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Scientific Research

Evidence of Synergistic Activity of Medicinal Plant Extracts against Neuraminidase Inhibitor Resistant Strains of Influenza Viruses

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Abstract

The frequent emergence of drug resistant influenza viral strains emphasizes the urgent and continual need to develop new antiviral drugs. Given the encouraging findings of previous studies on antiviral compounds from plant sources, this study focused on medicinal plants from Borneo that were traditionally used to treat symptoms of influenza infection. Following the promising results of earlier investigations, four plant extracts that demonstrated multiple modes of viral inhibition were studied against wild-type and neuraminidase (NA) inhibitor-resistant strains of Types A and B influenza viruses. The extracts exhibited more pronounced activities against the wild-type viruses than the NA inhibitor-resistant strains. Variations in the antiviral potential of the extracts collected from different parts of the same plant were also evidenced in the *in vitro* micro-inhibition assays. Even though all plant extracts affected NA activity of all viruses, only two extracts demonstrated hemagglutination inhibitory (HI) activities against Type A pandemic H1N1 and Type B viruses. Furthermore, Receptor Destroying Enzyme (RDE) treatments of extracts exhibiting HI activities indicated the presence of sialic acid (SA)-like component(s) that may be responsible for HI activity. Since the antiviral potential of extracts was not completely suppressed by RDE, the possibility of non SA-like antiviral components cannot be ruled out. Therefore, synergistic activity between SA-like and non SA-like components contained in the plant extracts may be responsible for the demonstrated antiviral potential. The results also indicated the presence of non SA-like components that may act against other viral proteins apart from hemagglutinin (HA) and NA.

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Hence, this study supports the presence of multiple antiviral components that act against different viral proteins or interfere with different stages of viral replication. Our results suggest that these plant extracts have the potential to be developed as therapeutic agents for the treatment of influenza and could be a solution to the global occurrence of viral strains resistant to NA inhibitors.

Keywords

Influenza, Antivirals, Medicinal Plant Extracts, Hemagglutination Inhibition, Neuraminidase Inhibition, Sialic Acid-Like, RDE

1. Introduction

Infections caused by the influenza virus are a critical public health issue which is reflected by significant mortality in humans during annual epidemics or pandemics occurring typically every 10 to 40 years [1]. The most recent, caused by pandemic human influenza A (H1N1), resulted in 18,449 laboratory-confirmed deaths globally within a period of one and a half years from early April 2009 to August 2010 [2]. There are unceasing concerns that the 2009 H1N1 virus may cause life-threatening illness in the near future [3].

Influenza viruses belong to the family *Orthomyxoviridae*, a family of RNA viruses, and are classified into three types, A, B and C, all of which are known to affect vertebrates, including birds, humans and other mammals [4]. Types A and B viruses are the predominant causes of human influenza infections with Type A being the major causative agent of epidemics [5]. Hemagglutinin (HA) and neuraminidase (NA) are the main glycoproteins of Types A and B viruses [6]; depending on the antigenicity of the two surface glycoproteins, there are currently 17 HA (H1 - H17) and 9 NA (N1 - N9) subtypes recognized in Type A virus, with most subtypes present in waterfowl and shorebirds [7] [8].

Although annual vaccination is the principal approach for the prevention of infections, influenza antiviral drugs also play a vital role in the control of illnesses and transmission of the virus. Two classes of antiviral drugs, namely M2 ion channel inhibitors (Amantadine and Rimantadine) and neuraminidase inhibitors (NAIs) have been approved for the treatment and prophylaxis of influenza. However, the use of M2 ion channel inhibitors has been limited due to the rapid emergence of drug resistance, the ready transmissibility of drug-resistant viruses and the occurrence of central nervous system side effects [3]. Currently, NAIs including Zanamivir, Oseltamivir, Laninamivir, and Peramivir are generally used to treat influenza [2]. However, influenza viruses with low susceptibility to NAIs have been isolated both *in vitro* and *in vivo*. Mutations in the active sites of the NA or in HA, which alter its sensitivity to inhibition, are the prime reasons for resistance towards the drugs [3].

New influenza viruses arise through variations called antigenic drift and antigenic shift. Antigenic drift refers to a minor change, such as an amino acid substitution in either HA and/or NA that causes an antigenic site change. The majority of seasonal influenza infections are caused by antigenic drift. On the other hand, the predominant cause for influenza pandemics is antigenic shift, which results in the formation of new viral strains through the combination of HA and NA from diverse subtypes [9]-[11]. Although the structure of the NA active site is highly conserved, making it a prominent target for NAIs in antiviral therapy, mutations in the NA may alter the shape of the catalytic site, thereby reducing the binding potential of the inhibitor. Due to differences in the chemical structures of the inhibitors, many of the mutations do not confer reduced sensitivity to all NAIs.

Oseltamivir, an NAI drug, has a hydrophobic chain; therefore, viral NA must undergo rearrangements to accommodate drug binding and the efficiency of Oseltamivir may be lowered by mutations that affect this rearrangement. Hydrophobic binding of oseltamivir carboxylate (the active metabolite of Oseltamivir) relies on glutamic acid residue 276 present within the viral NA active site. This residue is in close proximity to histidine 274. Oseltamivir resistance is conferred on the virus by a single amino acid change: histidine to tyrosine at position 274 (H274Y). Hence, the H274Y mutation (H275Y in N1 numbering) confers resistance in only N1 subtype viruses. Similarly, resistance to Zanamivir may result from mutations in framework or catalytic residues of the NA, thereby affecting the binding affinity between the enzyme and the inhibitor [12] [13].

Type B viruses are also often responsible for a significant proportion of seasonal influenza infections. The

D197E mutation in Type B viruses confers cross-resistance to all NAIs. A greater than 10 - 50 fold change in IC_{50} values compared with the wild-type virus is evident with Oseltamivir and Peramivir, affecting the drug's potential at a medium rate, whereas a lower than 10-fold change in the NAI potential of Zanamivir is shown against Type B virus with the D197E mutation [14]. In addition to NA mutations, NAI resistance could also emerge *in vitro* due to mutations in or near the HA receptor binding site, reflecting the importance of functional balance between HA and NA [15]. The increasing appearance of resistant variants of influenza virus highlights the urgent need to identify new antiviral drugs [3].

Natural products (NPs), predominantly those obtained from plant sources, have received increasing attention for their antiviral potential and numerous conventional drugs obtained from NPs are widely used in mainstream medicine for several conditions, including influenza. Norsesquiterpenoids isolated from the roots of *Phyllanthus emblica*, the flavone 4',5,7-trihydroxy-3',5'-dimethoxyflavone (tricin) isolated from bamboo (*Sasa albo-marginata*) [16], germacrone, a major component of the essential oils extracted from *Rhizoma curcuma* [3] and several Chinese herbal formulations, such as Jinhua Qinggan granule and Shufeng Jiedu capsule [2], have all been shown to possess anti-influenza activity.

In the earlier investigations with fifty medicinal plant extracts from the tropical rain forests of Borneo, all extracts indicated minimal cytotoxicity owing to the presence of cytoprotective components that support the safety of compounds linked to ethnomedical background. Out of the fifty extracts, eleven plant extracts were shown to demonstrate potent antiviral activity against H1N1 and H3N1 subtypes of influenza virus. All extracts inhibited the enzymatic activity of viral NA and four extracts were also shown to act through the hemagglutination inhibition (HI) pathway. Moreover, the samples that acted through both HI and neuraminidase inhibition (NI) evidenced more than 90% reduction in virus adsorption and penetration, thereby indicating potent action in the early stages of viral replication [17].

In an effort to identify new antivirals for influenza, four plant extracts that were shown to inhibit the viruses through HA and NA inhibition pathways were chosen for further investigation against Types A and B viruses, including Oseltamivir resistant seasonal and pandemic H1N1 viruses and a Type B virus with the D197E mutation. As shown in **Table 1**, the extracts collected from the tropical rainforests of Borneo were traditionally used to treat symptoms of influenza infection [17]-[22]. Wild-type viruses corresponding to the mutant strains that are susceptible to the NAI inhibitors were also included in the study. These studies were designed to further investigate the effect of extracts on NAI drug resistant strains and also help in the understanding of whether synergistic effects of components in the extracts are responsible for the multiple modes of action exhibited.

2. Materials and Methods

2.1. Cells

Madin Darby Canine Kidney (MDCK) cells were obtained from the American Type Culture Collection (Manassas, VA) and grown at 37˚C with 5% CO_2 in Roswell Park Memorial Institute medium (RPMI; Invitrogen, No: 22400-105), supplemented with 10% foetal bovine serum (FBS; Invitrogen, No: 16140-071) and 1% Penicillin-Streptomycin (Invitrogen, No: 15140-122). Before the addition of compounds or the virus, or while determining the results, the monolayers of MDCK cells were washed twice with phosphate buffered-saline (PBS, pH 7.4 at room temperature). The following controls were included in all experiments: cell control (cells that were neither infected with the virus nor treated with the plant extracts), virus control (cells that were infected

Table 1. Medicinal plant extracts from Sarawak demonstrating antiviral activity against influenza viruses.

