KMITL Sci. Tech. J. Vol. 12 No. 1 Jan. - Jun. 2012

Antiviral Activity of Crude Hexane Extracts from *Allamanda cathartica* on the Replication of *Autographa californi*ca Multiple Nucleopolyhedrovirus

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Abstract

The purpose of this research was to investigate the antiviral activity of crude hexane extracts from the stem and leaf of *Allamanda cathartica* L. on the replication of *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV) in *Spodoptera frugiperda* cell line (Sf9). The cytotoxic effect of crude hexane extracts from *A. cathartica* on Sf9 cell line was studied using neutral red assay and the results show that the 50% cytotoxicity concentration (CC_{50}) values of crude hexane extracts from the stem and leaf of *A. cathartica* against Sf9 cells after 96 hours of exposure were 326.44 and > 1,000 µg/ml, respectively.

The effects of crude hexane extracts from the stem and leaf of *A. cathartica* at the concentration of 31.25 µg/ml on the replication of *Ac*MNPV grown in Sf9 cell line were investigated by observing the reduction percentage of virus titer, the percentage of infected cells and the number of occlusion bodies (OBs)/ml). When the extracts were added after 1 h post-infection of *Ac*MNPV at the multiplicity of infection (MOI) of 1, in Sf9 cell line cultivated *in vitro*, the results show that the average virus titer was 1.56 x 10^8 PFU/ml for the infected control cells (cell incubated with virus), 4.56 x 10^7 PFU/ml for stem extract and 5.82 x 10^7 PFU/ml for leaf extract. Based on the productive control, the average percent reduction of virus titer produced by *Ac*MNPV grown in Sf9 cell line was 60.79% for stem extract and 59.08 % for leaf extract. The average percentage of infected cells in the infected control cells the stem extract and the leaf extract were 89.59, 92.80 and 90.59, respectively. The results of the average number of occlusion bodies (OBs)/ml of infected control cells, the stem extract and the leaf extract were 7.49 x 10^5 OBs /ml, 4.95 x 10^5 OBs /ml and 5.96 x 10^5 OBs /ml, respectively. It can be concluded that hexane crude extract from the stem and leaf of *A. cathartica* affected DNA replication of *Ac*MNPV in Sf9 cell line when the extract was added at 1 h post- infection.

Keywords: *Allamanda cathartica*, *Spodoptera frugiperda* cell line, multiplicity of infection, *Ac*MNPV, crude hexane extract, CC_{50}

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1. Introduction

The Baculoviridae are divided into four genera. The viruses of lepidopteran insects are known as Alphabaculovirus (Nucleopolyhedrovirus, NPV) and Betabaculovirus (Granulovirus, GV). The viruses of hymenopteran and dipteran insects are known as Gammabaculovirus and Deltabaculovirus, respectively [1-2]. Baculoviruses are large, enveloped DNA viruses that are characterized by rod-shaped nucleocapsids ranging in size from 30-60 nm x 250-300 nm and circular double stranded DNA genomes with various sizes ranging from 80 to over 180 kbp [1-4]. Baculoviruses are arthropod-specific viruses and are commonly used as biopesticide for controlling insect pests of agricultural crops [4]. As for their life cycle, they produce two types of virus particles, the occlusion-derived virion (ODV) and the budded virion (BV) [2, 5]. Although the two types of virions are genetically identical, they differ in the origin and composition of their envelope and their roles in the virus life cycle [1, 4].

