimal dilutions of the tested phytochemical extracts and/or compounds in DMEM (supplemented with 4% BSA, 2% pen/strep mixture, and 1 mg/mL TCPK-treated trypsin) were prepared and incubated with the predetermined viral dilutions for each virus for 1 h at room temperature (RT). After 1 h incubation, the old media covering the cultured cells were then discarded and the cells washed with 1X PBS. The plate was then designated to assess both IC\textsubscript{50} and CC\textsubscript{50}, where virus/extracts or compound mixtures were added to the MDCK cell monolayers in a volume of 100 μL/well in triplicates to determine the IC\textsubscript{50} and a variety of the compound dilutions without any virus treatment in the same volume were also added in triplicates to determine the CC\textsubscript{50} and the cell control and virus control were included, and the plates were incubated at 37 °C/5% CO\textsubscript{2} in a humified incubator for 72 h. After 3 days, the cells were then fixed with 100 μL/well 10% fixing solution and incubated for 2 h at RT, and then the supernatants were discarded and 50 μL/well 0.1% crystal violet stain was added for 10 min. Following staining procedures, 180 μL/well of absolute methanol was added to the plates and was shaken for 30 min, and then the optical density (OD) was measured at 570 nm using an ELISA plate reader. A plot of cell viability % and viral inhibition % versus concentration for each extract/compound was presented using GraphPad prism 5 software.

2.6. Plaque Reduction Assay. The most promising extracts according to their IC\textsubscript{50} values were selected to further confirm their antiviral efficacy. Different safe concentrations of each extract were incubated with countable virus dilution in DMEM (supplemented with 4% BSA, 2% pen/strep mixture, and TCPK-treated trypsin) for 1 h at RT. In a 12-well plate, MDCK cells (1×10\textsuperscript{5} cells) (previously cultured for 24 h at 37 °C in 5% CO\textsubscript{2} with humidity) were incubated with triplicates of virus extracts or compound mixtures (100 μL/well) for 1 h at 37 °C/5% CO\textsubscript{2} in a humified incubator to give chance for virus adsorption. After the second incubation, we added 1.5 mL of 2% agarose overlay in 2X DMEM supplemented with 1% FBS and 1 μg/mL TPCK and allowed the plates to set, and then, we incubated the plates at 37 °C/5% CO\textsubscript{2} in a humified incubator for 72 h. The plates were fixed with 10% formaldehyde (fixing solution) for 2 h, and then, we removed the overlay and stained the plates with 0.10% crystal violet in distilled water for 10 min. The number of viral plaques for each concentration of the tested extracts were determined manually and compared with the virus control for each plate as in ref \textsuperscript{23b} with minor modifications. The viral reduction % was calculated for each extract using the formula below and plotted against extract concentration using GraphPad prism 5 software.

\[
\text{viral inhibition(%) = } \frac{\text{count of untreated virus (control) } - \text{ count of treated virus}}{\text{count of untreated virus (control)}} \times 100
\]

2.7. Mechanism of Action. To determine the exact step at which the highly promising phytochemical extracts (extracts with high SI values) exhibited their antiviral effect (1) viral replication step, (2) viral adsorption step, or (3) the extracts exert their virucidal effect against H1N1 and H5N1 viruses, the plaque reduction assay was carried out as previously described.\textsuperscript{23b}

2.7.1. Viral Replication Interference. The MDCK cells were cultured in six-well plates at a density of 1×10\textsuperscript{5} cell/well and incubated for 1 day at 37 °C/5% CO\textsubscript{2} in a humified incubator. Predetermined viral dilutions for each virus (H1N1 and H5N1) were added to monolayer cells with a confluency of 80–90% after removal of the old media and washing; and the plates were incubated for 1 h at 37 °C/5% CO\textsubscript{2}; the cell control and virus control wells were included. The non-adsorbed particles were discarded, and the cells were washed with 3 mL/well of 1X PBS. A variety of different safe concentrations for each extract were added, and the plates were incubated for another 1 h at 37 °C. After the second incubation, the plates undergo the same washing procedures, 3 mL/well of 2% agarose overlay in 2X DMEM supplemented with 10% FBS and 1 μg/mL TPCK was added, and the plates were allowed to set, and then, the solidified plates were incubated at 37 °C/5% CO\textsubscript{2} for 3 days. The cells were then fixed with 10% fixing solution for 2 h, and the plaques were visualized using 0.10% crystal violet. The plaques are manually counted regardless of their sizes, and the % of reduction was calculated using the same formula used in the plaque reduction assay.

2.7.2. Viral Adsorption Interference. MDCK cells were cultured in six-well plates at a density of 1×10\textsuperscript{5} cell/well and incubated for 1 day at 37 °C/5% CO\textsubscript{2} in a humified incubator. A variety of different safe concentrations for each extract were
added to 80–90% confluent monolayer cells (200 μL/well), and the plates were incubated for another 1 h at 4 °C; cell control and virus control wells were included. The non-adsorbed compounds were discarded, and the cells were washed with 1×
units/L = (ΔM sample − ΔM blank − ΔM water) 
/(slope × T)

where ΔM sample, ΔM blank, and ΔM water are the changes in absorbance intensity of the sample, sample blank, and water (standard blank), respectively. Slope represents the slope of the standard curve in μM⁻¹, while T represents the time of reaction between readings (30 min).

2.9. Chemoinformatic Studies. 2.9.1. Molecular Docking. The X-ray crystal structure coordinates of neuraminidase (PDB ID: 6hp0) were retrieved from PDB with its co-crystallized bound ligand. The docking study was performed by using OpenEye Scientific Software version 2.2.5 (Academic License 2021, Yaseen Elshaier lab, Santa Fe, NM, USA, http://www.eyesopen.com). To validate the docking study, the co-crystal bound ligand was redocked.