No	Voucher specimen no.	Plant part	Botanical name (Family)	Medicinal use*
8	SABC 0782	Whole plant	*Mussaenda elmeri* (Rubiaceae)	Conjunctivitis, headache
41	SABC 1528	Whole plant	*Calophyllum lanigerum* (Clusiaceae)	Headache, eye related disorders, potential to inhibit HIV
42	SABC 1528	Stems	*Calophyllum lanigerum* (Clusiaceae)	Headache, eye related disorders, potential to inhibit HIV
43	SABC 4492	Stems	*Albizia corniculata* (Fabaceae)	Sore throat

*Information derived from; Chai 2006 [18], Salleh 2002 [21], Yaacob 2009 [22], Maji 2010 [20], Focho 2010 [17] [19].

only with the virus but not with the plant extracts), and the positive controls (virus-infected cells treated with NA inhibitor drugs, Zanamivir or Oseltamivir).

2.2. Viruses

Six influenza viruses (**Table 2**) that were isolated, plaque purified and cultured in MDCK cells were provided by Dr. Aeron Hurt, WHO Collaborating Centre for Reference and Research on Influenza, Victorian Infectious Diseases Reference Laboratory (VIDRL), Melbourne, Australia. Virus stocks were developed in MDCK cells using RPMI medium supplemented with 4 µg/mL trypsin (Sigma, No: T1426) at 37˚C in 5% CO_2 for three days as described elsewhere [23]. Supernatants containing virus were collected after cytopathic effects (CPE) were observed and antiviral titres were determined using 50% Tissue Culture Infectious Dose ($TCID_{50}$), according to Reed and Muench's endpoint method [24], and a colorimetric endpoint was used to obtain quantitative results as described elsewhere [25]. All aliquots of virus stocks that were collected were stored at −80˚C until use.

2.3. Plant Extracts

The medicinal plant extracts were originally extracted with a mixture of dichloromethane and methanol in a 1:1 (v/v) ratio and subsequently concentrated using a rotary evaporator. The yield was dependent on the part of plant used; approximately 0.05 to 0.10 g of extract was obtained from 6 g of whole plant, whereas the yield was reduced to 0.01 to 0.05 g when extracts were obtained from stems only. Prior to use, the extracts were reconstituted in PBS with 10% dimethyl sulfoxide (DMSO, SIGMA No: D 5879) and filtered using a 0.45 µm filter (Sartorius Stedium Australia No: 16533K). Selection of plant extracts for this study was based on the pathways of viral inhibition involving inhibition of hemagglutination demonstrated in previous studies [17].

2.4. *In Vitro* Micro-Inhibition Assay

The antiviral activities of plant extracts against influenza viruses were evaluated according to a method described elsewhere [17]. Briefly, 96-well plates seeded with 3×10^4 cells/well were incubated for 24 h at 37˚C with 5% CO_2 until a confluent monolayer was attained. The cells were washed twice with PBS, and two-fold serial dilutions of plant extracts (3.13 - 100 µg/mL) in RPMI medium challenged with 100 $TCID_{50}$ of influenza virus were added to the wells in triplicate for each concentration. Wells containing 10% DMSO with media only and virus only, devoid of plant extract were also included in the study. All wells were supplemented with virus growth medium (100 µL of RPMI medium containing 2 µg/mL trypsin). After three days of incubation at 37˚C/5% CO_2, the results were quantified as previously explained. The antiviral activity curve was then constructed by plotting percentages of virus inhibition against concentrations of extracts. The inhibitory concentration (IC_{50}) of extract required to reduce virus-induced CPE by 50% was expressed relative to the virus control employing dose-response curves. A trend line that best suited the curve was selected using regression analysis of antiviral activity curves (in Microsoft excel) and the corresponding equation was used to calculate IC_{50} values [26].

Table 2. List of influenza viruses used in the antiviral assays.

Viruses	Description
Seasonal A (H1N1)	A/MISSISSIPPI/3/2001 wild-type virus (former seasonal H1N1; A/New Caledonia/20/99-like) - containing histidine at position 275 (275H) of the neuraminidase glycoprotein
Seasonal A (H1N1) (H275Y)	A/MISSISSIPPI/3/2001 variant virus (former seasonal H1N1; A/New Caledonia/20/99-like) - containing tyrosine at position 275 (275Y) of the neuraminidase glycoprotein—*i.e.* a H275Y substitution
Pandemic A (H1N1)	A/PERTH/265/2009 wild-type virus (H1N1pdm09; A/California/7/2009-like) - containing histidine at position 275 (275H) of the neuraminidase glycoprotein
Pandemic A (H1N1) (H275Y)	A/PERTH/261/2009 variant virus (H1N1pdm09; A/California/7/2009-like) - containing tyrosine at position 275 (275Y) of the neuraminidase glycoprotein—*i.e.* a H275Y substitution
Type B	B/PERTH/211/2001 wild-type virus (B/Sichuan/379/99-like) - containing aspartic acid at position 197 (197D) of the neuraminidase glycoprotein
Type B (D197E)	B/PERTH/211/2001 variant virus (B/Sichuan/379/99-like) - containing glutamic acid at position 197 (197E) of the neuraminidase glycoprotein—*i.e.* a D197E substitution

2.5 Neuraminidase (NA) Inhibition Assay

In order to test the effects of extracts on the viral NA of Types A and B viruses, the NA-Fluor™ Influenza Neuraminidase Assay Kit (Life Technologies, No: 4457091) was employed as per the manufacturer's instructions. The optimum virus dilution for the NA inhibition assay was selected by titration of the virus stock, employing the NA activity assay. Two-fold serial dilutions of plant extracts (0.3 - 25 µg/mL) were tested for NA inhibitory activity. NAIs, Zanamivir and Oseltamivir, were included as positive controls in the assay and tested at nanomolar concentrations (10^{-2} to 10^{4} nM). A POLARstar Omega fluorescence polarization microplate reader (excitation 355 nm, emission 460 nm) was used to measure fluorescence. A sigmoidal curve generated by dose-response data and analysed using GraphPad Prism Software was used in the determination of IC_{50} values.

2.6. Hemagglutination Inhibition (HI) Test

The effect of extracts on virus adsorption was determined by an HI assay [27]. Briefly, two-fold serial dilutions of the extract (12.5 - 100 µg/mL) prepared in PBS and an equal volume (25 µL/well containing 4 HAU) of the virus stock were added to each well in a round-bottomed 96-well microtitre plate in triplicate, followed by the addition of 50 µL of 5% chicken red blood cells (CRBC). The following controls were included in every plate i) CRBC without virus; ii) CRBC with virus devoid of extract and iii) CRBC with extracts devoid of virus. The hemagglutination reactions were observed after 30 minutes incubation at room temperature.

2.7. RDE Treatment

In vitro micro inhibition and HI assays were employed to study the effect of Receptor Destroying Enzyme (RDE, Denka Seiken Co. Ltd., Tokyo, Japan) treatment upon the antiviral activity of the extracts. The extracts added to RDE solutions in the ratio 1:3 were incubated at 37˚C for 20 hours according to the manufacturer's instructions. The objective of this experiment was to eliminate the activity of compounds that may possess sialic acid (SA)-like structures which imitate the receptors of RBC and compete for hemagglutinin [28]. The RDE and extract mixture was inactivated at 56˚C for 60 minutes before performing the *in vitro* micro inhibition and HI assays.

2.8. Gas Chromatograph Mass Spectrometer (GC-MS) Analysis of Extract and Fractions

Crude extracts 8, 41, 42 and 43 were analysed using a gas chromatograph coupled to a mass spectrometer as detector (GCMS-QP2010 Ultra, Shimadzu), equipped with an Rxi®-5SIL-MS column. Helium gas was used as a carrier gas with total flow, column flow and purge flow set to 7.5 mL/min, 1.50 mL/min and 3.0 mL/min respectively; 2 µL of the sample was injected. Injector temperature was set to 200˚C and split injection mode (1:2) was selected at pressure 88.9 kPa. The column oven temperature was programmed as follows; held at 50˚C for the first two minutes and ramping at a rate of 20˚C/min to a final temperature of 250˚C, then held for eight minutes. Total GC run time was 20 min. The chromatogram obtained after the run was used to generate a qualitative table of the 50 most abundant compounds using peak integrate option. A similarity search was then performed to determine the possible structural units. The compounds were identified by comparison with the NIST and Wiley 8.0 MS libraries. Only peaks with a match greater than 75% and area % greater than 0.5% were reported.

2.9. Statistical Analysis

All treatments were performed in triplicate and each experiment was independently repeated at least twice. The data were expressed as mean ± standard error of the mean (SEM). The results of the antiviral activity assays were analysed with a one-way ANOVA test and a significance level (*p* value) of 0.05 or 0.01 was considered to compare the means.

