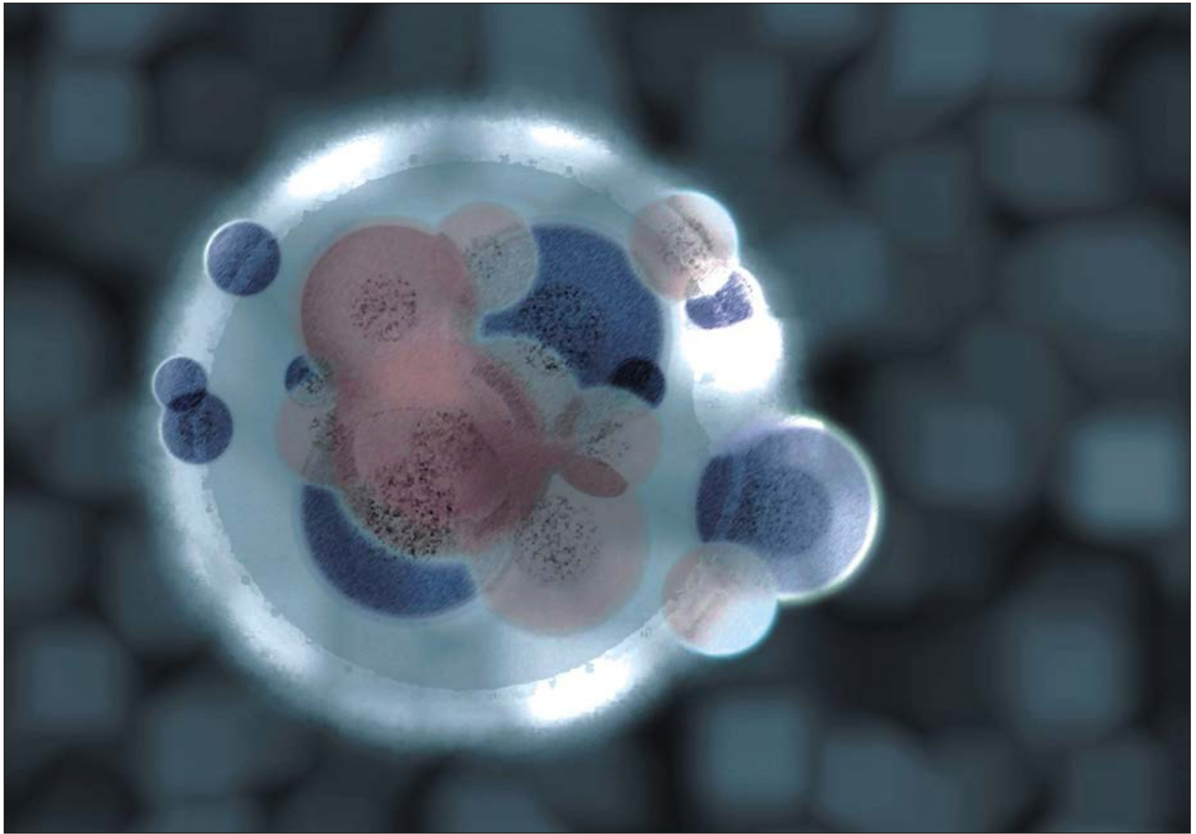

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Screening for Antiviral Activity of Sweet Lemon Grass (*Cymbopogon nardus* (L.) Rendle) Fractions

M.N. Nurul Aini, M.I. Said, I. Nazlina, M.N. Hanina and I.B. Ahmad
School of Bioscience and Biotechnology, Faculty of Science and Technology,
Universiti Kebangsaan Malaysia, Bangi, 43600, Selangor, Malaysia

Abstract: A study was carried out to test the cytotoxicity and antiviral effects of sweet lemon grass (*Cymbopogon nardus* (L.) Rendle) fractions. A total of 51 fractions were obtained after two sequential runs of flash chromatography. Further fractionation on selected fractions by preparative Thin Layer Chromatography (TLC) resulted in 40 sub fractions. The cytotoxicity towards Vero cells of the different fractions ranged from highly toxic (N3 and N29; 30 μg mL^{-1}) to considerably non toxic (N16, N17 and N19; 1 mg mL^{-1}). For antiviral activity test the cells were inoculated with 10 μL measles virus 1000 $TCID_{50}$ and treated with 1.0 LC_{50}, 0.1 LC_{50} or 0.01 LC_{50} concentrations of each fraction. Most fractions protected cell death due to measles inoculation when compared with the untreated controls, but were less effective when compared to the control guanidine hydrochloride treatment of 0.1 mg mL^{-1}. Treatment of Vero cells with fractions at 0.1 LC_{50} gave higher cell survival when compared to the other concentrations, which were 1.0 LC_{50} or 0.01 LC_{50}. At 0.1 LC_{50}, the *C. nardus* fractions were most effective when cells (C) were treated with the fractions (F) before being inoculated with the virus (V), which was treatment {(C+F) + V}. Using this method, more fractions with higher efficacy (+++) was obtained when compared with the methods {(C+V) + F} or {C + (V+F)}. Similar, treatment of cell culture with sub fractions before virus inoculations showed better antiviral effect that treatment after viral inoculations. This suggests that fractions and sub fractions were more effective in protecting cells against entry of virus particles into inoculated cells than other phases in the viral replication.

