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Antiviral Activity of *Aspalathus linearis* against Human Influenza Virus

Ratika Rahmasari[a], Takahiro Haruyama[a,b], Siriwan Charyasriwong[a], Tomoki Nishida[a] and Nobuyuki Kobayashi[a,b*].

[a]*Laboratory of Molecular Biology of Infectious Agents, Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan*
[b]*Central Research Center, AVSS Corporation, 1-22 Wakaba-machi, Nagasaki 852-8137, Japan.*

nobnob@nagasaki-u.ac.jp

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Influenza A viruses are responsible for annual epidemics and occasional pandemics, which cause significant morbidity and mortality. The limited protection offered by influenza vaccination, and the emergence of drug-resistant influenza strains, highlight the urgent need for the development of novel anti-influenza drugs. However, the search for antiviral substances from the library of low molecular weight chemical compounds is limited. Thus, because of their natural diversity and accessibility, plants or plant-derived materials are rapidly becoming valuable sources for the discovery and development of new antiviral drugs. In this study, crude extracts of *Aspalathus linearis,* a plant reported to have anti-HIV activity, were evaluated *in vitro* for their activity against the influenza A virus. Of the extracts tested, an alkaline extract of *Aspalathus linearis* demonstrated the strongest inhibition against influenza A virus and could also inhibit different types of influenza viruses, including Oseltamivir-resistant influenza viruses A and B. Our time course of addition studies indicated that the alkaline extract of *Aspalathus linearis* exerts its antiviral effect predominantly during the late stages of the influenza virus replication process.

Keywords: *Aspalathus linearis*, Alkaline extract, Anti-influenza activity, Natural product.

Influenza viruses are enveloped, single-stranded RNA viruses, belonging to the *Orthomyxoviridae* family. The genetic reassortment of influenza virus has resulted in the emergence of novel antigenic subtypes of the influenza virus such as 2009 H1N1 swine and 2013 H7N9 avian influenza virus, which can cause infection in humans [1a-c]. To prevent infection, annual vaccination is highly recommended. However, the relative benefits of the "flu" vaccine are significantly reduced when the antigenic component of this vaccine is miss-matched with the circulating virus [1a-e]. Consequently, the widespread use of an appropriate anti-influenza virus drug is essential to control the spread of the influenza virus. Currently, two main classes of anti-influenza drug are available for the prevention and treatment of influenza virus; the neuraminidase (NA) inhibitor and the matrix 2 protein (M2) ion channel inhibitor. However, because of the high mutation rates of influenza genomes, reports already describe the rapid and widespread emergence of resistance to the M2 ion channel inhibitor; furthermore, resistance to the NA inhibitor has also been reported for seasonal influenza virus A [for review, see 2a-b]. The emergence of such drug resistance is a major driving factor for the development of novel anti-influenza drugs.

However, the discovery of new drugs is facing significant challenges, largely due to a reduction in the number of approved new drugs, and the increasing costs associated with this practice. While low molecular libraries, based on combinational chemistry, have provided fresh hope for better success, this has generally failed to improve success rates in terms of the number of new drugs discovered [for review see 3a-b]. Due to their natural diversity and accessibility, plants and plant-derived phytochemicals are rapidly emerging into the limelight in the search for new antiviral [for review see 3c]. Consequently, the antiviral activity of traditional medicines, and their constituents, is becoming of increasing interest for study.

Aspalathus linearis is a leguminous plant belonging to the fabacea family which is native to the western-cape of South Africa. Mixtures of dried leaves and flowers from this particular plant, known commonly as Rooibos tea, have been used as traditional medicines and a common health beverage in Europe, Africa, and Japan [for review see 4a]. Traditionally, *A. linearis* is used to cure infant colic, improve appetite, reduce nervous tension and heartburn, and alleviate dermatological problems such as eczema. Its current use as a common health beverage is particularly popular because of the absence of alkaloids, particularly caffeine, and a low concentration of tannin [for review see 4b-c].

A. linearis has been reported to contain high amounts of phenols such as aspalathin, nothofagin, isoorientin, orientin, vixetin and rutin, along with a much lower content of quercetin and luteolin [5a-c]. Numerous studies have demonstrated the biological activity of *A. linearis.* Of particular note is that this plant has been reported to exhibit anti-mutagenic activity of aflatoxin B1 and 2-acetylaminofluorene in *Salmonella typhimurium* [6a], anti-cancer activity in rat liver by reducing the number of loci in liver cells [6b], and anti-rotavirus activity [6c] which was attributed to its phenol content. It has also been reported that an aqueous extract of *A. linearis* exhibits antioxidant activity which interferes with stress-reducing activity in rats in which stress was induced by immobilization [6d]. Nakano et al. further showed that the polysaccharide content of the alkaline extract of *A. linearis* could interfere with the binding of HIV-1 to MT-4 cells [7]. In addition, previous studies have reported that quercetin was able to interfere with influenza virus infection [8a-b]. In the present study, considering the emergence of novel antigenic subtypes of the influenza virus and anti-influenza drug resistance, we investigated the anti-influenza virus activity of *A. linearis* extracts *in vitro*.