3. Results

3.1. Antiviral Activity against Seasonal Influenza Viruses

The plant extracts 8, 41, 42 and 43 were active against both the wild-type seasonal Type A (H1N1) and mutant seasonal Type A (H1N1) (H275Y) viruses. As shown in **Table 3**, IC_{50} values for extract 8 were <3.13 µg/mL

Table 3. Inhibitory concentrations of anti-influenza extracts.

Extract	IC_{50} (µg/ml)					
	Seasonal A (H1N1)	Seasonal A (H1N1) (H275Y)	Pandemic A (H1N1)	Pandemic A (H1N1) (H275Y)	Type B	Type B (D197E)
8	<3.13	<3.13	<3.13	<3.13	<3.13	<3.13
41/42	14.86 ± 2.47/ 14.46 ± 6.89	22.87 ± 3.33/ 21.06 ± 5.79	<3.13/ <3.13	9.99 ± 2.02/ <3.13	11.16 ± 5.88/ 4.6 ± 2.13	11.29 ± 4.2/ <3.13
43	16.45 ± 6.00	27.63 ± 4.67	<3.13	12.2 ± 4.99	13.55 ± 1.40	9.19 ± 3.06
Zanamivir	<3.13	96.62 ± 23.18	<3.13	<3.13	<3.13	<3.13
Oseltamivir	<3.13	>100	<3.13	>100	6.2 ± 0.1	22.67 ± 6.89

IC_{50} represents the concentration of plant extract needed to reduce the viral inhibition by 50% relative to virus control wells without test compound, calculated from dose-response data of virus inhibition. Plant extracts (3.13 - 100 µg/mL) in RPMI medium were challenged with 100 $TCID_{50}$ of influenza viruses.

against both viruses. Extracts 41 and 42, obtained from different parts of the same plant, showed similar IC_{50} values against the wild-type virus and a relatively higher IC_{50} value was noted when the mutant virus was tested. Extract 43 was shown to have an IC_{50} of 16.45 µg/mL against the wild-type virus, while 27.63 µg/mL were required to reduce the activity of the mutant virus to 50%. Among the NAI drugs that were included as controls, Zanamivir showed antiviral inhibition against both viruses while Oseltamivir was active only against the wild-type virus and showed an IC_{50} value > 100 µg/mL against mutant seasonal A virus.

As shown in **Figure 1**, Extract 8 demonstrated similar activity against both the wild-type and mutant seasonal A (H1N1) viruses between 25 - 100 µg/mL, but at concentrations <12.5 µg/mL a lower percentage of viral inhibition was exhibited against the mutant strain; this result was statistically significant ($p < 0.05$). Extracts 41, 42 and 43 also showed a similar trend in viral inhibition against both wild-type and mutant seasonal Type A viruses, however inhibition against the mutant strain was relatively lower. DMSO, at a concentration of 10% in media did not affect the cell viability of cells with media only and virus only, devoid of plant extracts and cell viability obtained was similar to that of cell control and virus control respectively without 10% DMSO.

3.2. Viral Inhibition against Pandemic Influenza Viruses

Extracts 8, 41, 42 and 43 demonstrated antiviral activities against both the wild-type pandemic A (H1N1) and the mutant pandemic A (H1N1) (H275Y) viruses. As evidenced in **Figure 2**, Extract 8 showed similar activities against the wild-type and mutant pandemic viruses with a demonstrated IC_{50} value of <3.13 µg/mL. Although similar trends in virus inhibition were shown by extracts 41, 42 and 43, at concentrations below 6.25 µg/mL a lower percentage of virus inhibition was demonstrated against the mutant virus compared to the the wild-type. As shown in **Table 3**, IC_{50} values were <3.13 µg/mL against the wild-type for all the extracts while against the mutant virus IC_{50} values of 9.99 µg/mL and 12.2 µg/mL were obtained for extracts 41 and 43, respectively. Even though extracts 41 and 42 were obtained from different parts of the same plant, the IC_{50} value against the mutant virus was <3.13 µg/mL for Extract 42, while it was 9.99 µg/mL for Extract 41; this result was statistically significant ($p < 0.05$).

Since the H275Y mutation confers Oseltamivir-resistance, the NAI inhibitor Oseltamivir was only active against the wild-type virus with an IC_{50} value of <3.13 µg/mL while the IC_{50} was >100 µg/mL against the Oseltamivir-resistant mutant virus. Zanamivir was shown to be active against both viruses with IC_{50} values of <3.13 µg/mL.

3.3. Inhibitory Effects against Type B Viruses

Extracts 8, 41, 42 and 43 were also shown to be active against both the wild-type and the mutant Type B (D197E) viruses. As evidenced in **Figure 3**, Extract 8 showed similar trends in viral inhibition against both wild-type and mutant viruses; this behaviour was also observed with extracts 41 and 43. However, Extract 42 was active between 6.25 - 100 µg/mL against both viruses; while at 3.13 µg/mL the antiviral activity against the wild-type virus was only 17% which was significantly less than the activity against mutant virus. As shown in **Table 2**, Extract 8 demonstrated an IC_{50} value of <3.13 µg/mL against both viruses while Extract 43 showed

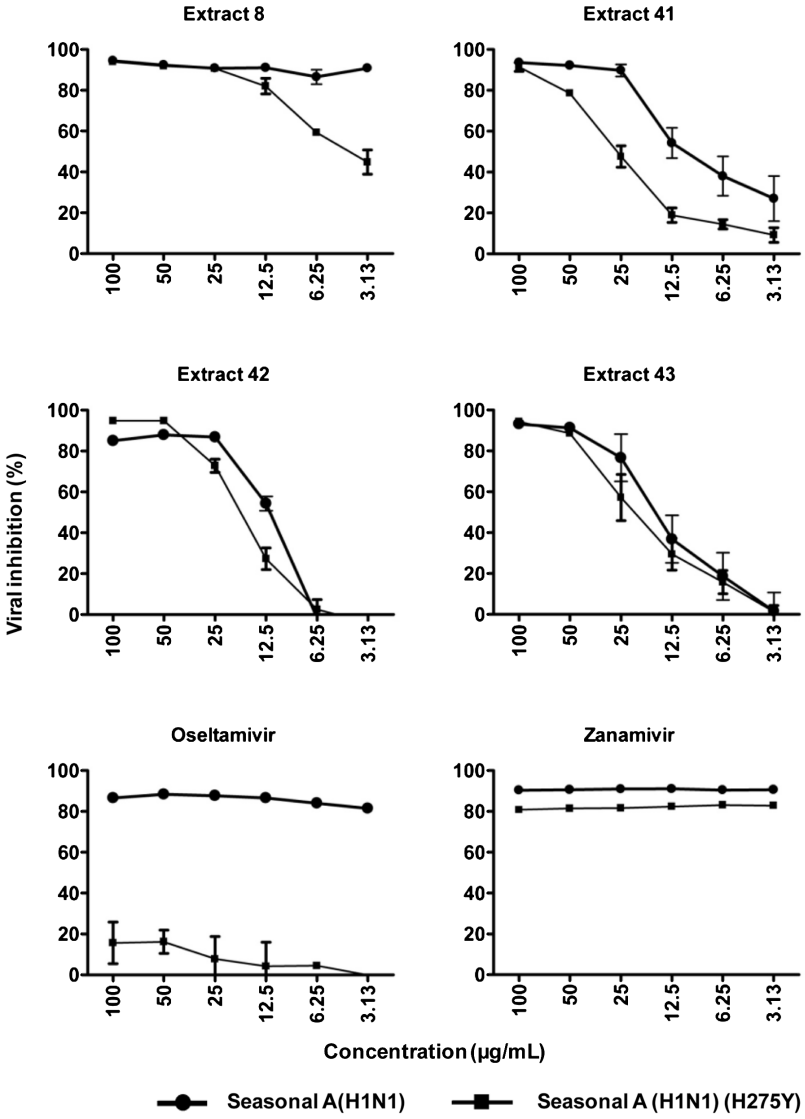

Figure 1. Inhibitory effects of plant extracts against seasonal H1N1 viruses. Cells at 80% confluency were treated with two-fold serial dilutions of plant extracts (3.13 - 100 µg/mL) and 100 $TCID_{50}$ of either seasonal H1N1 or mutant seasonal H1N1 (H275Y) Type A influenza viruses. All wells were provided with 100 µL of RPMI medium supplemented with 2 µg/mL trypsin (virus growth medium). Cell viability was evaluated using MTT and viral inhibition percentage calculated relative to virus control wells. Representatives of two independent experiments performed in triplicate are shown. Statistical analysis showed that data were significant and the differences among means for each concentration were statistically significant with $p < 0.05$ (one way ANOVA).

