

Full Length Research Paper

Effect of crude extracts from the root of *Stemona tuberosa* Lour. on the replication of *Autographa californica* multiple nucleopolyhedrovirus

Ounruan Petcharawan*, Dusanee Thanaboripat, Tippanya Karunasoong, Kunlanit Chalernsuk and Jira Sakolvaree

Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand.

Received 16 April, 2013; Accepted 23 August, 2016

The purpose of this research was to investigate the effect of crude extracts from the root of *Stemona tuberosa* Lour. on the replication of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV). Cytotoxicity of crude hexane, dichloromethane and ethanol extracts from the root of *S. tuberosa* was tested against *Spodoptera frugiperda* cell line (Sf9) using MTT assay. The cytotoxic effect, represented as CC_{50} ($\mu\text{g/ml}$) was observed after 48 and 96 h. It was shown that dichloromethane extract was more toxic than hexane and ethanol extracts and 96-h CC_{50} for the dichloromethane extract was 1,708.98 $\mu\text{g/ml}$. Crude dichloromethane extract at the concentration of 31.25 $\mu\text{g/ml}$ was then tested on AcMNPV. The extract was added after 1 h post-infection of AcMNPV at the multiplicity of infection (MOI) of 2, in Sf9 cell line cultivated *in vitro*. No significant difference between the percentage of infected cells in the control and the test sample with crude dichloromethane extract was found. The average number of polyhedra (OBs/ml) in the control ($5.11 \times 10^6 \pm 0.63$ OBs/ml) was not significantly different from the test sample ($4.19 \times 10^6 \pm 0.31$ OBs/ml). However, there was significant difference between the average virus titer (budded virus, BV) in the control ($2.06 \times 10^8 \pm 0.71$ PFU/ml) and the test samples ($2.65 \times 10^8 \pm 0.79$ PFU/ml). Crude dichloromethane extract (31.25 $\mu\text{g/ml}$) did not toxic to Sf9 cells but it could enhance AcMNPV replication in Sf9 cell line cultivated *in vitro*. It could be concluded that *S. tuberosa* can be a good candidate for developing the insect virus production *in vitro* for controlling insect pests.

Key words: AcMNPV, crude extract, cytotoxicity test, by 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

INTRODUCTION

In Thailand, *Stemona* species are known as Non Tai Yak. *Stemona* spp. can be used as insect repellent and

insecticides such as scabicide and pediculocide (Greger, 2006; Chanmahasathien et al., 2011). The total alkaloid

*Corresponding author. E-mail: ksounrua@kmitl.ac.th or oun5@hotmail.com. Tel: 66818182186.

profiles could be grouped into four types represented as the major component by stenine-type *Stemona* alkaloids such as tuberostemonine and neotuberostemonine or by non-stenine types such as croomine and stemoninine. The biological activities of *Stemona* alkaloids were insecticidal and larvicidal activities (Brem et al., 2002; Kaltenegger et al., 2003; Xu et al., 2006).

For the replication of baculovirus in insect 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) (O'Rielly et al., 1992). 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 (Olszewski and Miller, 1997). 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 (Blissard, 1996). The *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), which is the type species and the most widely studied of the Baculoviridae (Wu et al., 2006). 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 (Hitchman et al., 2009). The size of AcMNPV is 25×250 nm, and contains approximately 128 kbp double-stranded DNA (Arif, 1986). The replication of AcMNPV 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 (Fraser, 1986). In the second phase, after nucleocapsids 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 insect in nature (Bilimoria et al., 1992).

The extract of *Stemona tuberosa* roots was found to have antibacterial, antifungal and insecticidal activities. Thus, the objective of this study was to evaluate the

cytotoxicity of crude hexane, dichloromethane and ethanol extracts of *S. tuberosa* roots against insect cell line (Sf9) using MTT assay. The crude extract that showed the highest cytotoxicity to Sf9 cells was to investigate the efficiency of the extract on the replication of insect virus (AcMNPV) using the endpoint dilution assay.