2.9.2. Shape Similarity and ROCS Analysis. The basic method to represent shape and color features in ROCS is using ROCS application OpenEye scientific software. Santonin was selected as the query molecule and other compound library was adopted as the database file. Both query and database files were energy-minimized by Omega applications. Personal PC in very fast mode using the vROCS interface employed ROCS runs. The vROCS was employed to run and analyze/visualize the results. ROCS application searched the database with the query to find molecules with similar shapes and colors. The result was visualized by Vida application. Compound conformers were scored based upon the Gaussian overlap to the query, and the best scoring parameter is Tanimoto Combo scores (shape + color); the highest score is the best matched with the query compound.

3. RESULTS

3.1. Cytotoxicity and Viral Inhibitory Effect of the Selected Plant Extracts. To investigate the anti-influenza activity of the selected plant extracts (Table 1), the cytotoxic concentration values of these extracts were primarily assessed in MDCK cells. Almost all the tested concentrations (concentration range = 1 ng/mL to 10 mg/mL) of the plant extracts showed no toxic effect in the MDCK cells, even at high concentrations (>10 mg/mL) (Figure 1). Furthermore, a range of non-toxic concentrations (Figure 1) were used to investigate the viral inhibitory concentration for each plant extract against avian influenza A/H5N1 virus using the neuraminidase inhibitor oseltamivir carboxylate (active drug form) as a positive control. Interestingly, santonica flower extract (A. cina) showed potent activity with an IC₅₀ value of 3.42 μg/mL with a high selectivity index (Figure 1 and Table 2).

On the same hand, coffee seed extract, E. sativa juice extract containing glucosinolate hydrolysis products, and E. sativa juice extract containing intact glucosinolates showed potent activity. The basic method to represent shape and color features in ROCS is using ROCS application OpenEye scientific software. Santonin was selected as the query molecule and other compound library was adopted as the database file. Both query and database files were energy-minimized by Omega applications. Personal PC in very fast mode using the vROCS interface employed ROCS runs. The vROCS was employed to run and analyze/visualize the results. ROCS application searched the database with the query to find molecules with similar shapes and colors. The result was visualized by Vida application. Compound conformers were scored based upon the Gaussian overlap to the query, and the best scoring parameter is Tanimoto Combo scores (shape + color); the highest score is the best matched with the query compound.

### Table 2. Selectivity Indices “SI” for Tested Plant Extracts against Avian Influenza A/H5N1 Virus in MDCK Cells

| plant extract                        | CC₅₀ (mg/mL) | IC₅₀(mg/mL) | SI (CC₅₀/IC₅₀) |
|--------------------------------------|--------------|-------------|----------------|
| C. fistula flower extract            | >10          | >1.0        | NA             |
| T. laxiflora leaf extract            | >10          | >1.0        | NA             |
| S. terebinthifolius extract (fruit)  | >10          | >10         | NA             |
| S. terebinthifolius extract (stem)   | >10          | >10         | NA             |
| radish seed extract                  | 11.24        | CC₅₀ < IC₅₀ | <1             |
| black mustard seed extract           | 16.9         | CC₅₀ < IC₅₀ | <1             |
| cinchona bark extract                | >10          | CC₅₀ < IC₅₀ | <1             |
| T. cordata flower extract            | 19.22        | CC₅₀ < IC₅₀ | <1             |
| santonica (A. cina) flower extract   | >10          | 0.00342     | >2924          |
| black tea leaf extract               | >10          | >1.0        | NA             |
| coffee seed extract                  | 7.96         | 0.084       | 94.76          |
| E. camaldulensis leaf extract        | >10          | >1.0        | NA             |
| vinca herb extract                   | 0.78         | CC₅₀ < IC₅₀ | <1             |
| E. sativa juice containing glucosinolate hydrolysis products | >10 | 0.0562 | >178 |
| E. sativa juice containing intact glucosinolates | >10 | 0.0647 | >155 |
| E. sativa methanolic extract containing intact glucosinolates | 5.07 | 0.451 | 11.24 |
| E. sativa methanolic extract containing glucosinolate hydrolysis products | 9.22 | 1.215 | 7.59 |
| oseltamivir carboxylate              | >10          | 0.000865    | >11,561        |

The neuraminidase activity test is an enzymatic assay in which the standard curve in (standard blank), respectively. Slope represents the slope of the same formula in the plaque reduction assay.

2.7.3. Virucidal Mode. MDCK cells were cultured in six-well plates at a density of 1 × 10⁵ cells/well and incubated for 24 h at 37 °C/5% CO₂ in a humified incubator. A total of 100 μL from each extract (previously selected on the basis of SI values) was mixed with 100 μL of virus stock (for both viruses) and incubated for 1 h at RT. After 1 h, the mixtures were serially diluted in DMEM supplemented with 4% BSA, 2% pen/strep mixture, and 1 mg/mL TCPK-treated trypsin until the required viral dilutions were obtained. Following the washing procedures described above, the MDCK cell monolayers (80–90% confluency) were inoculated with 100 μL from virus/compound mixtures (cell control and virus control were applied) and incubated at 37 °C for 1 h. 2× DMEM/agarse overlayers were added after removal of the inocula, and after the solidification, the plates were incubated at 37 °C under 5% CO₂ under humified conditions for 3 days. After plaque formation, the plates underwent the same fixation and visualization procedures described above and the % reduction was calculated utilizing the same formula in the plaque reduction assay.