The ODV type is embedded within paracrystalline protein matrix (polyhedrin protein) of newly formed the occlusion bodies (OBs) or polyhedra that range in size from 0.15 to 3 μm and is required for ODV protection from the environmental elements [2, 4-6]. The ODV type is responsible for primary infection. The OBs of Nucleopolyhedroviruses (NPVs) contain a large number of virion embedded within the matrix and there are two subgenera depending on the number of nucleocapsids surrounded by a common membrane, MNPVs and SNPVs contain multiple and single nucleocapsids, respectively [5]. The OBs are ingested by insect larvae and dissolved in the alkaline environment of larval midgut, thereby releasing ODVs and then they penetrate into the midgut cells by fussion process [2]. These infected cells produce the BV type which is required for secondary infection. The BVs exit the cells in the direction of basement membrane and spread the infection throughout the body of insect [1]. The budded virion contains a single rod-shaped nucleocapsid which is surrounded by an envelope with the prominent spike-like structure or peplomer at the end of mature virion [3]. During the late of infection, ODVs are produced and packaged within OBs, which are located in nuclei of infected cells and the OBs are reseased into the environment after the host's death that can lead to infection of another host [1-2, 7].

For the replication of baculovirus in cell culture, there are three phases in the infection cycle: early (reprogramming the cell for virus replication), late (producing BV), and very late (producing ODV) [8]. The transition from the early phase to the late phase of infection is dependent upon viral DNA replication and occurs between 6 and 12 h after the initiation. During the late phase of infection, newly replicated viral DNA is condensed and packaged within the nucleus, in association with the virogenic stroma, into capsid structures to form nucleocapsids. From about 12 to 20 h, these nucleocapsids leave the nucleus, travel through the cytoplasm, and bud through a modified plasma membrane to acquire a loosely fitting envelope important for BV infectivity. Beginning at about 20 h, there is a transition from the late phase to very late phase of infection, nucleocapsids remain within the nucleus, become bundled together, and are enveloped by a membrane elaborated within the nucleus [4]. The BVs enter insect cells by endocytosis that include: 1) virion binding to a host cell receptor, 2) invagination of the host plasma membrane, 3) formation of endocytic vesicle containing the enveloped virion, 4) acidification of the endosome, 5) activation of the viral envelope fusion protein, 6) fusion of the viral and endosomal membranes, and 7) release of the viral nucleocapsid into the cytoplasm [3].

The *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), which is the type species and the most widely studied of the Baculoviridae [6]. It has a wide host range, replicates well in the commonly used insect cell culture systems (*Spodoptera frugiperda*) and there is a wide range of commercially available transfer vectors [9]. The size of *Ac*MNPV is 25 x 250 nm, and contains approximately 128 kbp double-stranded DNA [10]. The replication of *Ac*MNPV occurs in the nuclei of infected cells and takes place in two phases. In the first phase, nucleocapsids are formed in the nucleus. These nucleocapsids reach the cytoplasm by passing through nuclei pores.

The nucleocapsids gain envelope during the budding through the plasma membrane, and the particles released from the cell [11]. In the second phase, after nuclocapsids acquire envelope (apparently *de novo*) within the nucleus, viral occlusion bodies of NPV are known as polyhedral inclusion bodies (PIBs), and there are infective particles among insects in nature [12].

Althrough baculovirus does not infect human, *Ac*MNPV has a similar replication cycle to double-stranded DNA viruses that cause infection in people. Therfore, *Ac*MNPV is a good model to search antiviral effect of any natural or chemical substances [13]. Petcharawan *et al*. [14] has been reported that the crude hexane extracts from the stem and leaf of *Allamanda cathartica* L. exhibited to be more cytotoxic than dichloromethane and ethanolic extracs on Sf9 cell line according to CC_{50}. In this study, the effect of crude hexane extracts from the stem and leaf of *Allamanda cathartica* L. on the replication of *Ac*MNPV grown in *Spodoptera frugiperda* cell line (Sf9) were investigated by observing the percentage of reduction of virus titer, the percentage of infected cells and the number of occlusion bodies (OBs/ml).

