International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 12 (7), pp. 001-007, July, 2018. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Antiviral activity and mode of action of *Dianthus caryophyllus L.* and *Lupinus termes* L. seed extracts against *in vitro* herpes simplex and hepatitis A viruses infection

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Accepted 28 January, 2018

Crude extracts of sixteen seeds belonging to different plant species were tested for their antiviral activity against herpes simplex virus-1 (HSV-1) and hepatitis A virus-27 (HAV-27). Non-toxic concentration (20 ∝g/ml) of *Dianthus caryophyllus* and *Lupinus termes* seed extracts to both Vero and HepG2 cells showed potent antiviral activity against HSV-1 and HAV-27 using plaque infectivity count assay. The mechanism of action *D. caryophyllus* revealed its virucidal activity against HSV-1 and HAV-27 as 92.3 and 92.6%, respectively, while, the virucidal activity of *L. termes* was observed only against HAV-27 giving 93.7% of inhibition. No effect was detected for both extracts on adsorption or on the stages of virus replication. A comparison has been done between the antiviral activity of two therapeutic drugs (Acyclovir and Amentadine used as controls for HSV-1 and HAV-MBB, respectively) and the two tested seed extracts. The results revealed that these seed extracts were more efficient in their inhibitory activity than synthetic chemical drugs against the same viruses. This may open the way to give more attention to use the natural botanical origin in treatmenting viral infection with or without therapeutic agents to obtain better recovery with least side effects.

Key words: Antiviral seed extract, herpes virus infection, hepatitis virus infection, amantadine, acyclovir.

INTRODUCTION

There is currently a large and ever-expanding global population base that prefers the use of natural products in treating and preventing medical problems. This has influenced many pharmaceutical companies to produce new antimicrobial formulations extracted from plants or herbs. At present, plant and herb resources are unlimited, have provided mankind remedies for many infectious diseases and continue to play a major role in primary health care as therapeutic remedies in developing countries (Sokmen et al., 1999). The search for biological active extracts based traditionally used plants is still relevant due to induction of resistance of pathogens to chemical drugs and the prevalence of the fatal different infections (Rabindran et al., 2003). Human herpes viruses are found worldwide and are among the most frequent causes of viral infections in immunocompetent as well as in immunocompromised patients. During the past two decade a better understanding of the replication and disease causing state of herpes simplex virus type 1 and 2 (HSV-1 and HSV-2), has been achieved due the development of potent antiviral compounds that target these viruses. While some of the antiviral therapies are considered safe and efficacious (acyclovir, pencyclovir), others have toxicities associated with them (gancyclovir and foscarnet). In addition, the increased and prolonged use of these compounds in clinical setting, especially for the treatment of immunocompromised patients, has led to the emergence of viral resistance against most of these drugs (Villarreal, 2001).

Fulminant hepatitis is a severe complication of hepatitis A virus infection (HAV). Its mechanism is unknown but spontaneous recovery is frequent. There are no data on the level of viral replication according to the clinical form of HAV (Rezende et al., 2003). A high fatality rate among

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chronic hepatitis B or C patients with HAV super-infection was observed (Lee, 2003). Although there are no commercial antiviral drugs specifically licensed for treating HAV infection, ribavirin, amentadine, and 2-deoxy-D-glucose are among several antiviral substances known to interfere with HAV replication (Hollinger and Emerson, 2001).