2.8. NA Inhibition Assay. To determine the neuraminidase activities following treatment with various concentrations of santonin and zanamivir as a reference neuraminidase inhibitor, a colorimetric neuraminidase inhibition assay was conducted using an MAK121 Neuraminidase Activity Assay Kit (Sigma Aldrich) according to the manufacturer’s instructions. Briefly, the neuraminidase activity test is an enzymatic assay in which the sialic acid released by neuraminidase results in a colorimetric/fluorometric product, directly proportional to the neuraminidase activity in the sample as previously described. Aliquots of 20 μL of supernatant samples were mixed with 80 μL of the Master-Mix reaction provided in the kit in 96 μL well plates and incubated at 37 °C. The absorbance of the samples, reference drug, and standards was measured at 20 and 50 min (interval T = 30 min) with λex = 570 nm using a SPARK 10M instrument. The neuraminidase activity (units/L) against each sample concentration was calculated as recommended by the manufacturer using the following equation:

units/L = (ΔM sample − ΔM blank − ΔM water) 
/(slope × T)
heated juice extract containing intact glucosinolates showed moderate activity with IC\textsubscript{50} values of 84.02, 56.24, and 46.67 μg/mL, respectively. However, \textit{E. sativa} heated methanol extract containing intact glucosinolates, black tea leaf extract, \textit{T. cordata} flower extract, and \textit{S. terebinthifolius} fruit extract and stem extract showed poor antiviral activities when compared to the reference oseltamivir carboxylate drug. Meanwhile, the rest of the extracts including \textit{C. fistula} flower extract, radish seed extract, black mustard seed extract, and \textit{T. laxiflora} leaf extract showed no antiviral activity against influenza A/H5N1 virus.

To select an effective and safe drug candidate for further investigations, the selectivity index (SI), which is the ratio between the half-maximal toxic concentration of the tested candidate drug (CC\textsubscript{50}) against its effective half-maximal bioactive concentration (IC\textsubscript{50}), was calculated. Interestingly, the reference oseltamivir carboxylate drug showed the highest SI value (>11,561) followed by santonica extract (>2924), \textit{E. sativa} juice containing intact glucosinolates (SI > 178), and \textit{E. sativa} juice containing intact glucosinolates (SI > 155).

To confirm that the anti-influenza activity for the most potent plant extract is not strain-specific, the antiviral activity of santonica flower extract was further assessed against seasonal human influenza A/H1N1 virus (Figure 2a) and compared to oseltamivir carboxylate (Figure 2b) as a reference drug. Likewise, santonica flower extract showed potent anti-influenza activity against influenza A/H1N1 virus, with a potent IC\textsubscript{50} value of 0.0215 μg/mL.

3.2. Concentration-Dependent Viral Reduction of the Selected Phytochemicals. To confirm the antiviral activity against both influenza viruses, A/H5N1 and A/H1N1, santonica flower extract was further tested against the predefined viruses using a plaque reduction assay. Consistent with our previous results, the tested extract revealed its ability to induce viral inhibition at low concentrations against influenza A/H5N1 (Figure 3a) and A/H1N1 (Figure 3b) viruses.

3.3. Mechanism of Action. To specify the affected compartments during viral infection, three main targets were assessed: the direct virucidal effect, interference with viral adsorption, and interference with viral replication. Herein, the selected plant extract showed its ability to reduce viral titer in the tested mechanisms but with different ratios. Santonica flower extract (the chief constituent is santonin) was mainly able to antagonize the viral propagation by directly affecting the viral particles “virucidal” (Table 3).

3.4. Cytotoxicity and Viral Inhibitory Effect of Santonin as a Major Component of Santonica Flower Extract. The terpenoid santonin, a powerful anthelmintic drug, is the main constituent of \textit{A. cina}.\textsuperscript{26} To confirm that the santonin as a major component of the \textit{A. cina} flower extract directly contributes to the overall activity of the santonica flower extract,
the antiviral potential of pure santonin has been determined against the highly pathogenic A/H5N1 and seasonal influenza A/H1N1 viruses. (Figure 4). The cytotoxic concentration value of santonin was assessed in MDCK cells, and it showed a slightly toxic effect in MDCK cells. Furthermore, the nontoxic concentrations were used to investigate the viral inhibitory concentration for santonin against influenza A/H5N1. Interestingly, santonin showed potent anti-influenza activity against influenza A/H5N1 and A/H1N1 with IC\textsubscript{50} values of 1.701 μg/mL (Figure 4a) and 2.91 μg/mL (Figure 4b), respectively. These results confirm the in vitro neuraminidase inhibition activity of santonin and highlights its potential application as an NA inhibitor.

3.5. Experimental Validation of Neuraminidase Inhibition Activity of Santonin. To investigate the possible neuraminidase inhibition activity of santonin, different concentrations of santonin were assayed for their neuraminidase inhibition activities. Compared to the reference zanamivir drug, santonin showed dose-dependant neuraminidase inhibition activities at all tested concentrations (10 mM to 1 μM) (Table 4). These results confirm the in vitro neuraminidase inhibition activity of santonin and highlights its potential application as an NA inhibitor.

3.6. Chemoinformatic Studies. 3.6.1. Molecular Docking Study with Neuraminidase (PDB ID: 6ph0). The standard ligand was redocked with neuraminidase enzyme active sites using Openeye software, and it was superimposed with its co-crystallized downloaded complex. Both structures overlayed each other with the same binding mode and pose (Figure 5a). Santonin adopted a crooked shape in which the carbonyl of 1,4-cyclohexene formed a hydrogen bond (HB) with Asp:151 A and two HBs with Arg:239 A as an acceptor. The remaining part of the santonin molecule formed a hydrophobic–hydrophobic interaction (Figure 5b). These HBs as the standard ligand formed with the receptor. For comparative analysis of santonin with standard drugs, oseltamivir and zanamivir, santonin revealed similarity to both drugs in the formation of HBs with essential amino acids (Figure 5c,d, respectively).