MATERIALS AND METHODS

Preparation of *S. tuberosa* extracts

Fresh *S. tuberosa* roots were purchased from Chatuchak market in Bangkok, Thailand. Fresh roots were dried at 45°C until they were quite dried and were ground to powder using a milling machine. The powdered plant material (30 g) was macerated for 7 days sequentially with 450 ml of the following solvents: hexane, dichloromethane and ethanol. The hexane, dichloromethane and ethanol extracts were carefully filtered through Whatman® No. 1 filter paper and were evaporated to dryness under reduced pressure using rotary evaporator to give 0.155, 0.256 and 0.596 g, respectively.

Cell line and virus

The *S. 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 cm² culture flasks at 28°C (Petcharawan et al., 2009; Petcharawan et al., 2012). The AcMNPV 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 TCID₅₀/ml (tissue culture infectious dose per ml) and converted to pfu/ml (plaque forming unit per ml) (Reed and Muench, 1992), virus stock was stored at -20°C until needed.

Cytotoxicity assay

The extracts of *S. tuberosa* root were tested for *in vitro* cytotoxicity, using Sf9 cell line by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Each crude extract was separately dissolved in 1 ml of dimethyl sulfoxide (DMSO) and volume was made up to 10 ml with maintained medium to obtain a stock solution of 5 mg/ml concentration, sterilized by filtration to make stock solution, then diluted to various concentrations (2,000, 1,000, 500, 250, 125, 62.5, and 31.25 µg/ml) in TNM-FH medium. The cells were seeded in a 96-well plate with 2×10⁴ cells/well and incubated at 28°C for 24 h. After 24 h of seeding, the culture medium was replaced with fresh medium containing the different concentrations of the extracts. Each concentration was applied in 4 wells and within 3 independent experiments. Control cells were incubated without the extracts. After incubation at 28°C for 48 and 96 h, the MTT (10 µl of 5 mg/ml in PBS) was added in each well and the cells were incubated at 28°C for 4 h. Thereafter, the medium with MTT was removed from the wells and formazan crystal formed was dissolved by adding 150 µl/well a mixture of 10% SDS:DMSO (1:9). The plates were then shaken for 5 min and the absorbance of the solution in each well was measured at 570 nm on a microtiter plate reader (Siddiqui et al., 2010).

The percentage of cytotoxicity was calculated as $\{(A-B)/A\} \times 100$, where A and B are the absorbance of the control cells and treated

cells, respectively. All absorbance values were corrected against blank wells which contained only growth medium. For the evaluation of the concentration that induced 50% cytotoxicity relative to controls (CC₅₀ values) was calculated in each assay using GraphPad Prism software program (Chiba et al., 1998; Krzyminska et al., 2010; Duman, 2012; Petcharawan et al., 2012).

Effect of crude extracts on the replication of AcMNPV

The effect of *S. tuberosa* crude extract on the replication of AcMNPV was conducted using cell monolayers in 35-mm tissue culture dishes. Exponential phase Sf9 cells in TNM-FH medium were seeded into a 35-mm tissue culture dish at a density of 4×10^5 cells/dish and incubated at 28°C overnight. The medium in each dish was aspirated and carefully replaced with 1 ml of AcMNPV at a multiplicity of infection (MOI) of 2. 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°C for 4 days. At the end of the incubation period, the cells were scraped and counted, and then centrifuged at 3,500 rpm for 15 min to separate the supernatant and the pellet. The supernatant that contained budded virion was kept at -20°C until needed. The pellets were resuspended with 0.1% sodium dodecyl sulfate (SDS) and incubated at room temperature overnight, then suspended cells were centrifuged and the SDS was discarded. The pellets were resuspended in 2 ml of sterile distilled water and counted the number of polyhedra that liberated from the infected cells in the control and the test samples. Each panel included toxicity control (cells incubated with the extract), uninfected cell control (cells incubated with medium) and infected cell control (cells incubated with virus) and the corrected % mortality was calculated using Abbot's formula, corrected % mortality = $100 \times \{(T\% - C\%) / (100\% - C\%)\}$, where T% = the percentage of dead test cells and C% = the percentage of dead control cells (Abbot, 1925; Undeen and Vávra, 1997).