2. Materials and Methods

2.1 Preparation of plant extracts

A. cathartica plants were collected from the garden in Bangkok Province, Thailand. The plant materials were washed and completely dried in hot-air oven at 70^{o} C until the dried weight of samples (stems and leaves) were stable and then were finely ground. Suitable amounts of the powdered plant materials were soaked in hexane (1 :15) for 7 days. The extracts were then filtered through the cheesecloth and centrifuged at 3,500 rpm for 30 min. The filtrated were collected and filtered through Whatman No. 1 filter paper and evaporated at 40°C to dryness under reduced pressure using rotary evaporator. These extracts were stored at -20°C for further investigation.

2.2 Cell line and virus

The *Spodoptera frugiperda* cell line (Sf9) was used as the host cell for virus infection. Cells were cultivated in TNM-FH medium supplemented with 5% fetal bovine serum (FBS), 100 units/ml penicillin G, 100 µg/ml streptomycin in 25 cm^2 culture flasks at 28^{o} C [14]. *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV) stock was prepared by inoculating in Sf9 cells and incubated at 28 °C. The virus suspension was removed seven days post infection and the virus titer of stock was determined as $TCID_{50}$/ml and converted to pfu/ml [15], virus stock was stored at -20^{o} C until needed.

2.3 Cytotoxicity test by neutral red uptake assay

The neutral red uptake assay was carried out as previously described [14]. Briefly, Sf9 cells were seeded at 2 x 10^4 cells/well in each 96-well plate in 100 µl of fresh TNM-FH medium supplemented with 5% FBS and were allowed to attach overnight. When the cell cultures were confluent, the culture medium were removed from each well, 100 µl of plant extract dilutions (1,000, 500, 250, 125, 62.5 and 31.25 µg/ml) were added to each well. The well with 100 µl maintenance medium but without sample was prepared to act as a cell control. All cultures were incubated at 28^{o} C for 4 days. After the end of the incubation period, the media was replaced with medium containing 50 µg/ml neutral red. The plates were incubated at 28^{o} C for 3 h to allow for uptake of the vital dye into the lysosomes of viable and uninjured cells. After the incubation period, the media was removed and cells were washed with a fixative and then solubilised with 150 µl of destaining solution for 10 min. The 96-well plates were then shaked for 10 min and the absorbance of solution in each well was measured at 540 nm on a microtitre plate reader [16].The percentage of cytotoxicity was calculated as [(A-B)/A] x 100, where A and B are the absorbances of control and treated cells, respectively [17]. CC_{50} values were expressed as µg of compound/ml

that caused a 50% growth inhibition as compared to controls. Experiments were carried out in triplicate and in three independent experiments.

2.4 Antiviral activity assay

Two ml of TNM-FH medium supplemented with 5% FBS and 4 x 10^5 Sf9 cells were seeded into each dish of 35 mm tissue culture dish. After leaving cell for overnight attachment, the medium was removed and washed with phosphate-buffer saline (PBS). After washing, cells in three 35 mm tissue culture dishes were infected with *Ac*MNPV at a multiplicity of infection (MOI) of 1. After inoculation of virus, the dishes were placed on a rocker platform and rocked for 1 h. The inoculum was removed and washed with PBS, then fresh medium was added to each dish. At this time it was considered as 0 h post infection (p.i.). The dishes were incubated at 28^o C for 4 days. At the end of the incubation period, the cells were scraped and centrifuged at 3,500 rpm for 15 min to separate the supernatant and the pellet. Each panel included toxicity control (cells incubated with leaf extract and cells incubated with stem extract), uninfected cell control (cells incubated with medium) and infected cell control (cells incubated with virus).