Key words: *Cymbopogon nardus*, cytotoxicity, antiviral activity, measles virus

INTRODUCTION

Measles virus infects approximately 30-40 millions people in the year 2000, causing 777,000 deaths (WHO, 2001). For the year 2003, World Health Organization estimated that more than half million people, with majority of them were children, have died of measles (WHO, 2005). The problem persists particularly in developing countries of Africa and Asia where vaccination is not efficiently carried out. The highly contagious virus continues to circulate within the population and causes infection among those lacking protective antibodies (Karp *et al*., 1996). In addition, the measles vaccine can cause problems to the recipients. For example, the vaccine has been reported to decrease lymphokin production (Ovsyannikova *et al*., 2003) or causes autism in children (Vijendra and Jensen, 2003). Therefore, under certain circumstances the use of antiviral drugs can be seen as complement and alternative to vaccination.

The use of drugs against viruses is still in the infancy. Many synthetic, semi synthetic or plant-derived drugs are being tested and found positive against viruses *in vitro*. However, the number of drugs that are effective against measles virus *in vitro* is limited and according to Wyde (1999) only α-interferon and ribavirin have been used clinically against measles virus. Grancher *et al*. (2004) reported improved *in vitro* antiviral activity of ribavirin against measles virus after complexation with cyclodextrins. Hope for further development exists when Santagati *et al*. (2003) showed that chemically synthesized isoquinoline derivatives have good anti-measles activity.

The use of plant products for alleviation of infections and diseases, including those caused by viruses has been documented in many laboratories around the world (Jassim and Naji, 2003). Preliminary studies in our laboratory showed that the crude extracts of sweet lemon grass, *Cymbopogon nardus* (L.) Rendle, were active against measles virus and Newcastle disease

Corresponding Author: M.N. Nurul Aini, School of Bioscience and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, 43600, Selangor, Malaysia

virus (Ahmad *et al.*, 1992, 1993) but not against polio virus (Ahmad *et al.*, 2003). The observations could indicate the presence of antiviral substances which are active against enveloped viruses. Recently, we further fractionated the hexane fractions and tested for its cytotoxicity against Vero cells and activity against measles virus.

MATERIALS AND METHODS

Fractionation of extract: Dried *C. nardus* leaves are minced and then extracted with hexane. The hexane extracts was run through flash chromatography using chloroform, ethyl acetate and methanol as the solvents. The fractions were then further fractionated with different ratios of chloroform-ethyl acetate mixtures to obtain fractions with decreasing polarity. The fractions were designated as N1 to N*n*. The fractions were let to dry by vacuum.

Further fractionation was done on selected fractions by preparative thin-layer chromatograpy using silica gel 60PF254 (Merck). The spots obtained on the chromatographic plates were isolated and extracted with ethyl acetate to derive new sub fractions designated as N*n*(1 to *n*).

Cytotoxicity test: Fractions and sub fractions were first reconstituted in 50 μL methanol and then further diluted with cell culture growth medium, which was Minimal Essential Medium (MEM) (Gibco) with 5% fetal bovine serum (FlowLab). Confluent Vero cells grown in 96-well microtitre plates were treated with the fractions or sub fraction that have been diluted to 1, 0.5, 0.25, 0.125 and 0.0625 mg mL^{-1}. After 48 h incubation, the microtitre plates were processed and treated with 0.3% crystal violet in 20% methanol (Schmidtke *et al.*, 2001). The amount of dye retained by each well was measured by first dissolving the dye with 100 μL cell lysis buffer and then measuring the absorbance with ELISA plate reader (Labsystems Multikan MultiSoft) at 562 nm. The fifty-percent lethal concentration (LC_{50}) values were calculated graphically according to Ahmad and Marini (1994) and Marini *et al.* (1998).

Antiviral assay: Three methods of treatments to detect antiviral activity in each of the fraction were used: (I) cells (C) were inoculated with virus (V) 1 h before treatment with fractions (F), that is (C+V) + F; (ii) virus was inoculated to cells one day after treatment with fractions, that is (C+F) + V; and (iii) the virus and fractions were added concurrently to the cells, that is C + (V+F). For the antiviral tests, the fractions were diluted at 1.0 LC_{50}, 0.1 LC_{50} and 0.01LC_{50}. The viral concentrations used for cell inoculations were fixed at 1000 $TCID_{50}$.

