韓藥作誌(Korean J. Medicinal Crop Sci.) 16(4) : 273 – 278 (2008)

인플루엔자 바이러스에 대한 *Zanthoxylum*속의 항바이러스 효과 검정

최화정* · 송재형** · 권두한* · 백승화** · 안용준***†

*한국생명공학연구원 천연물의약연구센터, **원광대학교 한의학전문대학원, ***서울대학교 농생명과학대학

Antiviral Activity of *Zanthoxylum* Species against Influenza Virus

Hwa Jung Choi*, Jae Hyoung Song, Dur Han Kwon*, Seung Hwa Baek**, and Young Joon Ahn***†**

*Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Korea.
**Department of Herbal Resources, Professional Graduate School of Oriental Medicine, Wonkwang University, Iksan 570-749, Korea.
***School of Agricultural Biotechnology, Seoul National University, Seoul 151–921, Korea.

ABSTRACT : We aimed to investigate the antiviral activity of *Zanthoxylum* species against influenza virus A/WS/33, A/PR/8 and B/Lee/40 used by sulforhodamine B (SRB) assay and the action of leaves extracts of *Zanthoxylum piperitum* on life cycle of influenza virus A/WS/33. Among the twelve extracts, only the leaf extract of *Z. piperitum* exhibited strong antiviral activity at low concentration of less than 10 ㎍/㎖ with no citotoxicity (50 ㎍/㎖) against all of three viruses. In addition, only oseltamivir showed antiviral activity with IC_{50} of 65.3 ㎍/㎖ against influenza A/WS/33 among the viruses. Furthermore, the leaf extract of *Z. piperitum* suppressed infection of influenza virus A/WS/33, when added just prior (–1 hr) or after virus inoculation (0 hr). Leaf extract of *Z. piperitum* directly affect the infectivity of influenza virus A/WS/33 particles. Therefore, Leaf extract of *Z. piperitum* exhibited higher antiviral activity against three influenza viruses than that of the oseltamivir, which directly interacts with influenza A/WS/33 particles, affecting the initial stages of infection such as receptor binding and virus entry.

Key Words : Influenza virus, *Zanthoxylum piperitum*, Oseltamivir, Cytotoxicity, Antiviral activity

INTRODUCTION

Highly pathogenic RNA viruses can be defined as those causing contagious or transmissible animal/or human diseases. Such diseases are characterized by the potential for severe and rapid transmission, irrespective of national borders, resulting in serious socio-economic and public health consequences. Influenza viruses infect humans and several animal species, causing respiratory complications with high morbidity and mortality rates (Webster *et al*., 1992). Two commercially available neuraminidase inhibitors, inhaled zanamivir and oral oseltamivir, have demonstrated clinical benefits in the prevention and treatment of seasonal influenza infections (Abed & Boivin, 2006; Moscona, 2006). However, their utility has been limited by side-effects and the emergence of resistant viral strains (Gubareva *et al*., 1998; Kiso *et al*., 2004; Le *et al*., 2005).

Medicinal plants have a variety of chemical constituents, which have the ability to inhibit the replication cycle of various types of DNA or RNA viruses, compounds from that are increasingly being investigated for their ability to combat viral infections, with successful cases being recorded for the treatment of Hepatitis viruses in animals and humans (Thyagarajan *et al*., 1988; Thyagarajan *et al*., 1990; Venkateswaran *et al*., 1987).

As many viruses are not yet curable, the development of safe, effective and inexpensive antiviral drugs are among top global health priorities. This includes the influenza virus. Many research groups in Asia, Europe and America have paid particular attention to the development of antiviral agents from traditional plant medicines (Jassim & Naji, 2003). The *Zanthoxylum* species has been used for centuries as source of spices in Asian cuisine and traditional Asian medicine (Bryant & Mezine, 1999; Cho *et al*., 2003;

†Corresponding author: (Phone) +82-2-880-4702 (E-mail) yjahn@snu.ac.kr
Received March 17, 2008 / Revised April 20, 2008 / Accepted July 31, 2008

Shibata *et al*., 1999). Species within this genus are generally characterized by the presence of compounds such as alkaloids, lignans, and coumarins, and known to have many benefits such as antibiotic and anti-inflammatory effects, antioxidative abilities, and antimalarial activities (Hisatomi *et al*., 2000; Matu & Staden, 2003; Talapatra *et al*., 1973; Weenen *et al*., 1990).