In order to determine the antiviral effect of plant extracts, the following experiments were performed with 31.25 µg/ml concentration of extracts for leaf and stem samples. Sf9 cells were seeded at a density of 2 x 10^5 cells/ml in 2 ml/dish and allowed to attach overnight at 28^o C. The medium was removed and washed with phosphate-buffer saline (PBS). After washing, cells in six 35 mm tissue culture dishes (three dishes prepared for each crude extract) were infected with *Ac*MNPV at a multiplicity of infection (MOI) of 1. After inoculation of virus, the dishes were placed on a rocker platform and rocked for 1 h at room temperature for adsorption. After adsorption, the inoculum was removed and washed with PBS, then 2 ml of crude extract was added to each dish and incubated at 28^{o}C for 4 days. At the end of the incubation period, the cells were scraped and centrifuged at 3,500 rpm for 15 min to separate the supernatant and the pellet and the virus titer of each treatment was determined as $TCID_{50}$/ml and converted to pfu/ml [15] to observe the antiviral effect of extracts on DNA replication or transcription.

Finally the virus concentration was determined as $TCID_{50}$/ml and converted to pfu/ml [15]. The percentage of infected cells, the number of occlusion bodies (OBs)/ml in the infected control cells, the stem extract and the leaf extract were determined. The percent reduction in the pfu/ml produced by virus in the presence of extracts was calculated as % reduction = 100 - [(pfu at given extract dose/pfu in control) x 100] [18].

2.4 Statistical analysis

The program GraphPad Prism 5.0 was used for the calculation of cytotoxicity curves and CC_{50} [19]. All the data were statistically evaluated with SPSS statistics 17.0 software. Hypothesis testing methods include one way analysis of variance (ANOVA) followed by Duncan's new multiple range test a post hoc or multiple comparison test, which is used to determine whether three or more means differ significantly in an analysis of variance. Differences were considered significant at $p < 0.01$ [20].

3. Results and Discussion

3.1 Cytotoxic effect of crude hexane extracts from *A. cathartica* on Sf9 cells

Effects of various concentrations of crude hexane extracts obtained from *A. cathartica* on Sf9 cells after 96 h of exposure are shown in Table1. The highest percent cytotoxicity was exhibited by crude hexane extract from the stem at concentration 1,000 and 500 µg/ml while less toxicity occurred at lower concentrations. High concentration of crude hexane extract from the stem and leaf of *A. cathartica* induced granularity, retraction and then lysis (Figure 1).

Based on these data, we used the concentration of stem and leaf extracts at 31.25 µg/ml to determine the antiviral activity of plant extracts on the replication of *Ac*MNPV.

The value of CC_{50} of crude hexane extracts from *A. cathartica* against Sf9 cell line after 96 h of exposure is shown in Table 2, the result showed that the stem extract (CC_{50} = 326.44 µg/ml) was more toxic than the leaf extract (CC_{50} >1000 µg/ml).

Table 1 Comparison of the cytotoxic effect of crude hexane extracts from *A. cathartica* on Sf9 cell line after 96 h of exposure

Crude hexane extracts From *A. cathartica*	**Concentration (µg/ml)**	**Mean percent cytotoxicity***
Stem	1,000	96.60[a]
	500	90.62[b]
	250	34.34[e]
	125	20.39[g]
	62.5	15.72[h]
	31.25	11.81[i]
	1.35% DMSO	2.44[j]
	0	0.00[k]
Leaf	1,000	41.24[c]
	500	37.96[d]
	250	32.28[e]
	125	25.11[f]
	62.5	16.70[h]
	31.25	10.72[i]
	1.28% DMSO	2.02[jk]
	0	0.00[k]

*Means within the column followed the same letter is not significantly different (p<0.01, Duncan's new multiple range test).