IC_{50} values of 13.55 µg/mL and 9.19 µg/mL against the wild-type and mutant viruses, respectively. Extract 41 demonstrated a similar IC_{50}, of 11.16 µg/mL and 11.29 µg/mL against the wild type and mutant viruses, respectively, while Extract 42 showed IC_{50} values of 4.6 µg/mL and <3.13 µg/mL for the same viruses.

Since the Type B virus with D197E mutation confers resistance against all NA inhibitors, both Oseltamivir and Zanamivir were shown to demonstrate significantly lower antiviral activity against the mutant virus relative to the wild-type virus (**Figure 3**), but the IC_{50} value of Zanamivir showed no significant difference for both viruses, even though the D197E mutation affects the NAI potential of the drug in the order of <10 fold. However, the IC_{50} value of Oseltamivir, which is known to be affected by the D197E mutation in the order of 10 - 50 fold or higher, was shown to have a calculated IC_{50} value greater than 22.67 µg/mL against the mutant virus while that against the wild-type virus was 6.23 µg/mL.

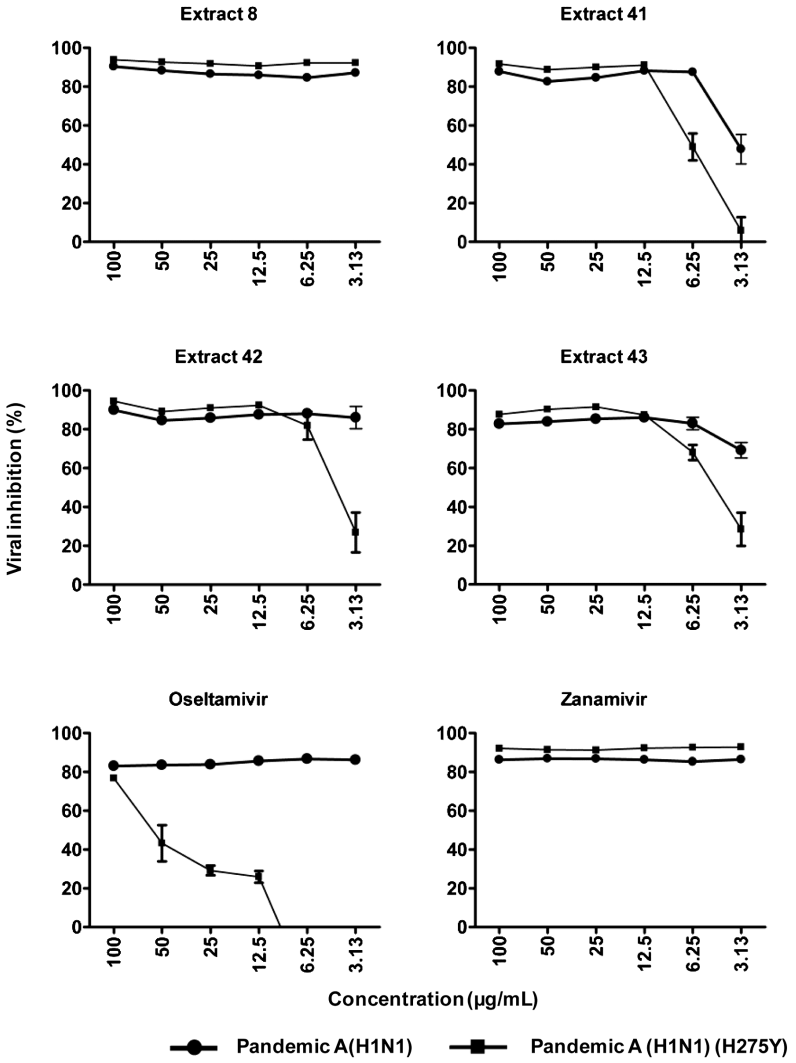

Figure 2. Inhibitory effects of plant extracts against pandemic H1N1 viruses. Cells at 80% confluency were treated with two-fold serial dilutions of plant extracts (3.13 - 100 µg/mL) and 100 $TCID_{50}$ of either pandemic H1N1or mutant pandemic H1N1 (H275Y) Type A influenza viruses. All wells were provided with 100 µL of RPMI medium supplemented with 2 µg/mL trypsin (virus growth medium). Cell viability was evaluated using MTT and viral inhibition percentage calculated relative to virus control wells. Representatives of two independent experiments performed in triplicate are shown. Statistical analysis showed that data were significant and the differences among means for each concentration were statistically significant with $p < 0.05$ (one way ANOVA).

3.4. Neuraminidase Inhibitory (NAI) Effects of Plant Extracts

Influenza NA serves as a principal drug target for the treatment of influenza [29]. Extracts 8, 41, 42 and 43 were tested for NA inhibitory activity against the six influenza viruses included in the study. Increasing concentrations of extracts were associated with decreased relative fluorescence, consistent with the inhibition of NA activity (**Table 4**). Extract 8 reduced the NA activity of the six viruses at lower concentrations (3.14 - 7.99 µg/mL) compared to other extracts. The NAI potential of Extracts 41 and 42 collected from the same plant source were not similar. As reported in **Table 4**, Extract 41 showed lower IC_{50} values than Extract 42 against wild-type and mutant seasonal A (H1N1), pandemic A (H1N1) (H275Y) and Type B viruses, while Extract 42 showed lower IC_{50} values than Extract 41 against pandemic A (H1N1) and Type B (D197E) viruses; this result was statistically significant ($p < 0.05$). The IC_{50} value for Extract 43 ranged between 4.90 - 10.02 µg/mL against all the viruses that were tested.

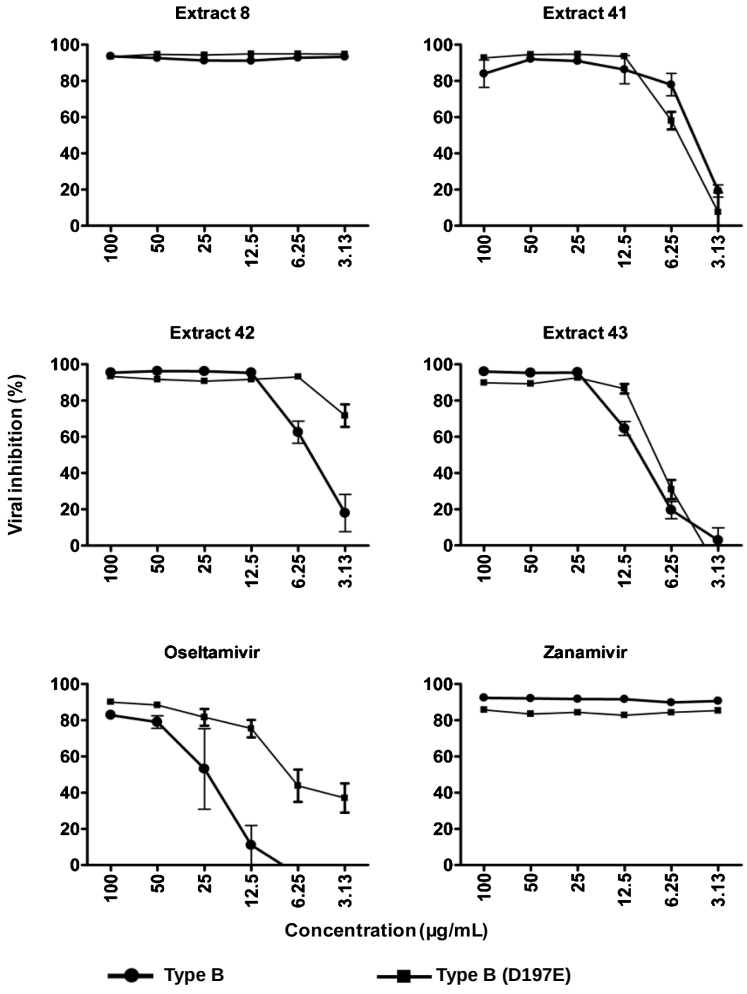

Figure 3. Inhibitory effects of plant extracts against Type B influenza viruses. Cells at 80% confluency were treated with two-fold serial dilutions of plant extracts (3.13 - 100 µg/mL) and 100 $TCID_{50}$ of either Type B or mutant Type B (D197E) virus. All wells were provided with 100 µL of RPMI medium supplemented with 2 µg/mL trypsin (virus growth medium). Cell viability was evaluated using MTT and viral inhibition percentage calculated relative to virus control wells. Representatives of two independent experiments performed in triplicate are shown. Statistical analysis showed that data were significant and the differences among means for each concentration were statistically significant with $p < 0.05$ (one way ANOVA).

Table 4. NAI activity of anti-influenza extracts.