Finally, the virus concentration was determined as TCID₅₀/ml and converted to pfu/ml (Reed and Muench, 1992). The percentage of infected cells, the number of occlusion bodies (OBs)/ml in the control and the test samples were determined. The percent of reduction in the pfu/ml produced by virus in the presence of extracts was calculated as % reduction = $100 - [(pfu \text{ at given extract dose} / pfu \text{ in control}) \times 100]$ (Sökmen, 2001).

Statistical analysis

The program GraphPad Prism 5.0 was used for the calculation of cytotoxicity curves and CC₅₀ (Motulsky, 2007). 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. A value of $p < 0.05$ was regarded as statistically significant differences (Custódio et al., 2011).

RESULTS

The effect of crude hexane, dichloromethane and ethanol extracts from *S. tuberosa* against Sf9 cell line was evaluated using MTT assay. Results of the cytotoxicity evaluation of these extracts against Sf9 cell line are shown in Table 1. The dichloromethane extract was more toxic than the others. The values of CC₅₀ of these

extracts against Sf9 cell line after 48 and 96 h of exposures are shown in Table 2. Based on 96 h exposure results obtained from hexane, dichloromethane and ethanol extracts from *S. tuberosa* against Sf9 cell line, the CC₅₀ values were >2,000, 1,708.98, and >2,000 µg/ml, respectively. Among the crude extracts tested, the dichloromethane extract gave the highest toxic to Sf9 cells. However, the cytotoxicity of these extracts can be categorized using the classification of the cytotoxicity of natural ingredients reported by Gad Shayne (1999) and Shirazi et al. (2004), we can conclude that these extracts had partially nontoxic to Sf9 cell line, because of the CC₅₀ were more than 1,000 µg/ml (Table 2).

The results (Table 3 and Figure 1) showed that there was no significant difference ($p > 0.05$) between the percentage of infected cells in the control sample (95.54%±3.18) and in the test sample with crude dichloromethane extract (98.51%±1.34). However, there was significant difference ($p < 0.05$) between the average virus titer in the control ($2.06 \times 10^8 \pm 0.71$ PFU/ml) and the test ($2.65 \times 10^8 \pm 0.79$ PFU/ml) samples. The average number of polyhedra (OBs/ml) in the control ($5.11 \times 10^6 \pm 0.63$ OBs/ml) was not significantly different ($p > 0.05$) from the average number of polyhedra in the test sample ($4.19 \times 10^6 \pm 0.31$ OBs/ml). However, there was significant difference ($p < 0.05$) between the percent reduction of virus titer in the control (0%±0) and in the test samples (-27.45%±4.36). These results showed that the crude dichloromethane extract of *S. tuberosa* at the concentration of 31.25 µg/ml was no toxic effect to Sf9 cells and could not inhibit AcMNPV infection cultivated *in vitro* but it could enhance the virus titer (budded virus, BV) of AcMNPV in Sf9 cells.

DISCUSSION

According to the results of this study, the crude dichloromethane extract of *S. tuberosa* root was more toxic to Sf9 cells than crude hexane and ethanol extracts, with the CC₅₀ values of 1708.98, 2,000 and 2,000 µg/ml, respectively. The result corresponded with the report of Phattharaphan et al. (2010), the highest insecticidal activity to the third instar larvae of *Plutella xylostella* was observed from dichloromethane extract of *Stemona collinsae* root with the LC₅₀ of 0.71% and the major active compound responsible for the insecticidal activity was hydroxystemofoline (alkaloid). Brem et al. (2002) reported that tuberostemonine was dominating alkaloid in the roots of *S. tuberosa* Lour., showing repellency but not toxic effect to neonate larvae of *Spodoptera litoralis*. Lee et al. (2006) reported that three new bibenzyl glycosides were isolated from *S. tuberosa* significantly protected human neuroblastoma SH-SY5Y cells from 6-hydroxy dopamine induce neurotoxicity.