The root bark and stems of one species within this genus, *Z. schinifolium*, have been reported to exhibit antiviral activity against Hepatitis B Virus (HBV) (Chen *et al*., 1997). In this study, we investigated the antiviral activity of four plants from the *Zanthoxylum* species against influenza viruses A/WS/33, A/PR/8 and B/Lee/40 via sulforhodamine B (SRB) assay, and the action of leaf extract of *Z. piperitum* on the life cycle of influenza virus A/WS/33.

MATERIALS AND METHODS

1. Virus, Cell line and Reagents Preparations

Influenza virus A/WS/33, A/PR/8 and B/Lee/40 were kindly provided by ATCC (American Type Culture Collection, Manassas, VA, USA) and were propagated in Madin-Darby canine kidney (MDCK) cells at 37℃. MDCK cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 0.01% antibiotic-antimycotic. Antibiotic-antimycotic, trypsin-EDTA, FBS and MEM were supplied by Gibco BRL (Grand Island, NY, USA). The tissue culture plates were purchased from Falcon (BD Biosciences, NJ, USA). Sulforhodamine B (SRB) was purchased Sigma-Aldrich (St. Louis, MO, USA). Oseltamivir (F. Hofmann-La Roche Ltd, Switzerland) were purchased from a pharmacy in Korea as prescribed by a medical doctor. All other chemicals were a reagent grade.

2. Plant and Sample Preparations

Four plants, *Zanthoxylum piperitum, Zanthoxylum schinifolium*, *Zanthoxylum coreanum*, and *Zanthoxylum planispinum* were collected from Mt. Gwanggyo (Suwon, Korea), air-dried in room temperature and pulverized. Each 100 g sample of the specimen plants was extracted twice with 600 ㎖ of methanol at room temperature for 3 days and filtered (Whatman No. 2). The combined filtrate was concentrated to dryness by rotary evaporation at 40℃. Each extract was solublized in 1 ㎖ DMSO and stored at −20℃.

3. Cytotoxicity and Antiviral Assay

MDCD (Madin-Darby canine kidney) cells grown to confluence in 96-well plates were exposed to different concentrations of the antiviral compounds (thee wells per compound concentration) in maintenance medium for 2 days at 37℃, in parallel with the virus-infected cell cultures. For each antiviral compound, three wells were used as controls (non-drug-treated cells). After 2 days of incubation, cytotoxicity was evaluated via SRB assay as previously described (Lin *et al*., 1999). The concentration of antiviral compound that reduced the viability of MDCD cells to 50% of the control was estimated as the 50% cytotoxic concentration (CC_{50}). To calculate the CC_{50} values, the results were transformed to percentage of controls and the CC_{50} values were graphically obtained from the dose-response curves (Microsoft Office, 2003).

Antiviral activity against influenza viruses were determined via SRB assay, as mentioned above. Infectivity of virus stock was determined as infectivity of the virus by SRB ID_{50} (50% infective dose). MDCD cells in 96-well tissue culture plates were used when confluent. Culture medium was removed and the cells were washed with 1 × phosphate buffered saline (PBS). Following this, 0.09 ㎖ of the diluted virus suspension containing $CCID_{50}$ (50% cell culture infective dose) of the virus stock were used to produce an appropriate cytopathic effects within 2 days after infection and 0.01 ㎖ of medium supplemented with trypsin-EDTA containing an appropriate concentration of the antiviral compound. The antiviral activity of each test material was determined with four concentrations ranging from 0.1 to 100 ㎍/㎖. A ten-fold dilution scheme for each compound was employed. Three wells were used as virus controls (virus-infected non-drug-treated cells) while three wells were used as cell controls (non-infected non-drug-treated cells). The culture plates were incubated at 37℃ in 5% CO_2 for 2 days and observed for appropriate cytopathic effects (CPE). Subsequently, the 96-well plates washed 1 times with 1 × PBS, while 100 ㎕ of cold (−20℃) 70% acetone were added to the top of each well and left for about 30 min at −20℃. After 70% acetone was removed, 96-well plates were left to dry in an oven for 30 min. 100 ㎕ of 0.4% (w/v) SRB in 1% acetic acid solution were added to each well and left at room temperature for 30 min. SRB were removed and the plates washed 5 times with 1% acetic acid before oven drying. 96-well plates were then left to dry in