Extract	IC_{50} (µg/mL)					
	Seasonal A (H1N1)	Seasonal A (H1N1) (H275Y)	Pandemic A (H1N1)	Pandemic A (H1N1) (H275Y)	Type B	Type B (D197E)
8	7.26 ± 0.16	6.21 ± 0.31	3.14 ± 0.04	5.54 ± 0.14	7.99 ± 0.48	4.95 ± 3.60
41/42	6.33 ± 2.08/ 9.64 ± 0.50	6.28 ± 0.23/ 10.57 ± 0.07	6.96 ± 0.07/ 5.15 ± 0.25	8.19 ± 0.26/ 9.78 ± 0.16	8.79 ± 0.43/ 11.36 ± 2.04	7.69 ± 0.10/ 6.43 ± 0.81
43	9.28 ± 0.68	10.02 ± 1.13	6.74 ± 0.05	9.363 ± 0.86	9.26 ± 1.37	4.90 ± 1.50
NAI drugs	IC_{50} (nM)					
Oseltamivir	4.52 ± 1.14	1517 ± 93.31	11.92 ± 0.08	1413.5 ± 197.28	22.03 ± 0.10	1335.5 ± 234.0
Zanamivir	5.152 ± 0.78	1.88 ± 0.12	1.07 ± 0.01	1.01 ± 1.41	4.56 ± 0.10	50.18 ± 2.93

NAI activity of the plant extracts was measured at concentrations ranging from 0.3 to 25 µg/mL, while the controls, Zanamivir and Oseltamivir, were measured at 0.01 to 10,000 nM, as recommended by the manufacturer. The optimum virus dilution for the NAI assay was selected by titration of virus stock in an NA activity assay; 1:4 dilutions of the virus strains were selected in the NA activity assay to perform NAI assay.

Zanamivir and Oseltamivir were included as positive controls in the assay and tested at nanomolar concentrations, as recommended by the manufacturer; therefore the results should not be compared directly with the crude plant extracts that were tested at µg/mL concentrations. IC_{50} values obtained in our studies for the NAIs were in agreement with those reported in the literature except for minor variations in the NAI potential of Oseltamivir against pandemic A (H1N1) and Type B (D197E) viruses and Zanamivir against seasonal A (H1N1) (**Table 5**).

3.5. Inhibitory Effects of Plant Extracts on Viral HA

The critical step in the initiation of influenza infection is the binding of viral HA to the sialic acid (SA) residues expressed by host cell glycoproteins and glycolipids [30]. The viral HA also binds to SA residues expressed on the surface of erythrocytes, resulting in hemagglutination. The ability of plant extracts to inhibit influenza virus-induced hemagglutination was studied in an HI assay.

As shown in **Figure 4**, Extract 8 did not demonstrate HI activity against any of the six viruses studied. At concentrations ranging between 50 - 100 µg/mL, extracts 41 and 42 (collected from different parts of the same plant) inhibited virus-induced hemagglutination of both wild-type and mutant strains of pandemic A (H1N1). Although extracts 41 and 42 demonstrated HI activity against Type B virus only at 100 µg/mL, both extracts demonstrated effective HI activity against Type B (D197E) virus at 50 - 100 µg/mL; however, no HI activity

Table 5. NAI activity of neuraminidase inhibitors.

Viruses	IC $_{50}$ range (nM)*	
	Oseltamivir	Zanamivir
Seasonal A (H1N1)	0.4 - 10	0.3 - 1
Seasonal A (H1N1) (H275Y)	257 - 3455	0.3 - 2
Pandemic A (H1N1)	0.2 - 10	0.2 - 1
Pandemic A (H1N1) (H275Y)	132 - 2179	0.2 - 2
Type B	8 - 128	0.5 - 12
Type B (D197E)	79 - 966	3 - 290

*The IC_{50} range was based on results determined by seven different laboratories using a range of different fluorescence-based protocols [39].

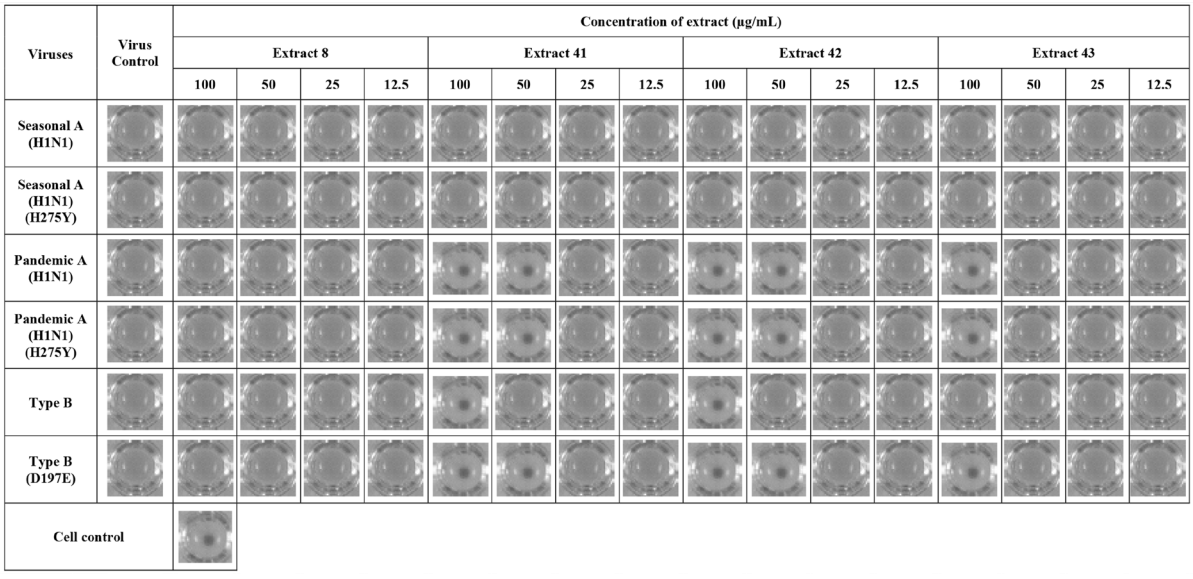

Figure 4. Inhibitory effects of plant extracts on influenza virus-induced hemagglutination. HI activities of four extracts (12.5 - 100 µg/mL) against 4HAU/25 µL of virus are shown. The following controls were included on each plate; i) extract controls with extract and CRBC only; ii) virus controls containing virus and CRBC and iii) cell controls containing only CRBC. Since extract controls were similar to cell controls, they are not included in the figure. Data are shown from one of three independent experiments, each performed in triplicate.

was evidenced against the seasonal A (H1N1) viruses. Extract 43 inhibited HI activity of both wild-type and mutant pandemic A (H1N1) viruses at 100 µg/mL while exhibiting HI activity only against Type B (D197E) at the same concentration. Nevertheless, Extract 43 did not exhibit HI activity against wild-type and mutant seasonal A (H1N1) viruses and wild-type B viruses. None of the plant extracts caused hemolysis of CRBC at 12.5 - 100 µg/mL as evidenced in previous studies that were performed on different batches of extracts 8, 41, 42 and 43 [17] [31].

3.6. Effect of RDE Treatment on the Antiviral Activity of Plant Extracts

Extracts 8, 41 and 42 and 43 were treated with RDE in order to eliminate SA mimics that may demonstrate antiviral potential against the viruses similar to the experiments against Mem-Bel and PR8 viruses in our previous study [17]. An *in vitro* micro-inhibition assay using the six viruses was performed with the RDE-treated extracts. As shown in **Figure 5**, antiviral activities originally exhibited by all four extracts were either eliminated or reduced