Phytochemical screening of *A. linearis* against the influenza virus was carried out using extracts of 1% sodium bicarbonate (alkaline)

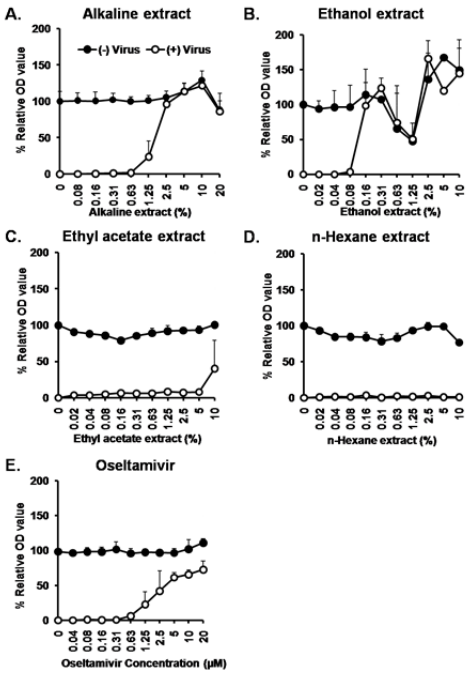

Figure 1: The effect of *Aspalathus linearis* extracts against influenza A virus infection. Evaluation of the activity of each extract against influenza A was performed as described in the experimental section. The extracts were added to MDCK cells in the absence (closed circles) or presence (open circles) of MOI 0.002 A/WSN/33 and incubated at 37°C for 72 h. The relative OD value (%) was also calculated as mentioned earlier. Data are represented as the mean ± standard error of mean from two independent experiments (each in triplicate). Extract concentration is represented as % (v/v).

solution, which was prepared as described in a previous publication [7], 70% ethanol (ethanol), ethyl acetate and n-hexane solvent, as described in the experimental section. The cellular toxicity and anti-influenza activity of *A. linearis* extracts were evaluated using the crystal violet (CV) method which represents a quick and simple technique for the quantification of cell death. This method uses Madin-darby canine kidney (MDCK) cells which undergo cell death following infection with the influenza virus; they then lose their adherence and are subsequently detached from the culture dish, thus reducing the amount of crystal violet staining. Inhibition of virus-induced cell death prevents cells from detachment, leading to high levels of crystal violet staining when compared to negative controls.

The antiviral activity of *A. linearis* extracts against influenza virus A/WSN/33 was tested using a multiplicity of infection (MOI) of 0.002. Data showed that alkaline and ethanol extracts could inhibit influenza virus A/WSN/33 infection with an IC_{50} of 1.6 ± 0.2% and 0.2 ± 0.1%, respectively (Figure 1). In addition, the second peak observed at 2.5% concentration of ethanol extract (Figure 1B) was caused by cellular toxicity. The cellular toxicity of *A. linearis* extracts was also determined; the CC_{50} of the alkaline was 34.6 ± 8.0 % (Supplementary data 1) and ethanol extracts was 1.3 ± 0.6%, respectively. However, no toxicity was observed when cells were treated with ethyl acetate and n-hexane extracts.

Table 1: Anti-influenza A virus activity of *Aspalathus linearis* extracts.

Extraction	Anti IFV A/WSN/33 activity		
	IC_{50} (%)[a]	CC_{50} (%)[b]	SI[c]
Alkaline	1.6 ± 0.2	34.6 ± 8.0	21.9
Ethanol	0.2 ± 0.1	1.3 ± 0.6	7
Ethyl acetate	-	> 10	-
n-Hexane	-	> 10	-
Oseltamivir	3.0 ± 0.0[d]	>1000[d]	>329

[a]IC_{50} is 50% inhibitory concentration of extract.[b]CC_{50} is 50% cytotoxic concentration of extract. [c]SI is selectivity index which is the ratio of CC_{50} to IC_{50}.[d]Oseltamivir unit is μM. All data are represented as mean ± standard error of mean of two independent experiments (each in triplicate).