oven for at least 24 hrs. Bound SRB was solubilized with 100 ㎕ of 10 mM unbuffered Tris-base solution and plates were left on a table for at least 30 min. Absorbance was read in a 96-well plate reader at 562 ㎚ subtracting the background measurement at 620 ㎚. To calculate the IC_{50} values, the results were transformed to percentage of controls and the IC_{50} values were graphically obtained from the dose-response curves (Microsoft Office, 2003). The percent protection achieved by the test compound in influenza virus A/WS-infected cells was calculated by the following formula:

$$\frac{(OD_t)_{\text{influenza virus}} - (OD_c)_{\text{influenza virus}}}{(OD_c)_{\text{mock}} - (OD_c)_{\text{influenza virus}}} \times 100 \text{ (Expressed in \%)}$$

where $(OD_t)_{\text{influenza virus}}$ is the optical density measured with a given concentration of the test compound in influenza virus-infected cells; $(OD_c)_{\text{influenza virus}}$ is the optical density measured for the control untreated influenza virus-infected cells; and $(OD_c)_{\text{mock}}$ is the optical density measured for control untreated mock-infected cells. The concentration achieving 50% protection according to the above formula was defined as the 50% inhibitory concentration (IC_{50}). The therapeutic index was defined as CC_{50}/IC_{50}.

4. Analysis of influenza virus A/WS/33 infectivity with *Z. piperitum* Leaves Extract

Influenza virus A/WS/33 was pre-incubated with 20 ㎍/㎖ leaves extract of *Z. piperitum* for 1 hr at 4℃. MDCD cells were infected with pretreated or untreated influenza virus A/WS for 1 hr. Unbound virus was washed away and cells were incubated in infection medium supplemented with 20 ㎍/㎖ leaf extract of *Z. piperitum* at 37℃. Antiviral activity was determined by SRB assay after 48 hrs.

5. Study of Time Course

The time-of-addition effect of *Z. piperitum* leaves extract was examined according to previously described procedures with minor modifications (Chiang *et al*., 2003). MDCK cells were seeded onto 96-well culture plates (Falcon; BD Biosciences, NJ, USA) at density of 2×10^4 cells per well and incubated for 24 hrs to reach at least 95% confluency. Ten ㎍/㎖ of *Z. piperitum* leaf extract of was then added onto the cells at either before (−1 hr), during (0 hr) or after (1, 2, 4, 6, 8, 12 and 24 hrs) the period of influenza virus A/WS infection. Following the antiviral assay, cells were washed twice by 1 × PBS to eliminate the compound prior to the inoculation of virus for the pre-infection (−1 hr) group, or the compound were added at different times for co-infection (0 hr) and post-infection (1, 2, 4, 6, 8, 12 and 24 hrs) groups. After 2 days, SRB assay test and antiviral activity were carried out as described above.

RESULTS

1. Cytotoxicity and Antiviral Activity of Specimen Extracts against Three Influenza Viruses

We used sulforhodamine B (SRB) assay in order to determine the cytotoxicity of test plant extracts. Oseltamivir exhibited no cytotoxicity at concentration of 50 ㎍/㎖ (Table 1). Only bark extract of *Z. schinifolium* and root extract of *Z. coreanum* exhibited ctytoxicity at concentration of 50 ㎍/㎖ (Table 1).