Table 2 The value of CC_{50} of crude hexane extracts from *A. cathartica* against Sf9 cell line after 96 h of exposure

Crude hexane extracts from *A. cathartica*	**CC_{50} (µg/ml)**
Stem	326.44
Leaf	>1,000

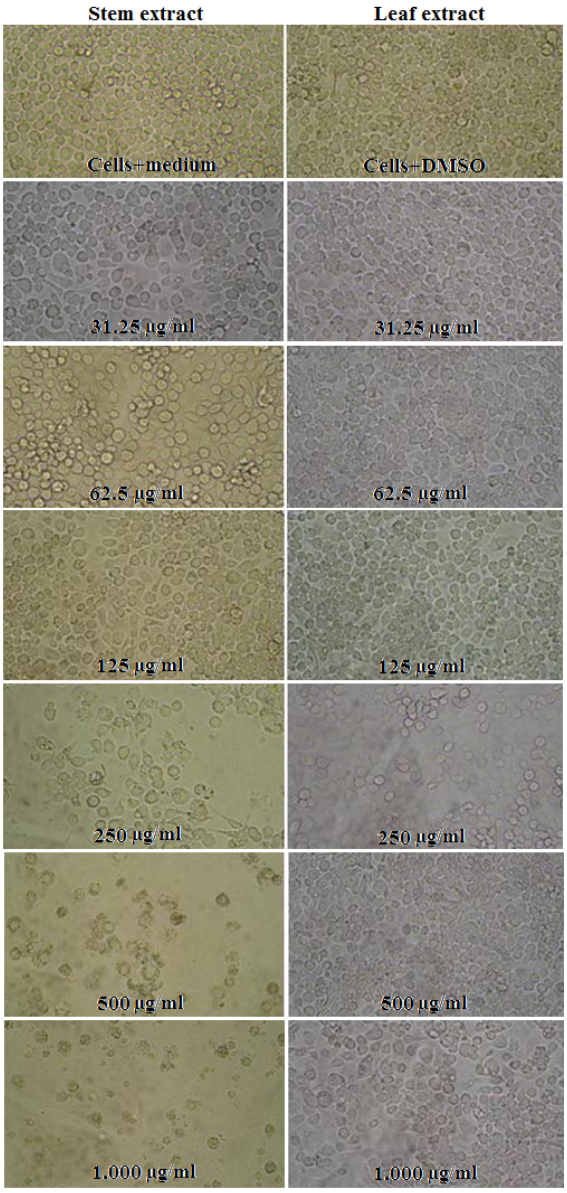

Figure 1 Phase-contrast micrograph of Sf9 cells, control cells in medium, control cells in medium with DMSO and Sf9 cells after 96 h of exposure in hexane extracts from the stems and leaves of *A. cathartica*.

3.2 Antiviral activity

The results of antiviral activity of crude hexane extracts from the stems and leaves of *A. cathartica* against *Ac*MNPV were shown in Table 3. The concentration of 31.25 µg/ml of both extracts was used to determine the antiviral activity of plant extracts on the replication of *Ac*MNPV. The extracts were added 1 h after infection with *Ac*MNPV at a multiplicity of infection of 1 and incubated at 28^{o} C for 96 h. The virus titer, the number of occlusion bodies (OBs)/ml and the corrected percent reduction of virus titer were determined. The results suggested that there is

highly significant difference in the mean virus titer between the infected control cells (1.56 x10^8) and the infected cells in the stem and the leaf extract solutions. When each extract was added at 1 h p.i, the virus concentration was determined as 4.56 x 10^7 pfu/ml for stem extract and 5.82 x10^7 pfu/ml for leaf extract. For the production of occlusion bodies in Sf9 cells, it was found that there is no significant difference between the average percentage of infected cells in the infected control cells, the stem extract and the leaf extract (89.59, 92.80 and 90.59%, respectively) and there is no significant difference between the mean number of occlusion bodies in the infected control cells and the infected cells in the leaf extract, when the extract was added 1 h p.i. the virus titer was reduced by 60.79% and 59.08% with stem and leaf extracts, respectively.

Table 3 Antiviral activity of crude hexane extracts from the stems and leaves of *A. cathartica*against *Ac*MNPV.