The alkaline extract of *A. linearis,* which possessed a high IC_{50} value, exhibited less cellular toxicity compared to the ethanol extract, resulting in a higher selectivity index value (SI), (Table 1). In the present study, the alkaline extract of *A. linearis* possessed an IC_{50} of 1.6 ± 0.2 % (v/v) with an SI value of 21.9. The alkaline extract of *A. linearis* was duly selected for further evaluation due to its favorable SI value. The alkaline extract of *A. linearis* also exhibited dose dependent activity against A/WSN/33 MOI 0.001 (IC_{50}= 1.4 ± 0.8 %) and MOI 0.1 (IC_{50}= 2.7± 1.4%) infection (data not shown). These results suggest that the alkaline extract of *A. linearis* had potent anti-influenza activity.

We further evaluated the antiviral activity of alkaline extract against different types of human influenza viruses in order to determine its spectrum of activity. For this purpose, a set of laboratory-adapted influenza viruses (A/PR/8/34, B/Brisbane/60/2008, B/Lee/40) and clinical isolates (A/Nagasaki/HA-6/2009, A/Nagasaki/HA-16/2009, A/Nagasaki/HA-33/2009, A/Nagasaki/HA-58/2009 Oseltamivir-resistant, B/Okinawa/4/2014 Oseltamivir-resistant, B/Okinawa/6/2014, B/Okinawa/7/2014 Oseltamivir-resistant, B/Okinawa/8/2014) were examined. These experiments showed that the alkaline extract of *A. linearis* was effective against all of the influenza A and B viruses tested. More importantly, the extract could also inhibit all clinical isolates of the influenza virus including Oseltamivir-resistant virus (Table 2). These results suggested that the *A. linearis* extract had a broad spectrum of antiviral activity.

Table 2: Activity of alkalineextract against different types of influenza virus.

	Influenza virus	IC_{50} (%)	SI[a]
Oseltamivir-Sensitive	A/Nagasaki/HA-6/2009	0.3 ± 0.0	134.6
	A/Nagasaki/HA-16/2009	1.3 ± 0.7	31.3
	A/Nagasaki/HA-33/2009	1.2 ± 0.6	32.4
	A/PR/8/34	0.5 ± 0.1	102.9
	A/WSN/33	1.6 ± 0.2	21.9
	B/Brisbane/60/2008	3.2 ± 0.0	13.5
	B/Okinawa/6/2014	3.0 ± 1.2	16.7
	B/Okinawa/8/2014	4.7 ± 0.8	12.8
	B/Lee/40	3.1 ± 0.3	17.3
Oseltamivir-Resistant	A/Nagasaki/HA-58/2009	0.9 ± 0.0	43.8
	B/Okinawa/4/2014	3.8 ± 0.7	12.4
	B/Okinawa/7/2014	3.2 ± 0.5	14.5

[a]SI was calculated as ratio of CC_{50} from table 1 to each IC_{50} value. All data are represented as mean ± standard error of mean of two independent experiments (each in triplicate).

Oseltamivir-resistant viruses have rapidly emerged across the world for both influenza viruses A and B [for review see 2a-b]. In the current study, the alkaline extract of *A. linearis* showed an inhibitory effect against three clinical isolates which had previously proved resistant to Oseltamivir: A/Nagasaki/HA-58/2009 which harbors the H257Y mutation in the NA glycoprotein [11] and B/Okinawa/4/2009 and B/Okinawa/7/2009 [12b], (Table 2). The ability of the alkaline extract to inhibit Oseltamivir-resistant virus is suggestive evidence that *A. linearis* would be a potential source for development as a novel anti-influenza therapeutic.

To characterize the point within the influenza virus infection cycle which is inhibited by the alkaline extract of *A. linearis*, it was necessary to perform a time course addition study, as reported previously [8a, 12] with some modifications. Five percent of alkaline extract, or 20 μM of Oseltamivir, was added into the cells at different time points after virus inoculation at a MOI of 0.001, as depicted in Figure 2.The viral yield from treatment, of after infection, was then compared to treatment during infection and Oseltamivir was used as a control drug. This showed that the addition of alkaline extract at 0 – 12 h could not significantly reduce the relative virus yield when compared to treatment during

infection. In contrast, there was a significant reduction in the yield of virus ($p < 0.05$) when the alkaline extract was added 9 – 12 h after infection (Table 3). As a control, treatment with 20 µM of Oseltamivir, which can inhibit NA activity during the later stages of influenza infection, at 0 – 12 h and 9 – 12 h, was shown to significantly reduce the yield of virus. These results indicated that the alkaline extract had the ability to inhibit the influenza virus at late stages of the viral life cycle.