The antiviral drug, oseltamivir was used as a positive control, while extracts were tested for their inhibitory activity against influenza virus type A/WS/33. Oseltamivir showed weak antiviral activity with IC_{50} of 65.3 ㎍/㎖ against influenza A/WS/33 (Table 1). Leaf extract of *Z. piperitum,* bark extract of *Z. coreanum,* stems, and fruit and leaf extract of *Z. planispinum* exhibited anti-influenza virus A/WS/33 activity with IC_{50} of 8.89, 93.6, 99.6, 8.88, 8.18 ㎍/㎖, respectively. Most notably, leaf extract of *Z. piperitum,* and fruit and leaf extract of *Z. planispinum* exhibited strong antiviral activity at less than 10 ㎍/㎖ (Table 1). Also, leaf extract of *Z. piperitum,* bark extract of *Zanthoxylum coreanum,* and stem and leaf extract of *Z. planispinum* exhibited anti-influenza virus A/PR/8 activity with IC_{50} of 9.28, 96.1, 85.52, 9.02 ㎍/㎖, respectively. Oseltamivir did exhibit antiviral activity of less than 50% at concentration of 100 ㎍/㎖ against influenza virus A/PR/8 (Table 1). Leaf extract of *Z. piperitum* and *Z. planispinum* exhibited strong antiviral activity at concentrations of less than 10 ㎍/㎖ (Table 1). Furthermore, oseltamivir did exhibit antiviral activity of less than 50% at concentration of 100 ㎍/㎖ against influenza virus B/Lee/40 (Table 1). Leaf extract of *Z. piperitum,* and fruit extract of *Z. planispinum* exhibited anti-influenza virus B/Lee/40 activity with IC_{50} of 0.44 and 6.24 ㎍/㎖, respectively. Leaf extract of *Z. piperitum* and fruit extract of *Z. planispinum* exhibited strong antiviral activity at concentration of less than 10 ㎍/㎖ (Table 1).

Table 1. Antiviral activity of *Zanthoxylum* species against influenza viruses in MDCK cells.

Plant species	Tissue sampled	CC_{50}[a]	A/WS IC_{50}[b]	A/WS TI[c]	A/PR IC_{50}[b]	A/PR TI[c]	B/Lee IC_{50}[a]	B/Lee TI[c]
Zanthoxylum piperitum	Roots	718.0	N.D.[d]	–	N.D.[d]	–	N.D.[d]	–
	Hearts	> 100	N.D.[d]	–	N.D.[d]	–	N.D.[d]	–
	Leaves	88.7	8.89±0.26	9.99	9.28±0.22	9.56	0.44±0.06	201.59
Zanthoxylum schinifolium	Barks	54.9	N.D.[d]	–	N.D.[d]	–	N.D.[d]	–
	Leaves	73.6	N.D.[d]	–	N.D.[d]	–	N.D.[d]	–
Zanthoxylum coreanum	Leaves	> 100	N.D.[d]	–	N.D.[d]	–	N.D.[d]	–
	Barks	1391.0	93.6±0.44	14.9	96.1±4.13	14.47	N.D.[d]	–
	Roots	41.2	N.D.[d]	–	N.D.[d]	–	N.D.[d]	–
Zanthoxylum planispinum	Stems	300	99.60±0.44	3.01	85.52±7.49	3.51	N.D.[d]	–
	Barks	416.0	N.D.[d]	–	N.D.[d]	–	N.D.[d]	–
	Fruits	98.6	8.88±0.32	11.1	N.D.[d]	–	6.24±0.92	15.80
	Leaves	72.1	8.18±1.47	8.82	9.02±0.18	7.88	N.D.[d]	–
Oseltamivir		178.98	65.3±15.2	2.74	N.D.[d]	–	N.D.[d]	–

[a] The 50% cytotoxic concentration for target cells (MDCK cells) in ㎍/㎖.
[b] Concentration of compound in ㎍/㎖ producing 50% inhibition of virus-induced cytopathic effects.
[c] Therapeutic index (TI) = CC_{50}/ IC_{50}.
[d] IC_{50} value within concentration of compound to test not calculated because maximum inhibition rate was arrived under 50%.