3.6.2. Shape Alignment and ROCS Analysis. To quantitatively determine santonin similarity to either of these drugs, rapid overlay chemical similarity (ROCS) was employed (Figure 6a). Shape similarity was determined by Tanimoto scores. Tanimoto combo (maximum value 2) is the summation of shape Tanimoto (maximum value 1) and color Tanimoto (maximum value 1). Considering santonin as a query, oseltamivir has a TC higher than zanamivir with values 0.81 and 0.77, respectively (Table 5). Interestingly, both drugs are equal in shape Tanimoto to santonin. However oseltamivir displayed color Tanimoto higher than zanamivir (0.14 and 0.09, respectively). The full description of santonin ROCS is displayed in Figure 6b.

4. DISCUSSION

Each year, annual estimations of 5–10% of adults and 20–30% of children are reported to be diagnosed positive for influenza. These annual influenza epidemics of influenza-associated lower respiratory tract infections in adults end up annually with more than 32 million cases including 5.7 million hospitalized severe influenza cases and one million deaths worldwide.

Recently, the increasing emergence of drug-resistant influenza strains becomes a remarkable phenomenon due to the continuous evolution of these viruses in their natural or intermediate reservoirs and/or the continuous leak of antiviral drugs into the environment due to uncontrolled drug prescription to humans and animals for treatment and prophylaxis purposes. Therefore, continuous screening for new anti-influenza candidates of low toxicity and potent activity is demanded.

Folk medicine and natural plant extracts play a key role in treating many life-threatening diseases such as influenza. Therefore, we aimed in this study to analyze the antiviral activity of a panel of natural plant extracts against two different influenza A virus subtypes (A/H1N1 and A/H5N1). Interestingly, out of the assayed 17 plant extracts, santonica (A. cina) flower extract showed potential anti-influenza activity against both IAV subtypes (influenza A/H5N1 and H1N1). Meanwhile, in the literature, rare or no information is available regarding the anti-influenza and/or antiviral activities of santonica flower extract (A. cina). In addition to santonica flower extract, coffee seed extract showed moderate antiviral activity against influenza A/H5N1 with an IC\textsubscript{50} value of 0.084 mg/mL. Consistent with a previous study, coffee seed extract revealed antiviral activities

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Table 4. Neuraminidase Enzyme Activities (Units/mL) Following Treatment with Various Concentrations of Santonin and Zanamivir

| Concentration | Neuraminidase Activity (units/mL) | IC\textsubscript{50} Value (μg/mL) |
|---------------|-----------------------------------|----------------------------------|
| 10 mM         | 0.14 ± 0.097                      | 0.91 ± 0.56                     |
| 1 mM          | 0.35 ± 0.11                       | 1.02 ± 0.57                     |
| 100 μM        | 0.41 ± 0.06                       | 1.03 ± 0.43                     |
| 10 μM         | 0.42 ± 0.08                       | 1.14 ± 0.06                     |
| 1 μM          | 0.65 ± 0.18                       | 1.25 ± 0.2                      |

Figure 4. Cytotoxicity and anti-H5N1 (a) and anti-H1N1 (b) activities of santonin in MDCK cells. The CC\textsubscript{50} and IC\textsubscript{50} of santonin were obtained by a crystal violet assay and calculated using nonlinear regression analysis of GraphPad Prism software (version 5.01) by plotting log inhibitor versus normalized response (variable slope).
against two strains of A/H1N1 (A/Puerto Rico/8/24 and A/Yokohama/77/2008) with IC\textsubscript{50} values of 0.019 and 0.054 mg/mL, respectively.\textsuperscript{17} 

\textit{E. sativa} juice containing glucosinolate hydrolysis products and \textit{E. sativa} heated juice containing intact glucosinolates also showed moderate antiviral activities against influenza A/H5N1 virus with IC\textsubscript{50} values of 0.0562 and 0.0647 mg/mL, respectively. Meanwhile, \textit{E. sativa} methanol extract containing intact glucosinolates and \textit{E. sativa} methanol extract containing glucosinolate hydrolysis products showed poor antiviral activity against influenza A/H5N1 with IC\textsubscript{50} values of 0.451 and 1.215 mg/mL, respectively. Compared with our findings, previous studies on glucosinolate derivatives showed their antiviral potential against seasonal human influenza (A/California/7/2009 (H1N1) NYMC X-179A) with comparable IC\textsubscript{50} values, ranging from 0.625 to 5.0 mg/mL.\textsuperscript{20} 

On the other hand, radish seed extract (extracted with MeOH) showed no activity against the studied avian influenza virus A/H5N1, while fermented radish was reported to have anti-influenza activity against the avian influenza virus H9N2.\textsuperscript{11} 