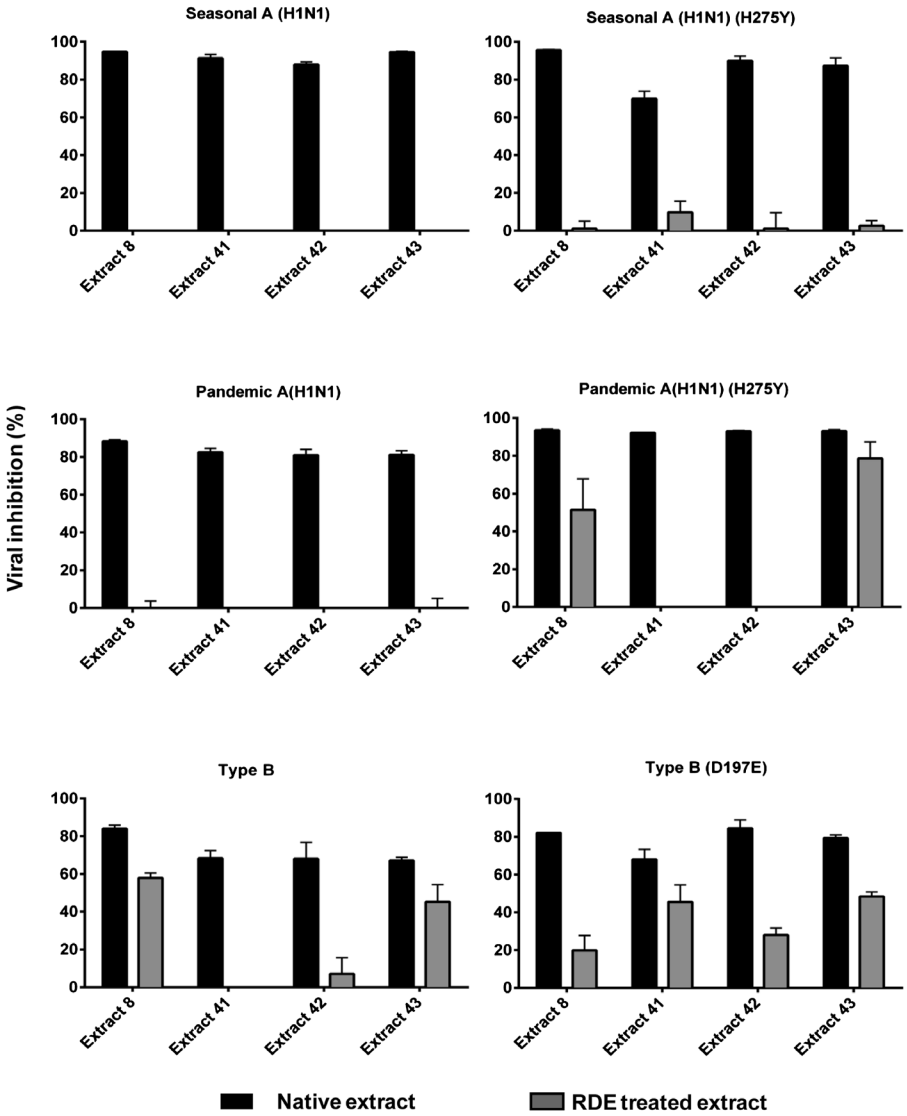

Figure 5. Effects of RDE treatment on the antiviral activity of plant extracts. An *in vitro* micro-inhibition assay was used to assess the ability of plant extracts (25 µg/mL, optimum concentration for antiviral activity) to inhibit influenza viruses (100 $TCID_{50}$). Extracts were either treated with RDE as per the manufacturer's instructions or left in their native form without RDE treatment. Data shown are representative of two independent experiments performed in triplicate. Statistical analysis showed that data were significant with p value < 0.05 (one way ANOVA).

following RDE treatment. The antiviral activity of Extract 8 was suppressed against all viruses following RDE treatment, with the exception of mutant pandemic A (H1N1) (H275Y) and Type B viruses where viral inhibitions of 51% and 57%, respectively, were exhibited. RDE-treated extracts 41 and 42 showed loss of antiviral activity against all the six viruses. On the contrary, the antiviral activity of RDE-treated Extract 43 was reduced by only 14% against pandemic A (H1N1) (H275Y) virus (down to 78% virus inhibition), 22% against Type B (45% virus inhibition) and 30% against Type B (D197E) virus (48% virus inhibition). However, the activity against Type B viruses was considered to be negligible since the 50% virus inhibition threshold was not reached. There was no evidence of antiviral activity against either the wild-type or the mutant seasonal A and pandemic A (H1N1) viruses; this result was statistically significant ($p < 0.05$).

3.7. Effect of RDE Treatment on the HI Activity of Plant Extracts

Extracts 41 and 42, which demonstrated HI activity against pandemic H1N1 and Type B viruses, were treated with RDE in order to eliminate SA-like components that may compete with the CRBC receptors for viral HA. Extract 43, which showed HI activity against pandemic H1N1 and mutant Type B (D197E) viruses was also subjected to RDE treatment. An HI assay was then performed with the RDE-treated extracts against wild-type and mutant strains of pandemic A (H1N1) and Type B viruses. As shown in **Figure 6**, RDE treatment suppressed the HI activity originally exhibited by extracts 41, 42 and 43.

3.8. Effects of RDE Treatment on the NAI Activity of Plant Extracts

RDE-treated extracts were also studied in an NAI assay to determine whether NAI SA mimics were present in the extracts. As shown in **Figure 7**, the NAI activity originally present in the extracts was also affected by RDE treatment. The NA activity of the virus-only wells were set to 100% and the loss in the NA activity of the virus by treatment with plant extracts in the native form and those that were treated with RDE is shown in **Figure 7**. In its native form, Extract 8 was shown to significantly reduce the NA activity to less than 30% against all but Type B viruses where the extract reduced the NA activity to 43%, while RDE-treated Extract 8 showed decreased NAI potential. Similarly, RDE treatment decreased the NAI potential of extracts 41 and 42; NA activity of virus in wells treated with RDE treated extracts was close to 100%, similar to the wells containing only the virus. Extract 43 also showed a similar trend in the loss of NAI activity upon RDE treatment. Only with Type B virus, the difference in the NAI activity of extracts 8, 41 and 42 in native and RDE-treated forms was less than 25%. RDE-treated extracts were shown to contain relatively lowered NAI potential than native extracts; the differences between means for each concentration were statistically significant ($p < 0.05$).

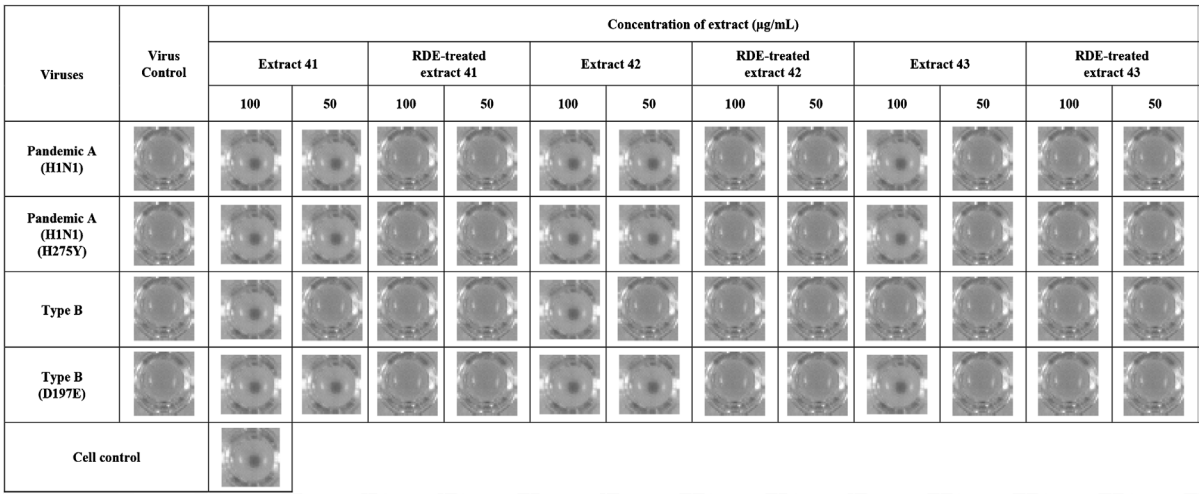

Figure 6. Effect of RDE treatment on the HI activity of plant extracts. HI activities of three extracts (50 - 100 µg/mL) treated with RDE against 4HAU/25 µL pandemic A (H1N1), pandemic A (H1N1) (H275Y), Type B and Type B (D197E) influenza viruses are shown. i) Virus controls containing virus and CRBC and ii) cell controls receiving CRBC only are shown. Extracts that mediate HI activity without RDE treatment were included in all plates as positive controls. The experiment was performed in triplicate.