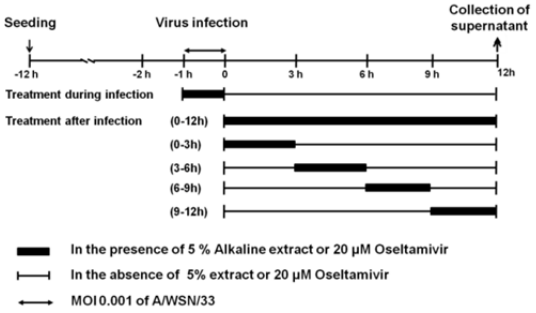

Figure 2: Time-of-addition assay used to identify the steps of the influenza A virus life cycle which can be inhibited by alkaline extract. A 5% alkaline extract or 20 µM of Oseltamivir was added into infected MDCK cells during infection or at different points of time after infection.

A previous report [7], which examined the antiviral activity of the alkaline extract of *A. linearis* against HIV-1 infection, concluded that the active component of this extract, in this particular instance, was polysaccharide. On the other hand, the active constituent of the alkaline extract of *A. linearis,* which is responsible for inhibiting influenza virus infection, is presently unknown. However, since anti-influenza virus activity could only be observed in ethanol and alkaline extracts (Figure 1), the phenolic content present in the alkaline extract could represent a promising candidate for the active compound [5c, 9a-c]. In addition, Nakano et al. mentioned that the alkaline extract of *A. linearis* contains uronic acid, which was also reported by Mann et al., which is effective in inhibiting influenza sialidase [7, 13]. Our present results suggested that the alkaline extract of *A. linearis,* extracted using the same method as that described by Nakano et al. [7], has potent inhibitory effects against the influenza virus at late stages of infection. Further studies are now needed to determine the active compound(s) involved and their precise mechanism of action.

Table 3: Relative virus yield of time of addition assay.

	Relative virus yield (%)[a]	
	5% Alkaline extract	20 µM Oseltamivir
During infection	100	100
0 - 12 hpi[b]	28 ± 17.7	2 ± 1.6*
0 - 3 hpi	>100	49 ± 24
3 - 6 hpi	>100	>100
6 - 9 hpi	61 ± 21	51 ± 28
9 - 12 hpi	7 ± 1.4*	1 ± 0.8*

[a]Relative virus yield was calculated as percentage ratio of treatment after infection to treatment during infection. [b]hpi is abbreviation of hours post infection. *The asterisks indicate the significant difference between treatment during infection and different time course treatment after infection, $p<0.05$.

Experimental

Cells, viruses and samples: MDCK cells were grown and maintained in Eagle's minimum essential medium (MEM), containing 100 units/ml of penicillin G, 100 µg/ml of streptomycin sulfate and supplemented by 5% fetal bovine serum (FBS), at 37ºC in 5% CO_2 atmosphere. The influenza A/Wisconsin/33 (A/WSN/33) was then propagated in MDCK cells. The influenza A/Puerto Rico/8/34 (A/PR/8/34) was propagated in MDCK cells in the presence of 2.5 µg/ml trypsin (Sigma-Aldrich Co., St. Louis, MO) and the culture supernatants were stored at -80°C. The clinical isolates of influenza virus from 2009 seasonal patient in Japan (A/Nagasaki/HA-6/2009, A/Nagasaki/HA-16/2009, A/Nagasaki/HA-33/2009, and A/Nagasaki/HA-58/2009 Oseltamivir-resistant) were propagated in a ten day-old embryonic egg for 48 h and then allantoic fluid was collected and stored at -80°C. The laboratory-adapted strains of influenza B/Brisbane/60/2008, B/Lee/40 and clinical isolates of influenza B virus from Japan (B/Okinawa/4/2014 Oseltamivir-resistant, B/Okinawa/6/2014, B/Okinawa/7/2014 Oseltamivir-resistant, and B/Okinawa/8/2014) were also propagated in MDCK cells and culture supernatants stored at -80°C. Viral titer was determined by calculating the 50% tissue culture infectivity dose ($TCID_{50}$). Oseltamivir sulfate was purchased from F. Hoffmann-La Roche. Ltd (Basel, Switzerland) and dissolved in ultrapure water to a concentration of 24 mM. Dried *A. linearis* leaves were purchased from All tea RTU Co. Ltd (Nagano, Japan).