To continue further investigations of the safe plant extract with promising anti-influenza activity, the most effective plant extracts were selected based on their potency and selectivity index. The selectivity index, defined as the ratio of the 50\% cytotoxic concentration (CC\textsubscript{50}) to the 50\% antiviral concentration (IC\textsubscript{50}), is a commonly used parameter to recommend a tested extract or purified compound based on its \textit{in vitro} efficacy in the inhibition of virus replication with no cytotoxic or cytostatic effects. Studies have recommended different SI values to endorse a compound or herbal extract for further investigation, whether \textit{in vitro} or preclinical.\textsuperscript{33} Awouafack \textit{et al}. recommended an SI that is greater than 10 as an acceptance criterion for selecting an active sample or potential therapeutic to be further investigated.\textsuperscript{34} Thus, we continued our investigations to define the potential mode of anti-influenza action for santonica flower extract (\textit{A. cina}). The extract could target viral particles and exert high antiviral activities at the three tested levels, virucidal, adsorption interference, and replication inhibition, especially the virucidal effect with almost full viral inhibition (>99.9\%). This finding is consistent with the predictable multitarget/nonspecific mode of antiviral action of plant extracts with extremely complex and multicomponent mixture against viral pathogens. To the best of our knowledge, rare or no studies have investigated the antiviral activity of
santonica flower extract. Several studies have shown that santonica flower extract has antibacterial, antioxidant, immunosuppressive, anthelmintic “anti-roundworm”, anti-inflammatory, and anti-malarial activities.\textsuperscript{15} The chief active ingredient in \emph{A. cina} is santonin that is widely applied as anthelmintic.\textsuperscript{26} To define the role of santonin in the overall anti-influenza activity of unexpanded santonica flower extract, santonin was tested and it showed potent anti-H5N1 and anti-H1N1 activities. Furthermore, santonin displayed virtual neuraminidase inhibition activity against N1-type neuraminidase and this activity has been validated in this study via a colorimetric \textit{in vitro} enzymatic assay. Santonin revealed potent activity as an anti-influenza candidate with IC\textsubscript{50} values of 1.701 \(\mu\)g/mL against influenza A/H5N1 and 2.91 \(\mu\)g/mL against seasonal influenza A/H1N1. Santonin is more similar to oseltamivir than zanamivir based on ROCS analysis. The neuraminidase inhibition activity against the reference zanamivir drug was validated \textit{in vitro}. This study highlights the applicability of santonica flower extract and its main active constituent santonin as non-toxic natural antiviral candidates against human and avian IAVs.

### Table 5. Tanimoto Scores of Zanamivir and Oseltamivir with Santonin as a Query

| drug     | Tanimoto combo | shape Tanimoto | color Tanimoto |
|----------|----------------|----------------|----------------|
| zanamivir| 0.77           | 0.68           | 0.09           |
| oseltamivir| 0.81          | 0.67           | 0.14           |

5. CONCLUSIONS

In regard to the elevated ratios of drug-resistance among influenza viruses “avian and seasonal” among humans, there is a continuous demand for effective anti-influenza candidates. In this study, the anti-influenza activity for several bioactive plant extracts was evaluated. Out of the tested extracts, santonica flower extract (\emph{A. cina}) and its major active constituent santonin showed potent anti-influenza activities against human IAV and/or avian IAV. The santonica flower extract could target IAV at the three major compartments: cell-free status (“viricidal effect”), while attaching to the host receptor (“adsorption interference”), and during viral replication (“replication inhibition”). As a major active ingredient, santonin revealed potent activity as an anti-influenza candidate with IC\textsubscript{50} values of > 100 \(\mu\)g/mL, a well-known side effect due to santonin overdose \textit{in vivo} is called xanthopsia, which is a visual disorder where bright objects appear as yellow and sometimes dark objects are perceived as violet. In serious cases, this may result in complete loss of vision.\textsuperscript{30} To this point, further derivatization of santonin is recommended to benefit from its potent anti-influenza activity and to amend its reported side effect. The antiviral potential of some santonin derivatives against hepatitis C virus (HCV) was reported with EC\textsubscript{50} values >10 \(\mu\)M.\textsuperscript{36} Eventually, the findings of these results emphasize the potential antiviral activities of natural santonica flower extract and its major active constituent santonin against avian and human influenza viruses that negatively impact the public health and lead to dramatic losses in the poultry sector. Further studies need to be done to elucidate the identity of other antiviral compounds in the santonica flower extract (rather than santonin) and the \emph{E. sativa} juice extracts, the inhibitory or virucidal potential for these extracts and their active ingredients in experimental animals (preclinical studies), and the possible synergistic effects among the effective extracts and their active ingredients in targeting avian and human IAVs.

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Notes

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REFERENCES

(1) (a) Richard, M.; van den Brand, J. M. A.; Bestebroer, T. M.; Lexmond, P.; de Meulder, D.; Fouchi, R. A. M.; Lowen, A. C.; Herfst, S. Influenza A viruses are transmitted via the air from the nasal respiratory epithelium of ferrets. Nat. Commun. 2020, 11, 766. (b) Mostafa, A.; Abdelwhab, E. M.; Mettenleiter, T. C.; Pleschka, S. Zoontotic Potential of Influenza A Viruses: A Comprehensive Overview. Viruses 2018, 10, 497. (2) Wu, Y.; Wu, Y.; Tefsen, B.; Shi, Y.; Gao, G. F. Bat-derived influenza-like viruses H17N10 and H18N11. Trends Microbiol. 2014, 22, 183–191.

(3) Spackman, E., A Brief Introduction to Avian Influenza Virus. In Animal Influenza Virus: Methods and Protocols, Spackman, E., Ed. Springer US: New York, NY, 2020; pp. 83–92.

(4) Tenforde, M. W.; Kondor, R. J. G.; Chung, J. R.; Zimmerman, R. K.; Nowalk, M. P.; Jackson, M. L.; Jackson, L. A.; Monto, A. S.; Martin, E. T.; Belongia, E. A.; McLean, H. Q.; Gaglani, M.; Rao, A.; Kim, S. S.; Stark, T. J.; Barnes, J. R.; Wentworth, D. E.; Patel, M. L.; Flannery, B. Effect of Antigenic Drift on Influenza Vaccine Effectiveness in the United States-2019-2020. Clin Infect Dis 2021, 73, e4244–e4250.