In order to determine the antiviral effect of crude dichloromethane extract of *S. tuberosa* root, the experiment was performed with the concentration of

Table 1. Comparison of the cytotoxic effect of crude hexane, dichloromethane and ethanol extracts on Sf9 cell line after 48 and 96 h of exposures.

Solvents	Concentration ($\mu\text{g/ml}$)	Mean percent cytotoxicity \pm SD*	
		48 h	96 h
Hexane	2000	19.86 \pm 1.35 ^{cd}	41.05 \pm 1.17 ^b
	1000	18.03 \pm 1.29 ^{de}	31.08 \pm 0.93 ^c
	500	11.65 \pm 0.91 ^{fg}	19.88 \pm 2.17 ^{efg}
	250	10.12 \pm 0.51 ^{gh}	17.19 \pm 2.12 ^{fgh}
	125	9.49 \pm 0.30 ^{ghi}	14.08 \pm 3.20 ^{gh}
	62.5	5.67 \pm 0.61 ^{jk}	12.50 \pm 1.88 ^{hi}
	31.25	3.13 \pm 1.38 ^{kl}	1.15 \pm 0.13 ^{jk}
	1% DMSO	1.54 \pm 0.49 ^{lm}	1.32 \pm 0.39 ^{ijk}
	0 (Control)	0 \pm 0 ^m	0 \pm 0 ^k
Dichloromethane	2000	42.68 \pm 1.61 ^a	52.80 \pm 2.13 ^a
	1000	35.03 \pm 4.55 ^b	39.43 \pm 4.24 ^b
	500	21.29 \pm 0.60 ^c	29.82 \pm 5.28 ^{cd}
	250	16.12 \pm 0.36 ^e	25.20 \pm 6.85 ^{cde}
	125	11.69 \pm 0.44 ^{fg}	20.74 \pm 8.60 ^{ef}
	62.5	7.68 \pm 0.18 ^{hij}	17.23 \pm 10.11 ^{fgh}
	31.25	2.25 \pm 0.73 ^{lm}	7.67 \pm 0.77 ⁱ
	0.4% DMSO	1.35 \pm 0.02 ^{lm}	1.22 \pm 0.10 ^{jk}
	0 (Control)	0 \pm 0 ^m	0 \pm 0 ^k
Ethanol	2000	20.15 \pm 2.14 ^{cd}	41.04 \pm 2.59 ^b
	1000	16.23 \pm 4.30 ^e	30.84 \pm 3.24 ^c
	500	13.43 \pm 2.68 ^f	24.17 \pm 1.33 ^{de}
	250	8.97 \pm 0.30 ^{ghi}	21.96 \pm 1.14 ^{ef}
	125	6.81 \pm 0.83 ^{ij}	16.41 \pm 1.21 ^{fgh}
	62.5	5.82 \pm 0.89 ^j	7.25 \pm 0.31 ^{ij}
	31.25	1.99 \pm 1.40 ^{lm}	0.47 \pm 0.44 ^k
	0.2% DMSO	0.81 \pm 0.56 ^{lm}	0.48 \pm 0.45 ^k
	0 (Control)	0 \pm 0 ^m	0 \pm 0 ^k

*Means within the column followed the same letter is not significant difference ($p < 0.05$, Duncan's new multiple range test).

Table 2. The values of CC₅₀ of crude extracts from *S. tuberosa* root against Sf9 cell line after 48 and 96 h of exposures.