Extract preparation: The 1% sodium bicarbonate extraction procedure was performed as described previously by Nakano et al. [7] with some modifications. In brief, 1 g of *A. linearis* was extracted with 10 ml of boiled ultrapure water and shaken at 85°C for 3 h. After filtration with a 0.45 µm filter, the residue was collected and further extracted in 15 ml of ultrapure water containing 1% sodium bicarbonate at 37°C for 4 h. After re-filtration using a 0.45 µm filter, the supernatant (alkaline extract) was finally stored at -80°C.

For ethanol, ethyl acetate and n-hexane extraction, 1 g of *A. linearis* was extracted with 10 ml of 70% ethanol (or ethyl acetate or n-hexane, as appropriate) and shaken for 20 h at room temperature, followed by three separate centrifugation steps at 8740 ×*g* (Beckman J2-21 centrifuge, JA-20 rotor) for 20 min. The collected supernatant was then evaporated and the pellet diluted with 1% DMSO to become ethanol (or ethyl acetate or n-hexane) extract. All extracts were stored at -80ºC to await further analysis.

Cellular toxicity and anti-influenza activity of A. linearis extracts: Cellular toxicity was evaluated using a 96 well-culture plate. Confluent MDCK cells were first seeded and incubated overnight. The cell supernatant was then aspirated and washed by serum-free MEM, followed by the addition of 100 µl of serum-free MEM containing 1% vitamins and 100 µl of serial two-fold dilution of extracts. The mixtures were then incubated at 37°C in an atmosphere containing 5% CO_2 for 3 days. The supernatant was then aspirated and the cells fixed with 70% ethanol and then stained with 0.5% CV solution. Cells were then dried and absorbance measured using an Infinite M200 Tecan plate reader. The CC_{50} was then calculated by linear regression analysis using Microsoft Excel software.

The anti-influenza assay was carried out using a 96 well-culture plate. Confluent MDCK cells were seeded and incubated at 37°C overnight. The supernatant was then aspirated and washed with serum-free MEM, followed by the addition of 100 µl serial dilutions of extracts and 100 µl of influenza virus A (A/PR/8/34 or A/WSN/33 or A/Nagasaki/HA-6/2009 or A/Nagasaki/HA-16/2009 or A/Nagasaki/HA-33/2009 or A/Nagasaki/HA-58/2009 Oseltamivir- resistant) or influenza virus B (B/Brisbane/60/2008 or B/Lee/40 or B/Okinawa/4/2014 Oseltamivir-resistant or B/Okinawa/6/2014 or B/Okinawa/7/2014 Oseltamivir-resistant or B/Okinawa/8/2014) solution equivalent to a MOI of 0.002. The mixtures were then incubated at 37°C in an atmosphere containing 5% CO_2 for 3 days. The supernatant was then aspirated and the cells fixed with 70% ethanol and stained with 0.5% CV. Cells were dried and absorbance measured as described above. The relative optical

density (OD) value was calculated from the ratio of absorbance in the presence of virus to absorbance in the absence of virus. The IC_{50} was then calculated by linear regression analysis using Microsoft Excel software. The SI was then determined by the ratio of CC_{50} to IC_{50} values.

The time-of-addition assay: MDCK cells were seeded at a concentration of 2×10^5 cells per well into 24 well-culture plates and incubated overnight. The cell monolayer was then infected with A/WSN/33 at MOI 0.001 and treated with a 5% alkaline extract of *A. linearis*, or 20 µM of Oseltamivir at different times of addition as follows: a) treatment during infection: 100 µl of 5% alkaline extract of *A. linearis* or 20 µM of Oseltamivir and virus equal to MOI 0.001 were added to the cells simultaneously and incubated at 37°C for 1 h. Unattached viruses were removed and the cells washed twice with MEM followed by the addition of 1 ml of MEM; b) treatment after infection: cells were infected by 100 µl of virus equal to MOI 0.001 at 37°C for 1 h. Unattached viruses were removed and cells washed twice with MEM. Following infection, 1 ml of MEM containing 5% alkaline extract of *A. linearis*, or 20 µM of Oseltamivir, were added at different time periods, as shown in Figure 2. Twelve hours after infection, the supernatant from different time points of alkaline extract or Oseltamivir treatment were collected and subjected to viral titration using the 50 % tissue culture infectious dose assay. The viral titer was then calculated using the Reed-Muench method. The relative viral yield value was calculated as the percentage ratio of treatment after infection relative to treatment during infection.

Statistical analysis: Data are represented as the mean ± standard error of the mean from two independent experiments (each in triplicate). The comparison of test samples and controls was performed using the student's *t* test. A p value < 0.05 was considered as statistically significant.

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