Although a significant number of studies have used known purified plant chemicals as antiviral drugs (Binnus et al., 2002; Guarino and Sciarrillo, 2003; Jassim and Naji, 2003), very few screening programmes have been initiated on crude plant materials. Crude extracts of plant seeds are also a promising source of systemic broad-spectrum antiviral that may cause less damage to host cells than do pharmaceuticals. Topical antiviral substances are also important areas of study for the treatment of viral lesions such as in HSV, and plant-based substances offer promise as virucidal alternates (Hudson, 1990). The seed extracts of *Phyllanthus amarus* (Euphorbiaceae) species are known to reduce or eliminate detectable hepatitis B virus surface antigen in humans and show *in vitro* inhibition of viral DNA polymerase (DNAp) (Unander and Blumberg, 1991). The repeated oral administration of extracts of *Strychnos potatrum* seeds appreciably suppressed the development of skin lesions induced by HSV-1 in mice (Hattori et al., 1995). The *Pinus nigra* seed cones extract has anti-HIV activity (Eberhardt and Young, 1996). Incubation of acyclovir-resistant HSV-1 (ACVr-HSV-1), during infection of the HEp-2 cell culture, with an extract prepared from the seeds of *Licania tomentosa* species impaired the productive replication of this virus in a concentration-dependent manner. The extract was able to inhibit extracellular virus (virucidal effect) and also interfered with a very early event of cell infection at a non-cytotoxic concentration (Miranda et al., 2002).

The current investigation was undertaken to test the extracts of 16 plant seeds for their antiviral activity against herpes simples virus -1 (HSV-1, a DNA virus) and hepatitis A virus (HAV, a RNA virus). The mode of action of the most promising extracts was also studied.

MATERIALS AND METHODS

Plant material

Sixteen species of seeds belonging to different families were collected from seed bank of botanical garden, Ain Shams University; Ministry of Agriculture and land reclamation, Giza, Egypt; Orman botanical garden, Giza, Egypt and Flora and phytotaxonomy research department, Agriculture museum, Giza, Egypt.

Preparation of seed extracts for bioassay

10 mg of each crushed seed was resuspended in 1 ml solvent (10% Dimethyl sulfoxide (DMSO) in deionized water). Decontamination was carried out by adding 1% antibiotic-antimycotic mixture (10,000 IU Penicillin G sodium, 10,000 µg Streptomycin sulfate and 250 µg Amphotericin B) and the extracts were incubated at 37°C for 30 min then stored at -20°C. Sterility test was performed to ensure the sterility of the prepared extracts.

Cells

Both Vero and HepG2 cells were propagated in minimum essential medium (MEM) and RPMI 1640 medium respectively. They were supplemented with 10% foetal bovine serum, 1% antibiotic-antimycotic mixture. The cell culture was kindly provided by faculty of medicine, El-azhar University as confluent monolayer in 25 cm^2 tissues culture flasks.

Cytotoxicity assays

The cell culture safety doses of the dissolved seed extracts were performed by cell morphology technique (Aquino et al., 1989). Seed extracts were inoculated (100 µL each) into both cell lines with concentration, 5, 10, 20, 30, 40, 50 µg /100 µL and observed microscopically for any morphological changes after 24 h incubation at 37^0 C in a humidified incubator with 5% CO_2 .

Viruses

Egyptian isolate of Herpes simplex virus type 1, was provided by Virology Lab., Department of water pollution, NRC. Hepatitis A virus-MBB strain was kindly provided by Prof. Dr. Verena gauss-Muller, Molecular virology Institute, Luebeck University for Medicine, Germany.

Antiviral bioassay

Plaque infectivity count assay is the most widely accepted method for determining the % inhibition of virus as a result of being subjected to a given material (Tebas et al., 1995). A 6 well plate was cultivated with the specific cell type (10^5cell/mL) and incubated for 1 - 2 days at 37°C. Virus was diluted to final concentration of 10^7 PFU/mL and mixed with the safe concentrations of each seed extract as mentioned previously and incubated for 1 h at 37°C.

Growth medium was removed from the multi-well plate and virus-extract mixture was inoculated (100 µl/ well). After 1 h contact time for virus adsorption, the inoculum was aspirated and 3 ml of cell-specific 2× medium 2% agarose was overlaid the cell sheet. The plates were left to solidify and incubated at 37°C until the development of the viral plaques. Formalin was added for two hours then plates were stained with crystal violet staining solution. Control virus and cells were treated identically without seed extract. Viral plaques were counted and the percentage of virus reduction was calculated.