Solvents	The incubation time (h)	Mean CC ₅₀ ($\mu\text{g/ml}$)
Hexane	48	>2000
	96	>2000
Dichloromethane	48	>2000
	96	1,708.98
Ethanol	48	>2000
	96	>2000

31.25 $\mu\text{g/ml}$ because of this concentration did not have any effect on the growth of Sf9 cells. When the extract

was added 1 h after infection with AcMNPV, the virus titer was increased and the polyhedra or occlusion bodies

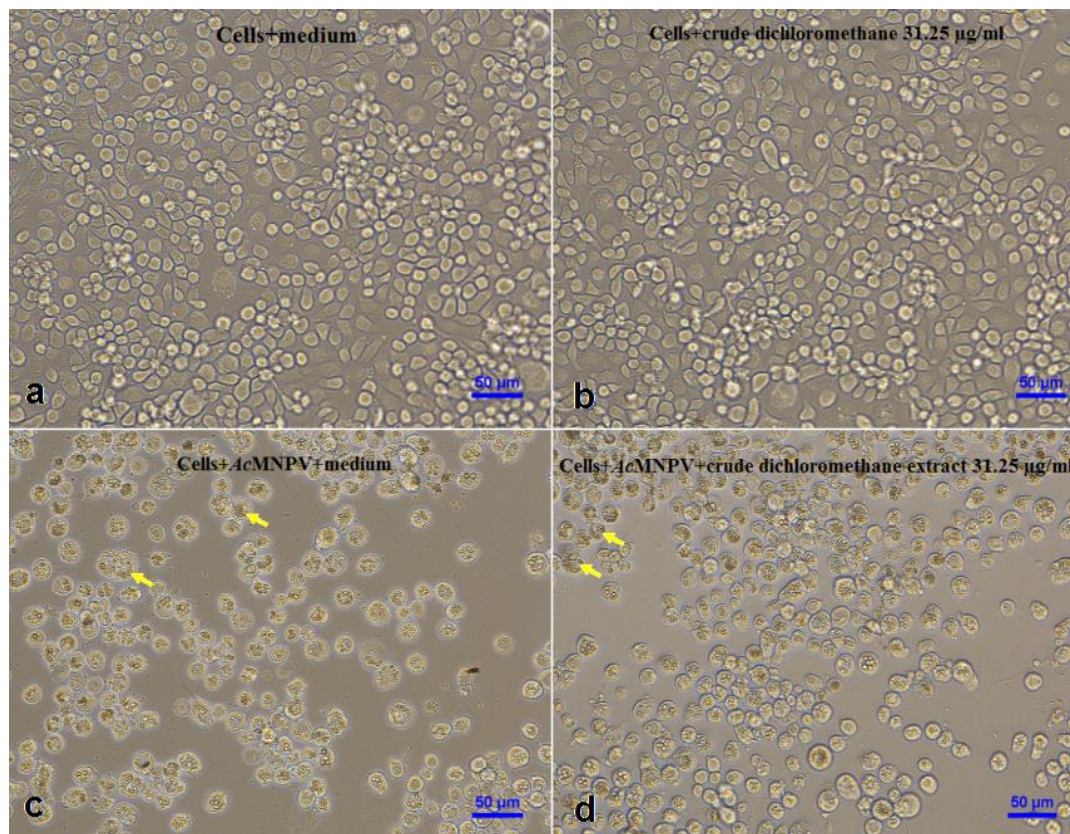


Figure 1. Phase-contrast micrographs of Sf9 cells cultivated in TNM-FH medium (a), Sf9 cells after 96 h exposure in dichloromethane extract (b), Sf9 cells after 96 h post infection with AcMNPV in culture medium (c), Sf9 cells after 96 h post infection with AcMNPV in 31.25 µg/ml of extract (d).

Table 3. Effect of crude dichloromethane extracts from *S. tuberosa* roots (31.25 µg/ml) on the replication of AcMNPV.