RESULTS AND DISCUSSION

Fractionation of sweet lemon grass: A total of 51 fractions were obtained after the second fractionation. For TLC separation, seven fractions were used, which were N6, N7, N23, N26, N28, N30 and N31. A total of 40 sub fractions were obtained with N6 having 14 sub fractions while the others were between two to six sub fractions. These sub fractions were N6(1-14), N7(1-4); N23(1-4), N26(1-6), N28(1-5); N30(1-5) and N37(1-2).

Cytotoxicity test: The cytotoxicity, measured in term of 50% lethal concentration (LC_{50}), of the different fractions ranged from highly toxic to considerably non-toxic (Table 1). For example, the highly toxic fractions N3 and N29 had LC_{50} values of 30 μg mL^{-1}, while the non-toxic fractions N16, N17 and N19 had LC_{50} values of 1 mg mL^{-1}. The determination of the LC_{50} values was important since the LC_{50} values obtained were used to calculate the actual concentration (w/v) of each fraction to be used in antiviral tests. In this study three concentrations of the same fraction or sub fraction corresponding to 1.0 LC_{50}, 0.1 LC_{50} and 0.01 LC_{50} were used.. For example, the amounts of N3 fraction corresponding to 0.1 LC_{50} and 0.01 LC_{50} values were 3 and 0.3 μg mL^{-1}, respectively, while the corresponding values for N16 were 100 and 10 μg mL^{-1}, respectively.

Varied response of cells to chemical agents have been correlated with the capability of the cells to multiply (Desselberger, 1995) and differences in cell growth rate, cell size and cell defenses (Schwobel *et al.*, 1979). A chemical which is toxic even at low level will not be a good candidate as a chemotherapeutic agent (Horvath, 1984). However, fractions which still contain many components cannot be totally ignored as there is a possibility of the active substance may be present in minute quantities amongst the more abundant toxic substances. Hence, in this study all fractions were tested for antiviral activity.

Antiviral activity: Most of the fractions tested confer some degree of protection to Vero cells against measles viral infection (Results not shown). Majority had moderate antiviral activity (++) when compared with negative control wells inoculated with virus alone (Table 2). However, most fractions showed lower cell survival when compared to the positive control treated with GHCl at 0.1 mg mL^{-1}. Generally, treatment of cells with fractions at the concentrations corresponding to 0.1 LC_{50} gave higher cell survival as compared to the other concentrations, which were 1.0 LC_{50} or 0.01 LC_{50} (results not shown). At 0.1 LC_{50} concentrations, the fractions were neither too toxic to the Vero cells as in 1.0 LC_{50} nor they were too dilute as in 0.01 LC_{50} concentration. The low

Table 1: The LC_{50} values for *C. nardus* fractions when tested on Vero cells

Fractions	LC_{50} (mg mL^{-1})	Fractions	LC_{50} (mg mL^{-1})	Fractions	LC_{50} (mg mL^{-1})
N1	Not done	N18	0.4	N35	0.2
N2	0.3	N19	1.0	N36	0.17
N3	0.03	N20	0.4	N37	0.25
N4	0.08	N21	0.3	N38	0.32
N5	0.05	N22	0.05	N39	0.16
N6	0.2	N23	0.05	N40	0.18
N7	0.4	N24	0.05	N41	0.1
N8	0.2	N25	0.05	N42	0.18
N9	0.2	N26	0.2	N43	0.2
N10	0.2	N27	0.1	N44	0.2
N11	0.2	N28	0.1	N45	0.2
N12	0.2	N29	0.03	N46	0.17
N13	0.1	N30	0.09	N47	0.19
N14	0.2	N31	0.15	N48	0.2
N15	0.2	N32	0.12	N49	0.18
N16	1.0	N33	0.18	N50	0.21
N17	1.0	N34	0.04	N51	0.19

Table 2: Fractions which show higher Vero cell survival after virus and fraction treatments at 0.1 LC_{50} concentration as compared with the negative (virus only) and positive (virus and guanidine hydrochloride) controls

Treatment methods	Comparison with Negative control[a] ++[b]	+++	Positive control ++	+++
(C+V)+F	2, 3, 4, 8, 14[c], 15, 23, 29, 30, 31, 42, 43, 45, 46, 47, 49, 50, 51	16, 17, 22, 33 33, 34, 35,	33, 34, 35, 37	None[d]
(C+F)+V	2, 3, 4, 11, 15, 18, 23, 38, 42, 45, 47	3, 5, 6, 7, 10, 12, 17, 18, 22, 25, 27, 29, 30, 33, 35, 36, 37, 41	36, 37	6, 7, 8, 11 16, 23, 26, 28, 30, 31, 34,
C + (V+F)	3, 14, 33, 37, 44, 45, 47, 49, 50	None	None	None

[a]Negative control = not treated with fractions; Positive control = treated guanidine hydrochloride (GHCl), [b]The relative efficacy of the fractions was evaluated based on the difference in absorbance between fraction-treated Vero cells with negative or positive controls. Indicators: + = 0.11-0.20; ++ = 0.21-0.30; +++ = 0.31-0.40; ++++ = 0.41-0.50, [c]The numbers refer to fractions N2, N3, N4 to N50, [d]None = no fractions having the activity

protection offered by the fractions at 0.01 LC_{50} showed that the antiviral action of the fractions was not sustained at low concentrations.