Treatments	Mean virus titer (PFU/ml)*± S.D.	Mean % infected cell	Mean no. of OBs/ml* ± S.D.	Corrected % reduction
Cells+ *Ac*MNPV	1.56 x10^{8a}±3.44	89.59^a±2.18	7.49 x10^{5a}±0.17	0.00^b
Cells+ *Ac*MNPV+stem extract	4.56 x10^{7b}±0.77	92.80^a±0.56	4.95 x10^{5b}±0.81	60.79^a
Cells+ *Ac*MNPV+leaf extract	5.82 x10^{7b}±3.74	90.59^a±1.37	5.96 x10^{5ab}±0.36	59.08^a

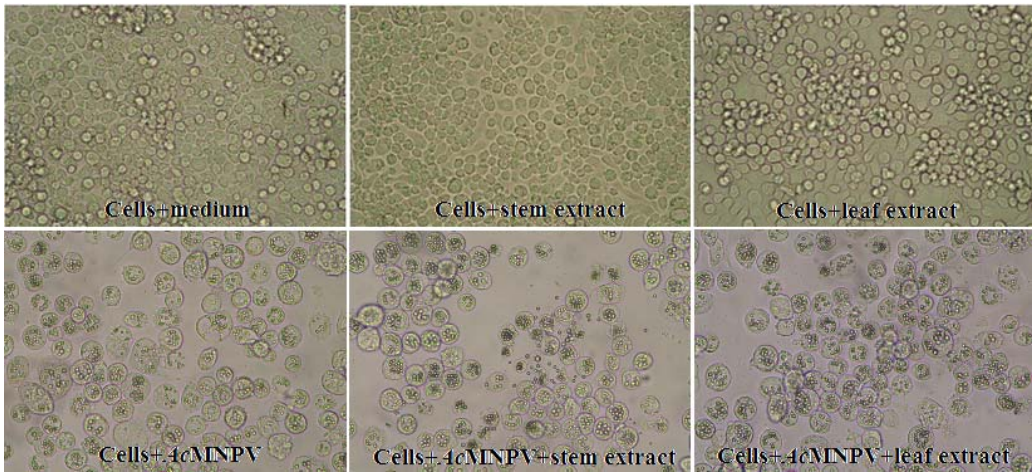

Figure 2 Phase-contrast micrographs of Sf9 cells cultivated in TNM-FH medium, Sf9 cells after 96 h exposure in hexane extracts from the stems and leaves of *A. cathartica*, Sf9 cells after 96 h post infection with *Ac*MNPV, Sf9 cells after 96 h post infection with *Ac*MNPV in 31.25 µg/ml of stem and leaf extracts.

These results showed that DNA replication of *Ac*MNPV was inhibited and stem extract of *A. cathartica* had strong antiviral effect. Saoo *et al*. [18] tested the antiviral effect of extract obtained from leaves of *Aloe barbadensis* plant. Their results showed that the antiviral effect of *Aloe* extracts occurred at the time of major DNA and protein synthesis, during the cause of cytomegalovirus infection. Kati *et al*. [19] reported that the used concentrations of root and stem extracts obtained from *Primula longipes* plant were 200 µg/ml, whereas, the used concentration of leaf extract was 20 µg/ml. They determined that extract obtained from leaf had higher antiviral effect, and it caused 66% decrease of the virus concentration. The results suggested that a major mechanism of inhibition of baculovirus infection by *Primula* extracts was through interference with gene expression.

4. Conclusions

Although baculovirus does not infect human, *Ac*MNPV has a similar replication cycle to double-stranded DNA viruses that cause infection in people. The concentration of 31.25 µg/ml for stem and leaf extracts of *A. cathartica* did not have the effects on the growth of Sf9 cells. However, the extracts at the same concentration caused highly significant inhibitory effect on the replication of *Ac*MNPV. When the stem and leaf extracts were added 1 h p.i., the virus concentration was reduced by 60.79% and 59.08%, respectively.

5. Acknowledgements

The authors are grateful to Prof. Dr. Serge Belloncik for providing *Ac*MNPV and Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Thailand for partial financial support to this study.

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