Treatments	*Mean % infectivity	*Mean no. of OBs/ml ($\times 10^6$)	*Mean virus titer (PFU/ml) \pm SD ($\times 10^8$)	*Corrected % reduction of budded virus
Cells+AcMNPV	95.54 \pm 3.18 ^a	5.11 \pm 0.63 ^a	2.06 \pm 0.71 ^a	0 \pm 0 ^a
Cells+AcMNPV+extract	98.51 \pm 1.34 ^a	4.19 \pm 0.31 ^a	2.65 \pm 0.79 ^b	-27.45 \pm 4.36 ^b

*Different letters indicate statistically significant differences between rows in the same column, $p < 0.05$.

(OBs) could be produced in the nucleus of Sf9 cells (Table 3). The results indicated that this extract could not inhibit the viral synthesis, the viral structural and the viral occlusion protein phases. Further studies are necessary to investigate the mechanism of crude dichloromethane extract of *S. tuberosa*, such as the enhancement activity of AcMNPV, purification and active compounds. These results corresponded with the report of Erturk et al. (2000), the effect of *Nerium oleander* L., *Prunus laurocerasus* L., *Punica granatum* L., *Olea europaea* L. and *Daphne glomerata* Lam. extracts could increase the concentration of progeny virus of AcMNPV.

This study showed that *S. tuberosa* can be a good candidate for developing the insect virus production *in*

vitro for controlling insect pests.

Conflicts of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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