(5) Mtmamo, S. E.; Amoako, D. G.; Somboro, A. M.; Agoni, C.; Lawal, M. M.; Gumeded, N. S.; Khan, B. B.; Kumalo, H. Influenza Viruses: Harnessing the Crucial Role of the M2 Ion-Channel and Neuraminidase toward Inhibitor Design. Molecules 2021, 26 (), DOI: 10.3390/molecules26040880.

(6) Mothatalgo, K. E.; Mehrbod, P.; Fotouhi, F.; Abdalla, M. A.; Ellof, J. N.; McGaw, L. J. Anti-influenza A virus activity of two Newtonia species and the isolated compound myricetin-3-o-rhamnoside. BMC Complementary Medicine and Therapies 2021, 21, 92.

(7) (a) Mostafa, A.; Mahmoud, S. H.; Shehata, M.; Müller, C.; Kandel, A.; El-Shenawy, R.; Nooh, H. Z.; Kayali, G.; Ali, M. A.; Pleschka, S.P.A from a Recent H9N2 (G1-Like) Avian Influenza A Virus (AIV) Strain Carrying Lysine 367 Confers Altered Replication Efficiency and Pathogenicity to Contemporary H5N1 in Mammalian Systems. Viruses 2020, 12 (), DOI: 10.3390/v120910406. (b) Petersen, H.; Mostafa, A.; Tantawy, M. A.; Iqbal, A. A.; Hoffmann, D.; Tallam, A.; Selvakumar, B.; Pessler, F.; Beer, M.; Rautenschlein, S.; Pleschka, S.N.S Segment of a 1918 Influenza A Virus-Descendant Endorses Replication of H1N1pdm09 and Virus-Induced Cellular Immune Response in Mammalian and Avian Systems. Front. Microbiol. 2018, 9, 526. DOI: 10.3389/fmicb.2018.00526.

(8) (a) Duraiapandian, V.; Ignacimuthu, S. Antibacterial and antifungal activity of Cassia fistula L. : an ethnomedicinal plant. J. Ethnopharmacol. 2007, 112, 590–594. (b) Mwangi, R. W.; Macharia, J. M.; Wagara, I. N.; Bence, R. L. The medicinal properties of Cassia fistula L: a review. Biomed. Pharmacother. 2021, 144, No. 112240.

(9) Allyn, O. Q.; Kusumawati, E.; Nugroho, R. A. Antimicrobial activity of Terminalia catappa brown leaf extracts against Staphylococcus aureus ATCC 25923 and Pseudomonas aeruginosa ATCC 27853. F1000Research 2018, 7, 1406.

(10) (a) Todirascu-Ciornea, E.; El-Nashar, H. A. S.; Mostafa, N. M.; Eldahshian, O. A.; Boiangiu, R. S.; Dumitrul, G.; Hritcu, L.; Singab, A. N. B. Schinus terebinthifolius Essential Oil Attenuates Scopolamine-Induced Memory Deficits via Cholinergic Modulation and Antioxidant Properties in a Zebrafish Model. J. Evidence-Based Complementary Altern. Med. 2019, 2019, 5256781. (b) Salles, T. S.; Meneses, M. D. F.; Yamamoto, K. A.; Sá-Guimarães, T. E.; Caldas, L. A.; Silva, J. H. S.; da Silva Ferreira, P.; Amaral, A. C. F.; Ventura, J. A.; Azevedo, R. C.; Kuster, R. M.; Soares, M. R.; Ferreira, D. F. Chemical composition and anti-Mayar virus activity of Schinus terebinthifolius fruits. VirusDisease 2021, 32, 526–534. (c) Torres, K. A.; Lima, S. M.; Ueda, S. M. Activity of the aqueous extract of Schinus terebinthifolius Raddi on strains of the Candida genus. Rev. Bras. Ginecol. Obstet. 2016, 38, 593–599.

(11) Sham, T. T.; Yuen, A. C.; Ng, Y. F.; Chan, C. O.; Mok, D. K.; Chan, S. W. A. A review of the phytochemistry and pharmacological activities of raphani semen. J. Evidence-Based Complementary Altern. Med. 2013, 2013, 636194.

(12) (a) Ahmed, A. G.; Hussein, U. K.; Ahmed, A. E.; Kim, K. M.; Mahmoud, H. M.; Hammouda, O.; Jang, K. Y.; Bishaye, A. Mustard Seed (Brassica nigra) Extract Exhibits Antiproliferative Effect against Human Lung Cancer Cells through Differential Regulation of Apoptosis, Cell Cycle, Migration, and Invasion. Molecules 2020, 25, 2069. (b) Guijarro-Real, C.; Plazas, M.; Rodriguez-Burruezo, A.; Prohens, J.; Fita, A. Potential In Vitro Inhibition of Selected Plant
Extracts against SARS-CoV-2. Chymotrypsin-Like Protease (3CL(Pro)) Activity. Foods 2021, 10, 1503.

(13) (a) Christensen, S. B.Natural Products That Changed Society. Biomedicines 2021, 9 (4), DOI: 10.3390/biomedicines9040742. (b) Ren, J.; Zeng, W.; Jiang, C.; Li, C.; Zhang, C.; Cao, H.; Li, W.; He, Q. Inhibition of Porcine Epidemic Diarrhea Virus by Cinchonine via Inducing Cellular Autophagy. Front. Cell. Infect. Microbiol. 2022, 12, No. 856711.