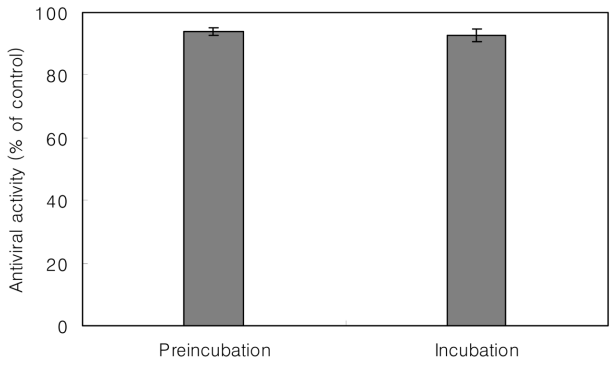

Fig. 1. The effects *Zanthoxylum piperitum* leaves extract on the infectivity of influenza virus A/WS particles. Influenza virus A/WS particles were incubated with 20 ㎍/㎖ of leaf extract at 4℃ for 1 hr. Subsequently, Vero cells were incubated with treated or untreated virus extracts at 4℃ for 1 hr. Unbound virus was removed by extensive washing and infection was continued by cultivating cells in infection medium without leaf extract at 37℃. Antiviral activity was determined by titration using SRB assays 2 days post-infection.

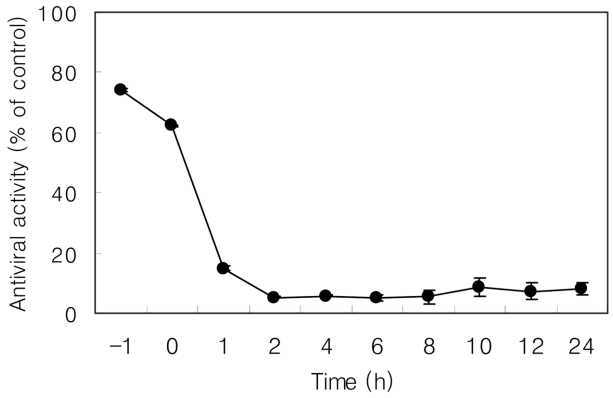

Fig. 2. The time course of *Zanthoxylum piperitum* leaves extract addition on influenza virus A/WS replication in Vero cells. Ten ㎍/㎖ of each compound was added at either before (−1 hr), during (0 hr), or after (1, 2, 4, 6, 8, 12 and 24 hrs) virus infection. After 2 days, inhibition was evaluated by SRB method and expressed as the inhibition rate. Each value is the result of mean ± S.D. of thee independent experiments.

2. Analysis of influenza virus A/WS/33 infectivity with *Z. piperitum* Leaves Extract

Analysis was performed to ascertain whether leaf extract of *Z. piperitum* interact with the viral particle, Antiviral activity of pre-incubation with leaf extract of *Z. piperitum* resulted in 94%. Similarly, continuous presence of the extract during infection led to 93% antiviral activity (Fig. 1).

3. Study of Time Course

To study the inhibitory effect of *Z. piperitum* leaf extract, which possess a broad spectrum of antiviral activities on the stages of virus infection, the compounds were added at different periods (before, during, and after) of influenza virus A/WS/33 infection. Results showed that the leaf extract suppressed influenza virus A/WS/33 infection, when

added just prior (−1 hr) or after the virus inoculation (0 hr). The inhibitory rate was higher than 60% (Fig. 2). However, the inhibitory rates declined to 15% or less when added at post (1, 2, 4, 6, 8, 12 and 24 hrs) infection (Fig. 2).