REFERENCES

- Abbot WS (1925). A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18:265-267.
- Arif MB (1986). The structure of the viral genome. The molecular biology of baculoviruses. *Curr. Top. Microbiol. Immunol.* 131:21-29.
- Bilimoria SL, Demirbag Z, Ng H, Rainisch AJ (1992). Abortive cell culture infection of nuclear polyhedrosis viruses as model system for host specificity. *Pesqui. Agropecu. Bras.* 27:123-141.
- Blissard GW (1996). Baculovirus-insect cell interactions. In *Insect Cell Culture: Fundamental and Applied Aspects* (pp. 73-93). Springer Netherlands.
- Brem B, Seger C, Pacher T, Hofer O, Vajrodaya S, Greger H (2002). Feeding deterrence and contact toxicity of *Stemona* alkaloids—a source of potent natural insecticides. *J. Agric. Food Chem.* 50(22):6383-6388.
- Chanmahasathien W, Ampasavate C, Greger H, Limtrakul P (2011). *Stemona* alkaloids from traditional Thai medicine increase chemosensitivity via P- Glycoprotein-mediated multidrug resistance. *Phytomedicine* 18:199-204.
- Chiba K, Kawakami K, Tohyama K (1998). Simultaneous evaluation of cell viability by neutral red, MTT and crystal violet staining assays of the same cells. *Toxicol. In Vitro* 12:251-258.
- Custódio L, Escapa AL, Fajardo A, Aligue R, Neng N, Floreirc JM, Romano A (2011). In vitro cytotoxic effects and apoptosis induction by a methanol leaf extract of carob tree (*Ceratonia siliqua* L.). *J. Med. Plants Res.* 5(10):1987-1996.
- Duman R (2012). Antiherpetic activity of some endemic *Hypericum* species in Turkey. *Afr. J. Biotechnol.* 11(5):1240-1244.
- Erturk O, Demirbag Z, Beldus AO (2000). Antiviral activity of some plant extracts on the replication of *Autographa californica* nuclear polyhedrosis virus. *Turk. J. Biol.* 24:833-844.
- Fraser MJ (1986). Transposon-mediated mutagenesis of baculovirus: Transposon shutting and implication for speciation. *Ann. Entomol. Soc. Am.* 79:773-786.
- Gad Shayne C (1999). Alternatives to in vivo studies in toxicology. In: Balantyne B, Marrs T, Syversen T (Eds.). *General and applied toxicology*, 1. Grove's dictionaries Inc., USA. 1999. P.178.
- Greger H (2006). Structure relationships, distribution and biological activities of *Stemona* alkaloids. *Planta Med.* 72:99-113.
- Hitchman RB, Possee RD, King LA (2009). Baculovirus expression systems for recombinant protein production in insect cells. *Recent Pat. Biotechnol.* 3(1):46-54.
- Kaltenegger E, Brem B, Mereiter K, Kalchhauser H, Kahlig H, Hofer O, Vajrodaya S, Greger H (2003). Insecticidal pyrido [1,2-a] azepine alkaloids and related derivatives from *Stemona* species. *Phytochemistry* 63:803-816.
- Krzyminska S, Raczowska M, Kaznowski A (2010). Cytotoxic activity of *Serratia marcescens* clinical isolates. *Pol. J. Microbiol.* 59(3):201-205.
- Lee KY, Sung SH, Kim YC (2006). Neuroprotective bibenzyl glycosides of *Stemona tuberosa* roots. *J. Nat. Prod.* 69:679-681.
- Mosmann T (1983). Rapid colorimetric assays for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65:55-59.
- Motulsky H (2007). GraphPad Prism® Version 5.0 Statistics Guide. San Diego CA, GraphPad Software, Inc. Available at: www.graphpad.com.
- O'Reilly DR, Miller LK, Luckow VA (1992). *Baculovirus Expression Vectors. A Laboratory Manual*. New York: W.H. Freeman and Company.
- Olszewski J, Miller LK (1997). Identification and characterization of a baculovirus structure protein, VP1054, required for nucleocapsid formation. *J. Virol.* 1(7):5040-5050.
- Petcharawan O, Paitoon N, Sripaiboon P, Saelee S (2012). Antiviral activity of crude hexane extracts from *Allamanda cathartica* on the replication of *Autographa californica* multiple nucleopolyhedrovirus. *KMITL Sci. Technol. J.* 12(1):21-29.
- Petcharawan O, Suriyasakol K, Vicharncharoensuk N (2009). Cytotoxicity of crude extracts from golden trumpet (*Allamanda cathartica* L.) on Sf9 and KMITL-HA-E1 cell lines. In: Yang Qian (Ed.). *Study on Biological Control and Biotechnology*, Harbin, Heilongjiang Science and Technology Press pp. 101-107.
- Phattharaphan N, Pitiyont B, Visetson S (2010). Potential of *Stemona* sp. For *Plutella xylostella* control. *J. Biopestic* 3(1 Special Issue):278-281.
- Reed L, Muench H (1992). A simple method for estimating fifty percent endpoints. In: O'Reilly DR, Miller LK, Luckow VA (Eds.). 1992. *Baculovirus Expression Vectors. A Laboratory Manual*, New York, W.H. Freeman and Company, pp. 124-138.
- Shirazi FH, Ahmadi N, Kamalinejad M (2004). Evaluation of Northern Iran *Mentha Pulegium* L. cytotoxicity. *DARU J. Pharm. Sci.* 12(3):106-110.
- Siddiqui MJ, Ismail Z, Aisha AFA, Abdul Majid AMS (2010). Cytotoxicity activity of *Catharanthus roseus* (Apocynaceae) crude extracts and pure compounds against human colorectal carcinoma cell line. *Int. J. Pharmacol.* 6(1):43-47.
- Sökmen A (2001). Antiviral and cytotoxic activities of extracts from the cell culture and respective parts of some Turkish medicinal plants. *Turk. J. Biol.* 25:343-350.
- Undeen AH, Vávra J (1997). Chapter IV Research Methods for Entomopathogenic Protozoa. In: Lawrence AL (Ed.). 1997. *Biological Techniques. Manual of Techniques in Insect Pathology*. Sandiagno, Academic Press pp. 117-151.
- Wu W, Lin T, Pan L, Yu M, Li Z, Pang Y, Yang K (2006). *Autographa californica* multiple nucleopolyhedrovirus nucleocapsid assembly is interrupted upon deletion of the 38K gene. *J. Virol.* 80(23):11475-11485.
- Xu YT, Hon PM, Jiang RW, Cheng L, Li SH, Chan YP, Xu HX, Shaw PC, But PPH (2006). Antitussive effects of *Stemona tuberosa* with different chem profiles. *J. Ethnopharmacol.* 108(1):46-53.