(14) (a) Fitiou, L.; Tsakou, O.; Hancianu, M.; Poia, A. Volatile Constituents and Antimicrobial Activity of Tilia tomentosa Moench and Tilia cordata Miller. J. Essent. Oil Res. 2007, 19, 183–185. (b) Cittan, M.; Altuntaş, E.; Celik, A. Evaluation of antioxidant capacities and phenolic profiles in Tilia cordata fruit extracts: A comparative study to determine the efficiency of traditional hot water infusion method. Ind. Crops Prod. 2018, 122, 553–558.

(15) (a) Ivanescu, B.; Miron, A.; Corcioca, A. Sesquiterpene lactones from Artemisia genus: biological activities and methods of analysis. J. Anal. Methods Chen. 2015, 2015, 1. (b) Kasmiyati, S.; Kristiani, E. B. E.; Herawati, M. M.; Sukmana, A. B. A. Antibacterial activity and flavonoids content of Artemisia cina Berg. ex Poljakov ethyl acetate extract. Biosintfaktika: J. Biol. Biol. Ed. 2021, 13, 106–112. (c) Karmi, E.; Kasmiyati, S.; Herawati, M. The cytotoxic and apoptotic effects of wild and polyploidy genotype of Artemisia cina extracts on the WiDr colon and HTB-183 lung cancer cell lines. Biodiversitas J. Biol. Div. 2021, 22 (4), DOI: 10.13057/biodiv220735. (d) Adewumi, O. A.; Singh, V.; Singh, G. Chemical composition, traditional uses and biological activities of artemisia species. Int J Pharmacogn Phytother. 2020, 9, 1124–1140.

(16) (a) Peluso, I.; Serafini, M. Antioxidants from black and green tea: from dietary modulation of oxidative stress to pharmacological mechanisms. Br. J. Pharmcol. 2017, 174, 1195–1208. (b) Chakraborty, K.; Dey, A.; Bhattacharyya, A.; Dasgupta, S. C. Anti-fibrotic effect of green tea in diabetes. Biomedicines 2021, 9, 1140. (c) Fitsiou, L.; Tzakou, O.; Hancianu, M.; Poiata, A. Volatile Chemical Compounds against Enterobacteria. Glória, M. B. A. Antibacterial Activity of Coffee Extracts and Selected Flavonoids. Henrick, K.; Azziz-Baumgartner, E.; Nuorti, J. P.; Widdowson, M. A. Global burden of resistant pandemic viruses via environmental drug exposure of resistant pandemic viruses. Environ. Sci. Technol. 2020, 54, 8743–8752. (d) Adeniyi, B. A.; Ayepola, O. O.; Adu, F. D. The antiviral activity of coffee extracts and selected flavonoids on influenza viruses A H1N1 and H5N1. Biog. Med. Chen. 2019, 27, 2935–2947. (b) Berman, H.; Henrick, K.; Nakamura, H. Announcing the worldwide Protein Data Bank. Nat. Struct. Mol. Biol. 2003, 10, 980–980.

(17) (a) Yaekub, H. S.; Mahmoud, A.; Mostafa, A.; Al-Karmalawy, A. A.; Zidan, A.; Abulkhair, H. S.; Mahmoud, S. H.; Shehata, M.; Elhefniawi, M. M.; Ali, M. A. The antiviral activity of artemisia species. Int J Pharmacogn Phytother. 2020, 9, 1124–1140.

(18) (a) Takahashi, T.; Kubo, R.; Sakaino, M. Antimicrobial activities of eucalyptus leaf extracts and flavonoids from Eucalyptus microcarpa. Lett. Appl. Microbiol. 2004, 39, 60–64. (b) Elsayar, H. O.; Salem, M. Z. M.; Ashmawy, N. A.; Yessoufou, K.; El-Settawy, A. A. A. In vitro antibacterial, antifungal and antioxidant activities of Eucalyptus spp. leaf extracts related to phenolic composition. Nat. Prod. Res. 2017, 31, 2897–2980. (c) Adeniyi, B. A.; Ayeole, O. O.; Adas, F. D. The antiviral activity of leaves of Eucalyptus camaldulensis (Dehn.) and Eucalyptus torelliana (R. Muell). Pak. J. Pharm. Sci. 2015, 28, 1773–1776.

(19) (a) Taher, M. A.; Nyeem, M. A. B.; Billah, M. M.; Ahammed, M. M. Vinca alkaloid-the second most used alkaloid for cancer treatment: A review. Int. J. Physiol. Nutr. Phys. Educ. 2017, 2, 723–727. (b) Ahmed, M. F.; Kazim, S. M.; Ghori, S. S.; Mehjabeen, S. S.; Ahmed, S. R.; Ali, S. M.; Ibrahim, M. Antidiabetic activity of Vinca rosea extracts in alloxan-induced diabetic rats. Int. J. Endocrinol. 2010, 10, 1.

(20) (a) Nie, L. X.; Wu, Y. L.; Dai, Z.; Ma, S. C. Antiviral activity of Isatisis Radix derived glucosinolates isomers and their breakdown products against influenza A in vitro/ovo and mechanism of action. J. Ethnopharmacol. 2020, 251, No. 115250. (b) Bayooshti, S. R.; Shokooohinia, Y.; Eftekhari, M. Glucosinolates and their hydrolysis products as potential nutraceuticals to combat cytokine storm in SARS-CoV-2. Daru, J. Pharm. Sci. 2022, 30, 245–252.

(21) (a) Reed, L. J.; Muench, H. A SIMPLE METHOD OF ESTIMATING FIFTY PER CENT ENDPOINT. Am. J. Epidemiol. 1938, 27, 493–497.