DISCUSSION

Influenza viruses are classified as types A, B, and C. The gradual antigenic drift of influenza virus types A and B, coupled with occasional antigenic shifts makes the control of infections problematic (Shaw *et al*., 1992). Only two classes of anti-influenza virus drugs are currently accessible. These are viral neuraminidase inhibitors (oseltamivir and zanamivir) and viral M_2 ion channel protein inhibitors (amantadine, rimantadine) (De Clercq, 2004). The viral M_2 ion channel protein inhibitors and neuraminidase inhibitors are not fully effective due to the frequent emergence of drug-resistant viral variants and their lack of activity against the influenza B virus (Betakova *et al*., 1996; Gubareva *et al*., 2001; Thompson *et al*., 2004; Stiver, 2003). As a result, there is an urgent need for broadspectrum antiviral drugs that exert inhibitory effects against currently active human viruses.

The objective of this study was to determine whether methanol extracts of different plant tissues would function as an antiviral agent against influenza A or B virus in vitro. Subsequently, we also attempted to elucidate the ability of *Z. piperitum* leaf extract on influenza virus multiplication. Oseltamivir, used for treatment of flu, exhibited weak antiviral activity against influenza virus A/WS/33 and no activity against influenza virus A/PR/8 and B/Lee/40. Among the specimen plant extract, only leaf extract of *Z. piperitum* exhibited antiviral activity against influenza viruses A/WS/33, A/PR/8 and B/Lee/40, higher than that of oseltamivir. Our results showed that leaf extract of *Z. piperitum* inhibit the growth of influenza strains A/WS/33, A/PR/8 and B/Lee/40 with differences in inhibitory efficacy among the strains.

To elucidate the ability of *Z. piperitum* leaf extract on influenza virus A/WS multiplication, we closely examined actions during the infection cycle. We demonstrated that leaf extract affected the early stages of viral infection cycle, such as receptor binding, virus entry, and internalization. Based on these result, it can be concluded that compounds in the leaf extract of *Z. piperitum* do interact with the particle of influenza virus A/WS/33, as pre-exposure did alter the infectivity of the influenza virus (Fig. 1). Based on the results of the time-of-addition, pre-incubation of the MDCK cells with *Z. piperitum* leaf extract followed by washing did protect the cells from A/WS/33 infection (Fig. 2). Furthermore, the extract can only inhibit A/WS infection when added on, pretreatment (−1 hr), and during virus inoculation (0 hr), but not after 1 hr or later (Fig. 2). This suggests that the mode of action is deriven from inhibiting the absorption of virus (Fig. 1 and Fig. 2).

CONCLUSION

We aimed to investigate the antiviral activity of *Zanthoxylum* species against influenza virus A/WS/33, A/PR/8 and B/Lee/40 used by SRB assay and only the leaf extract of *Z. piperitum* exhibited higher antiviral activity against three influenza viruses than that of the oseltamivir, which directly interacts with influenza A/WS/33 particles, affecting the initial stages of infection such as receptor binding and virus entry.

LITERATURE CITED

Abed Y, Boivin G (2006) Treatment of respiratory virus infections. Antiviral Res. 70:1-16.

Betakova T, Nermut MV, Hay AJ (1996) The NB protein is an integral component of the membrane of influenza B virus. J. Gen. Virol. 77:2689-2694.

Bryant BP, Mezine I (1999) Alkylamides that produce tingling paresthesia activate tactile and thermal trigeminal neurons. Brain Res. 842:452-460.

Chiang LC, Chiang W, Liu MC, Lin CC (2003) In vitro antiviral activities of *Caesalpinia pulcherrima* and its related flavonoids. J. Antimicrob. Chemother. 52:194-198.

Chen IS, Lin YC, Tsai IL, Chen IS (1997) Coumarins and anti-HBV constituents from *Zanthoxylum schinifolium*. Phytochem. 45:1419-1422.

Cho MG, Chang CS, Chae YA (2003) Variation of volatile composition in the leaf of *Zanthoxylum schinofolium* siebold et zucc. and *Zanthoxylum piperitum* DC. Korean J. Medicinal Crop Sci. 10:162-166.

De Clercq E (2004) Antiviral drugs in current clinical use. J. Clin. Virol. 30:115-133.