Except for method antiviral activity of the sub fractions was lost or reduced as compared to the activity shown by the fractions from which they were derived (Table 3). Although the sub fractions protect cells from viral killing of Vero cells, they perform inferior antiviral activity to positive controls, in which the cells were treated with guanidine hydrochloride. Hudson (1990) has pointed out that in order to be classified as a potentially useful antiviral agent, a compound must be able to reduce the virus titre by at least two log10 factors (that is 99%) at a non-cytotoxic concentration.

Table 3: Relative efficacy of sub fractions which show higher Vero cell survival after virus and sub fraction treatments 0.1 LC_{50} concentration as compared to the negative (virus only treatment) and positive (vims and guanidine hydrochloride) controls

Treatment methods	Comparison with Negative control[a] ++[b]	+++	Positive control ++	+++
(C+V)+F	6(1), 6(4 -5)[c] 6(10-12), 6(14) 6(14), N7(1-4), 23(1-4), 26(1-2) 26(4-6), 30(1-2)	6(2), 6(3), 6(6) 6(7-8),	None[d]	None
(C+F)+V	6(6), 6(8-11), 6(13), 7(1-4) 23(4), 28(1-2) 30(1), 30(4-5) 37(2)	6(7),23(4) 26(1), 26 (2) 28(3-5) 37(1)	None	None
C + (V+F)	None	6(1-5), 6(9-10), 7(1-4), 23(1-4), 30(5), 37(1-2)[e]	None 23(1-2),	6(1-3), 7(4),

[a]Negative control = not treated with sub fractions; Positive control = treated guanidine hydrochloride (GHCl), [b]The relative efficacy of the sub fractions was evaluated based on the difference in absorbance between fraction-treated Vero cells with negative or positive controls. Indicators: + = 0.11-0.20; ++ = 0.21-0.30; +++ = 0.31-0.40; ++++ = 0.41-0.50, [c]The numbers refer to fractions refer to sub fractions N6(1), N6(4), N6(5) etc., [d]None = no fractions having the activity, [e]A few 18 sub fractions showed antiviral activity indicated by ++++

Effect of virus treatments methods: The three treatment methods were designed to indicate whether the antiviral actions of fractions or sub fractions occur before, during or after viral entry into the cells. In the first method, cells were treated after viral inoculation, while in the second method cells were treated with fractions before viral inoculation and in the third method the virus was inactivated by the fractions before being inoculated to the cells.

At 0.1 LC_{50},, the *C. nardus* fractions were more effective when cells were treated with the fractions before being inoculated with the virus, which is {(C+F) + V} when compared with the methods {(C+V) + F} or {C + (V+F)}(Table 2). The first method of treatment where cells were treated for 24 h before viral infection {(C+F) + V} represents the capability of the test compound to protect the cells from viral attachment and hence giving us the idea of prophylactic effect of the compound. Thus there is a possibility that the main target of viral inhibition afforded by compounds found in *C. nardus* is the virus attachment step.

In contrast, the sub fractions did not give any particular trend as to the most effective method of treatment. However, with some fractions, treating the cells and the virus simultaneously, i.e. method {C + (V+F)} gave higher relative ELISA readings of ++++ when compared to the untreated controls. Simultaneous addition of virus and test compounds to cell culture represents the capability of the test compounds either to

modify cellular receptors for viral attachment or the viral attachment sites hence preventing successful attachment and penetration. It may also indicate that the sub fractions were directly affecting the virus particles. Therefore, both the fractions or sub fractions were less efficient in preventing multiplication of the virus after infection.

Replicating viruses will damage host and cell morphological changes is an indicator to viral multiplication (Desselberger, 1995). Therapy to viral diseases is aimed at three different aspects including I) enhancement of host defense mechanism ii) antiviral agent capable of inhibiting specific viral processes and iii) to relieve disease symptom (Bucknall, 1973). The second aspect is taken into account in this study using three different treatments as to give an overview of viral processes that could be the target of the compounds tested. The first treatment where the tested compounds were added after viral infection represents the capability of the compounds to modify viral attachment, penetration or replication processes.

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