(22) Gausch, C. R.; Smith, T. F. Replication and plaque assay of influenza virus in an established line of canine kidney cells. Appl. Microbiol. 1968, 16, 588–594.

(23) (a) Mahmoud, A.; Mostafa, A.; Al-Karmalawy, A. A.; Zidan, A.; Abulkhair, H. S.; Mahmoud, S. H.; Shehata, M.; Elhefniawi, M. M.; Ali, M. A. Telaprevir is a potential drug for repurposing against SARS-CoV-2: computational and in vitro studies. Helyon 2021, 7, No. e07962. (b) Mostafa, A.; Kandeil, A.; Elshaiyer, A.; Kutkat, O.; Moatasim, Y.; Rashad, A. A.; Shehata, M.; Gomaa, M. R.; Mahrous, N.; Mahmoud, S. H.; GabAllah, M.; Abbas, H.; Taweel, A. E.; Kayed, A. E.; Kamel, M. N.; Sayes, M. E.; Mahmoud, D. B.; El-Sheseny, R.; Kayalì, G.; Ali, M. A. FDA-Approved Drugs with Potent In Vitro Antiviral Activity against Severe Acute Respiratory Syndrome Coronavirus 2. Pharmaceuticals 2020, 13, 443.

(24) Salamanca, A.; Almodóvar, P.; Jarama, L.; González-Hedström, D.; Prodanov, M.; Inarejos-García, A. A. Anti-influenza virus activity of the elenolic acid rich olive leaf (Olea europea L.) extract Esolema®. Antiviral Chem. Chemother. 2021, 29, 20402066211063391. (c) Adeniyi, B. A.; Ayepola, O. O.; Adu, F. D. The antiviral activity of coffee extracts and selected flavonoids on influenza viruses A H1N1 and H5N1. Biog. Med. Chen. 2019, 27, 2935–2947. (b) Berman, H.; Henrick, K.; Nakamura, H. Announcing the worldwide Protein Data Bank. Nat. Struct. Mol. Biol. 2003, 10, 980–980.

(25) (a) Zima, V.; Albíñan, C. B.; Rojková, K.; Pokorná, J.; Pachl, P.; Rézová, P.; Hudlicky, J.; Navrátil, V.; Majer, P.; Konvalinka, J.; Kožíšek, M.; Machara, A. Investigation of flexibility of neuraminidase 150-loop using tamiflú derivatives in influenza A viruses H1N1 and H5N1. Biog. Med. Chen. 2019, 27, 2935–2947. (b) Ahmed, M.; Elsayar, H. O.; Salem, M. Z. M.; Ashmawy, N. A.; Yessoufou, K.; El-Settawy, A. A. A. In vitro antibacterial, antifungal and antioxidant activities of Eucalyptus spp. leaf extracts related to phenolic composition. Nat. Prod. Res. 2017, 31, 2897–2930. (c) Adeniyi, B. A.; Ayeole, O. O.; Adas, F. D. The antiviral activity of leaves of Eucalyptus camaldulensis (Dehn.) and Eucalyptus torelliana (R. Muell). Pak. J. Pharm. Sci. 2015, 28, 1773–1776.
amantadine-resistant variants among highly pathogenic avian influenza H5N1 viruses in Egypt. *Infect Genet Evol* 2016, 46, 102–109.

(32) Lenz, E.; Muller, C.; Mostafa, A.; Dzieciolowski, J.; Kanrai, P.; Dam, S.; Cwientzek, U.; Prenner, L.-N.; Pleschka, S. Authorised medicinal product Aspecton® Oral Drops containing thyme extract KMTv24497 shows antiviral activity against viruses which cause respiratory infections. *J. Herb. Med.* 2018, 13, 26–33.

(33) Indrayanto, G.; Putra, G. S.; Suhud, F., Validation of in-vitro bioassay methods: Application in herbal drug research. In *Profiles of Drug Substances, Excipients and Related Methodology*, Al-Majed, A. A., Ed. Academic Press: 2021; Vol. 46, pp. 273–307.

(34) Awouafack, M. D.; Kusari, S.; Lamshöft, M.; Ngamga, D.; Tane, P.; Spiteller, M. Semi-synthesis of dihydrochalcone derivatives and their in vitro antimicrobial activities. *Planta Med.* 2010, 76, 640–643.

(35) (a) Al-Harbi, M. M.; Qureshi, S.; Ahmed, M. M.; Raza, M.; Miana, G. A.; Shah, A. H. Studies on the antiinflammatory, antipyretic and analgesic activities of santonin. *Jpn. J. Pharmacol.* 1994, 64, 135. (b) Trifan, A.; Zengin, G.; Sinan, K. I.; Sieniawska, E.; Sawicki, R.; Maciejewska-Turska, M.; Skalikca-Woźniak, K.; Luca, S. V. Unveiling the Phytochemical Profile and Biological Potential of Five Artemisia Species. *Antioxidants* 2022, 11 ( ), DOI: 10.3390/antiox11051017. (c) Wang, J.; Su, S.; Zhang, S.; Zhai, S.; Sheng, R.; Wu, W.; Guo, R. Structure-activity relationship and synthetic methodologies of α-santonin derivatives with diverse bioactivities: A mini-review. *Eur. J. Med. Chem.* 2019, 175, 215–233.

(36) Hwang, D. R.; Wu, Y. S.; Chang, C. W.; Lien, T. W.; Chen, W. C.; Tan, U. K.; Hsu, J. T.; Hsieh, H. P. Synthesis and anti-viral activity of a series of sesquiterpene lactones and analogues in the subgenomic HCV replicon system. *Bioorg. Med. Chem.* 2006, 14, 83–91.