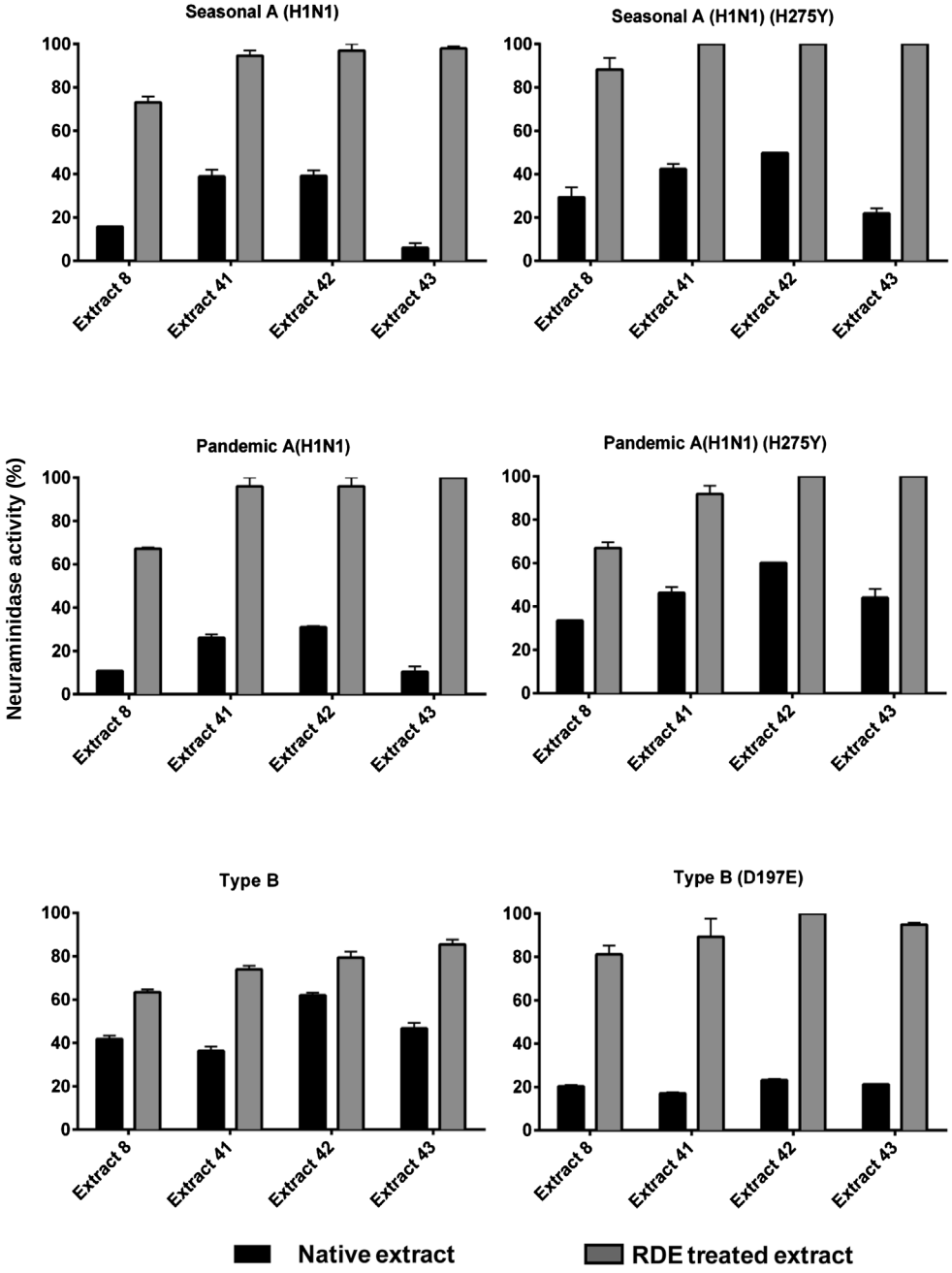

Figure 7. Effect of RDE treatment on the NAI activity of plant extracts. The NA activity of virus only wells were set to 100% and the NA activity of virus in the presence of native extracts (25 µg/mL) and those that were treated with RDE were calculated relative to the virus control. The optimum virus dilution for the NAI assay was selected by titration of virus stock in an NA activity assay; 1:4 dilutions of either of the viruses were selected in the NA activity assay to perform the NAI assay. Representatives of two independent experiments are shown. Statistical analysis showed that data were significant with $p < 0.05$ (one way ANOVA).

3.9. GC-MS Analyses of Crude Extract and Fractions

The crude extracts 8, 41, 42 and 43 were analysed using GC-MS in order to determine their chemical profile. Several components were present in the medicinal extracts and the first 50 peaks present in the fractions were identified using NIST and Wiley 8.0 MS libraries based on the area (%), however, only compounds that showed

greater than 75% matching and 0.5% area were reported. Since the crude extracts demonstrated the presence of several components with multiple peaks of minor area, 50 peaks were chosen for each sample in order to determine the chemical nature. The reason for choosing 50 peaks was to ensure that compounds which contributed only to a minor area were not neglected; hence compounds that contribute to the antiviral potential of the extract through synergy may be detected.

As shown in **Figure 8**, Extract 8 showed a major proportion of esters (20.63%), hydrocarbons (19.34%), cyclitols (16.06%) and alcohols (14.84%); Quinic acid constituted 9.25% of Extract 8. Although extracts 41 and 42 were obtained from different parts of the same plant, their chemical profile showed variations in the amount of chemical components contained. Extract 41 showed more amounts of alcohol (11.2%), aldehyde (4.8%) and ketone (19.37%) and Extract 42 showed relatively higher amount of esters (11.7%) and phenolics (8.2%) compared to Extract 41. Extract 43 showed major proportion of phenolic compounds (20.39%), esters (19.1%), alcohols (11.36%) and cyclitols (10.34%).

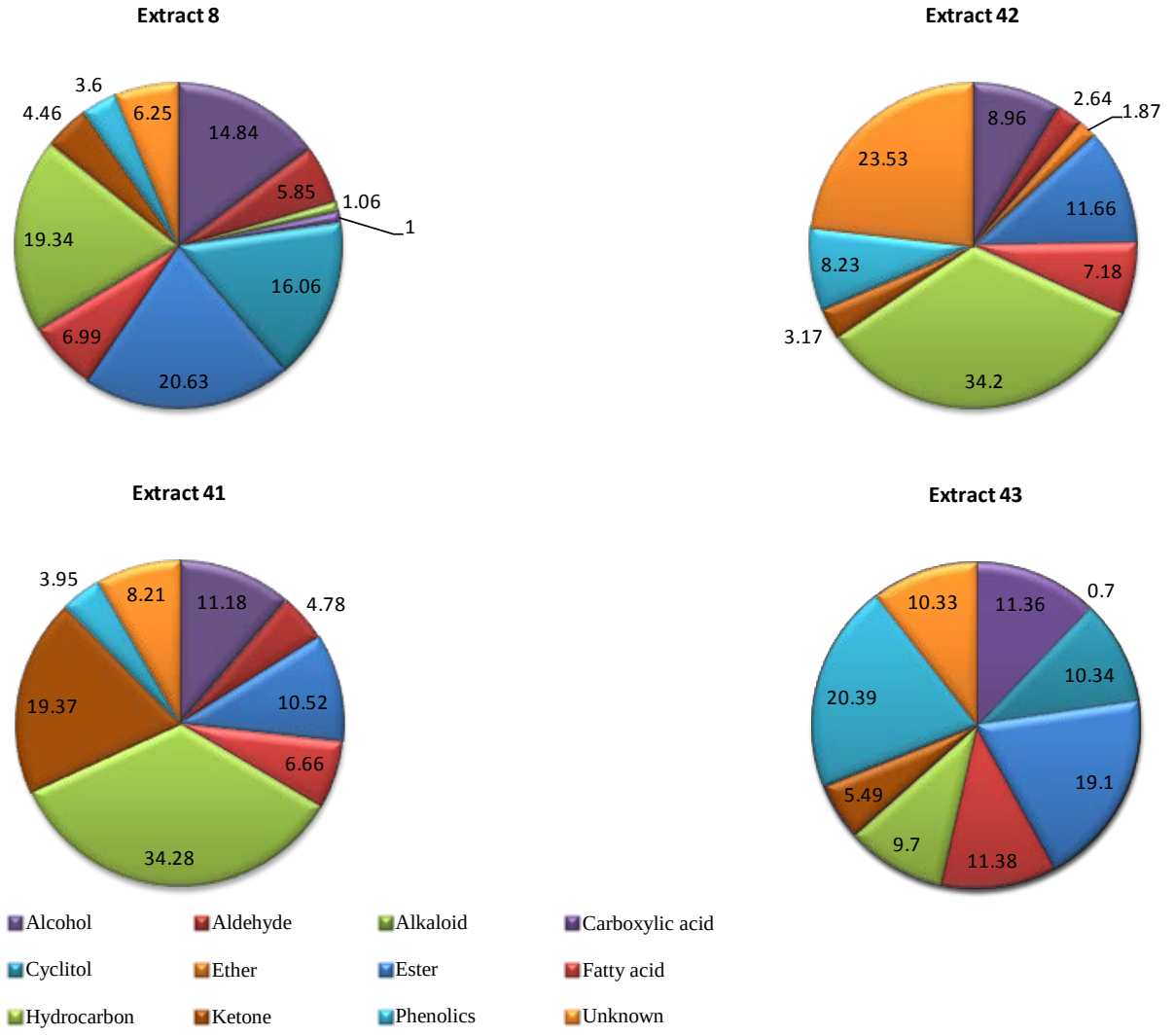

Figure 8. Components present in crude extracts 8, 41, 42 and 43. Extracts 8, 41, 42 and 43 were analysed using a gas chromatograph coupled to a mass spectrometer as detector (GCMS-QP2010 Ultra, Shimadzu), equipped with an Rxi-5SIL-MS column. Helium gas was used as a carrier gas with a total GC run time of 20 min. The compounds were identified by comparison with the NIST and Wiley 8.0 MS library. Components present (area %) in the extracts are shown in the figure, 93.75% of compounds in Extract 8, 91.79% in Extract 41, 76.47% in Extract 42 and 89.67% in Extract 43 showed similarity of more than 75% match to the compounds in the library. The remaining 6.25% in Extract 8, 8.21% in Extract 41, 23.53% in Extract 42 and 10.33% in Extract 43 constituted several compounds that showed less than 75% match with the library of compounds.