Gubareva LV, Matrosovich MN, Brenner MK, Bethell RC, Webster RG (1998) Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus. J. Infect. Dis. 178:1257-1262.

Gubareva LV, Webster RG, Hayden FG (2001) Comparison of the activities of zanamivir, oseltamivir, and RWJ-270201 against clinical isolates of influenza virus and neuraminidase

inhibitor-resistant variants. Antimicrob. Agents Chemother. 45: 3403-3408.

Hisatomi E, Matsui M, Kobayashi A, Kubota K (2000) Antioxidative activity in the pericarp and seed of Japanese Pepper (*Xanthoxylum piperitum* DC). J. Agric. Food Chem. 48: 4924-4928.

Jassim SAA, Naji MA (2003) Novel antiviral agents: a medicinal plant perspective. J. Appl. Microbiol. 95:412-427.

Kiso M, Mitamura K, Sakai-Tagawa Y, Shiraishi K, Kawakami C, Kimura K, Hayden FG, Sugaya N, Kawaoka Y (2004) Resistant influenza A viruses in children treated with oseltamivir: descriptive study. Lancet. 364:759-765.

Le QM, Kiso M, Someya K, Sakai YT, Nguyen TH, Nguyen KH, Pham ND, Ngyen HH, Yamada S, Muramoto Y, Horimoto T, Takada A, Goto H, Suzuki T, Suzuki Y, Kawaoka Y (2005) Avian flu: isolation of drug-resistant H5N1 virus. Nature 437:1108.

Lin ZX, Hoult JRS, Raman A (1999) Sulforhodamine B assay for measuring proliferation of a pigmented melanocyte cell line and its application to the evaluation of crude drugs used in the treatment of vitiligo. J. Ethnopharmacol. 66:141-150.

Matu EN, Staden J (2003) Antibacterial and anti-inflammatory activities of some plants used for medicinal purposes in Kenya. J. Ethnopharmacol. 87:35-41.

Moscona A (2006) Neuraminidase inhibitors for influenza. N. Engl. J. Med. 353:1363-1373.

Shaw MW, Arden NH, Maassab HF (1992) New aspects of influenza viruses. Clin. Microbiol. Rev. 1:74-92.

Shibata CI, Sasaki H, Naito TU, Matsuno S (1999) The herbal medicine Dai-Kenchu-Tou stimulates upper gut motility though cholinergic and 5-hydroxytryptamine 3 receptors in conscious dogs. Surg. 126:918-924.

Stiver G (2003) The treatment of influenza with antiviral drugs. Can. Med. Assoc. J. 168:49-56.

Talapatra SK, Dutta S, Talapatra B (1973) Alkaloids and terpenoids of *Zanthoxylum ovalifolium*. Phytochem. 12:729-730.

Thompson CI, Barclay WS, Zambon MC (2004) Changes in in vitro susceptibility of influenza A H3N2 viruses to a neuraminidase inhibitor drug during evolution in the human host. J. Antimicrob. Chemother. 53:759-765.

Thyagarajan SP, Jayaram S, Valliammai T (1990) *Phyllanthus amarus* and hepatitis B. Lancet 336:49-50.

Thyagarajan SP, Subramanian S, Thiranapasundaru T, Venkateswaran PS (1988) Effect of *Phyllanthus amarus* on chonic carriers of hepatitis B virus. Lancet. 2:764-766.

Venkateswaran PS, Millman I, Blumberg BS (1987) Effects of an extract from *Phyllanthus niruri* on hepatitis B and Woodchuck hepatitis viruses: in vitro and in vivo studies. Proc. Natl. Acad. Sci. USA 84:274-8.

Weenen H, Nkunya MHH, Bray DH, Mwasumbi LB, Kinabo LS, Kilimali VA, Wijnberg JB (1990) Antimalarial compounds containing an α, β-unsaturated carbonyl moiety from Tanzanian medicinal plants. Planta Med. 56:371-373.

Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992) Evolution and ecology of influenza A viruses. Microbiol. Rev. 56:152-179.