4. Discussion

The antiviral activities of extracts 8, 41, 42 and 43 against recent influenza viruses indicate that these extracts are a promising source for new anti-influenza drugs. Since the extracts were shown to act against the NAI drug resistant Type A and Type B influenza viruses, they might serve as potential candidates to treat infections caused by drug resistant viruses.

Extract 8 was shown to be the most potent among the plant extracts, as indicated by its IC_{50} values (<3.13 µg/mL) against all six viruses, irrespective of the strain (wild-type or mutant). This extract also displayed similar antiviral activities against seasonal A viruses at concentrations ranging between 25 - 100 µg/mL, however, the antiviral potential against seasonal A (H1N1) (H275Y) was relatively lower than that against seasonal A (H1N1). This may have been due to the presence of multiple active components at different concentrations and the decrease in potential may be attributed to the decrease in the synergism exhibited by the actives contained within the extract. Similarly, Extract 42 demonstrated antiviral activities at 3.13 µg/mL against pandemic A (H1N1) while being inactive against pandemic A (H1N1) (H275Y), hence synergy between the active components may be lost at lower concentration due to a decrease in their availability. Extract 8 was also shown to demonstrate similar activity against both the wild-type and mutant strains of pandemic A and Type B viruses indicating that similar active components in the extract may be inhibitory to the different viruses, as reflected by the similar trends of antiviral inhibition.

Although extracts 41 and 42 were collected from different parts of the same plant, Extract 42 (stems) was shown to be relatively more potent than Extract 41 (whole plant) in inhibiting pandemic A (H1N1) (H275Y) and Type B viruses, as reflected by the low IC_{50} (**Table 3**). Hence, the stems of the plant may be a better source, containing either higher proportions of active components or component(s) that exert pronounced antiviral activity even at a lower concentration than the whole plant. Despite showing potent activity against the wild-type A viruses, relative to the mutant strains, Extract 43 was less potent against the wild-type B virus than Type B (D197E) mutant virus. Hence, the type of virus and the plant part from which the extract is obtained, both play crucial roles in determining the antiviral potentials of the extracts. Generally, the extracts demonstrated higher antiviral activity against the wild-type viruses than against the mutant strains, indicating that amino acid changes in the virus may be responsible for the variations in the antiviral inhibition demonstrated by the plant extracts and higher concentrations of extracts may be required to inhibit the mutant viruses compared to wild-type strains.

The effects of the D197E mutation upon the antiviral potential of Zanamivir were more evident in the NAI assay than the *in vitro* micro inhibition assay since the antiviral potential of the drug showed only 5% loss in viral inhibition against mutant Type B (D197E) virus in comparison to the activity against the wild-type B virus. However a 10-fold increase in the IC_{50} against Type B (D197E) compared to the wild Type B virus was evidenced in the NAI assay. This was in agreement with those reported in the literature [14].

Though all plant extracts were shown to affect the enzymatic activity of viral NA in the NAI assay, Extract 8 demonstrated higher NAI potential compared to other extracts indicating the presence of compound(s) with potent NAI activity. Since NAIs are well tolerated compared to ion channel blockers [29], plant extracts with NAI activity warrants further research. The demonstrated NAI activity of these plant extracts against types A and B viruses resistant to NAIs further supports the need for research. The variations in the IC_{50} values obtained for extracts 41 and 42 in the NAI assay suggests a difference in the antiviral potential of extracts collected from different parts of the same plant. The diversity in the chemical make-up of whole plant (Extract 41) and stems (Extract 42) from which the extracts were prepared may contribute to the minor variations that were evident in the antiviral assays.

Since drugs with a unique mode of action, unlike the currently available NAIs, hold much promise in the fight against drug-resistant viruses, compounds demonstrating HI activity are particularly important agents. Although extracts 8, 41, 42 and 43 were included in this study, based on their previously reported HI activity against H3N1 and H1N1 viruses [17], only extracts 41, 42 and 43 demonstrated HI activities (**Figure 4**) and the batch of Extract 8 used in this study did not demonstrate any HI activity. The effect of batch variations of Extract 8 in the HI assay is unclear at this stage, however, the lack of HI active components in Extract 8 at the right concentration in order to detect HI activity cannot be ruled out. The lack of HI activity against seasonal Type A (H1N1) viruses may have resulted from the insufficiency of active component(s) in extracts 41, 42 and 43. This might also be the reason for the lack of HI activity of Extract 43 against the Type B virus. The concentration of HI ac-

tive component(s) in the plant extract is also crucial in exhibiting observable effects in the HI assay. Hence, chemical fractionation and isolation of the active components responsible for HI activity will be essential to support the demonstrated activity of extracts.

The suppression of antiviral potential of the extracts against both the seasonal (H1N1) viruses (**Figure 5**) indicates the presence of SA mimics that may be responsible for the demonstrated antiviral activity. Although RDE treatments suppressed the antiviral activities of the extracts against wild-type pandemic A (H1N1), extracts 8 and 43 demonstrated antiviral activity against pandemic A (H1N1) (H275Y) indicating the presence of non SA-like antiviral components, thus, the components that affect the wild-type and mutant viruses of pandemic A may be different. This result was also reflected in the NAI potential of RDE-treated Extract 8, where the extract retained the ability to reduce NA activity of pandemic A (H1N1) (H275Y), similar to the native extract (**Figure 7**), although the native extract was shown to demonstrate relatively greater NAI potential. This indicates the presence of non SA-like NAI components in Extract 8. However, RDE-treated Extract 43 did not show NAI activity like that of native Extract 43 against pandemic A (H1N1) (H275Y) (**Figure 7**), but demonstrated activity in the *in vitro* micro-inhibition assay (**Figure 5**). Hence, Extract 43 may contain non SA-like component(s) that affect other viral proteins or replication stages, while SA mimics act only against HA and NA.

RDE-treated Extract 42 failed to demonstrate antiviral activity in the *in vitro* micro-inhibition assay (**Figure 5**), nor any HI activity (**Figure 6**), however the difference in the NAI potential between RDE-treated and native Extract 42 showed minor variations (<10%) relative to the difference in the NA activity as exhibited by other native and RDE-treated extracts. Therefore the antiviral activity derived from the SA-like components is essential for the efficient functioning of extract against the virus to obtain detectable levels (more than 50% virus inhibition) of antiviral activity although some non SA-like components may affect the viral NA. None of the extracts exhibited antiviral activity against Type B (D197E) virus upon RDE treatment (**Figure 5**); this result was reflected in both HI (**Figure 6**) and NAI (**Figure 7**) assays where the antiviral potential of extracts was suppressed upon RDE treatment.

The suppression of HI activity by RDE treatment suggests that SA-like components in the extract may be responsible for the demonstrated HI activity (**Figure 6**). SA mimics affecting the HA of influenza virus have been reported previously [32] [33]. The loss in NAI potential of extracts upon treatment with RDE further supports the presence of NAI SA-like components (**Figure 7**). The two successful NAI inhibitors, Zanamivir and Oseltamivir, are structural mimics of SA that bind to the active site of the NA enzyme [34]. As such, the results of the current studies are promising for the development of more SA-like NA inhibitors as it is apparent that the plant extracts investigated also contain NAI SA mimics.

GC-MS studies of crude extracts 8, 41, 42 and 43 allowed the determination of the chemical profile of these medicinal plant extracts. Quinic acid, a cyclitol, which was shown to be dominant in crude extract 8 is known for its anti-influenza activity; it was once used to prepare the NAI drug, Oseltamivir, but was dropped in favour of shikimic acid [35]. Thus, cyclitols present in the crude extracts 8 and 43 may be responsible for the inhibition of influenza A and B viruses. Presence of alcohols and fatty acids in significant amount in the medicinal extracts are also evident from this study; alcohols and fatty acids have been shown to demonstrate anti-influenza activity in other studies [36] [37]. Since they are easily metabolized in the human body, fatty acid derivatives which are not easily metabolized may be good candidates in the development of anti-influenza agents. [37]. Hydrocarbons and esters were also present in extracts 8, 41, 42 and 43; plants with reported activity against influenza viruses have also shown to contain hydrocarbons and esters in their chemical profile [38]. Since the plant extracts present a blend of components that have been shown to contain anti-influenza activity in other studies, the possibility of synergy among the anti-influenza components is further supported.

Conclusion

Collectively, the plant extracts investigated contain both SA mimics and non SA-like components which appear to work in synergy against the viruses tested. Although components bearing structural similarity to SA seem to be mostly responsible for the demonstrated HI activity of all the plant extracts, NAI components may contain both SA-like and non SA-like components, both being responsible for the antiviral potential of the plant extracts. Hence, the results further support the need to study the chemical properties of the medicinal extracts. The activity of these extracts against the NA drug resistant strains following the HI pathway indicates that they could serve as promising candidates for the development of third generation anti-influenza drugs.

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