Mechanism of virus inhibition

Virus inhibition mechanism for the most potent crude seed extracts was studied in three categories:

A) Virucidal; tested by subjecting virus to extract directly (Schuhmacher et al., 2003).
B) Viral Adsorption; tested by subjecting cells to extract for 2 h before virus inoculation (Zhang et al., 1995).
C) Viral replication; tested by post inoculation of extract after virus application to cells (Amoros et al., 1994).

Table 1. Cytotoxicity of the sixteen seed extracts on Vero and HepG2 cells.

Seed extracts / Cell culture	Conc. of extracts (∝g)					
	5	10	20	30	40	50
	Vero/HepG2					
A. precatorius L.	+/+	+/+	+/+	+/+	+/+	+/+
A. cepa L.	-/-	-/-	-/-	-/-	-/-	-/-
A. nobilis L.	-/-	-/-	-/-	-/-	-/-	-/-
C. frutescens L.	-/-	-/-	-/-	-/-	-/-	-/-
D. caryophyllus L.	-/-	-/-	-/-	+/-	+/+	+/+
E. sativa Mill.	-/-	-/-	-/-	+/+	+/+	+/+
G. hispida L.	-/-	-/-	-/-	+/+	+/+	+/+
L. usitatissimum L.	-/-	-/-	-/-	+/+	+/+	+/+
L. termes L.	-/-	-/-	-/-	-/-	+/+	+/+
N. sativa L.	-/-	-/-	-/-	+/+	+/+	+/+
P. harmala L.	-/-	-/-	-/-	+/+	+/+	+/+
P. vulgaris L.	-/-	-/-	-/-	+/+	+/+	+/+
P. sativum L.	-/-	-/-	-/-	-/+	+/+	+/+
P. armeniaca Marshall	-/-	-/-	-/-	+/-	+/+	+/+
S. alba L.	-/-	-/-	-/-	+/+	+/+	+/+
T. f. graecum L.	-/-	-/-	-/-	-/-	-/-	-/-

(-): means safe to cells / (+): means toxic to cells.

RESULTS AND DISCUSSION

Cytotoxicity of tested seed extracts on VERO and HepG2 cells

Results as shown in Table (1) indicate that the accepted safe concentrations on both Vero and HepG2 cells were less than 30 µg/100 µl. The results also showed that the rate of cell death increased with increasing the concentration of the tested seed extract. However, 4 out of 16 tested seed extracts (*Allium cepa*, *Capsicum frutescens, Anthemis nobilis* and *Trigonella foenum graceum*) had no toxic effect on both Vero and HepG2 cells even when applied at high concentrations. On the other hand, *Abrus precatorius* showed high toxicity on both Vero and HepG2 cells even when applied at low concentrations.

The antiviral activity of seed extracts against HSV-1 and HAV-27

To evaluate the antiviral activities of 16 plant seeds, the inhibitory effects on the plaque formation were examined. The results in Table 2 show that out of sixteen seed extracts, *D. caryophyllus* has strong inhibitory activity against both HSV-1 and HAV-27 giving 92.3 and 92.6% inhibition at 20 µg, respectively. *L. termes* extract showed strong inhibitory activity against HAV-27 only giving 93.7% inhibition at 20 µg (Table 2). Except for these two seed species, all other tested seed extracts showed moderate or negligible inhibitory activity, giving us a green light to put both seeds under focus as promising natural extracts to be used for therapeutic purposes.

The effectiveness of seed extracts inhibiting several human viruses has been demonstrated. For examples, the hot water extract from seeds of *Arachis hypogaea* blocked HSV infection while, the hot water extract from seeds of *Pisum sativum* blocked adenoviruses (ADV) infection (Chiang et al., 2003). The hot-water extract of black soyabean showed significant antiviral activity against human adenovirus type 1 and coxsackievirus B1 (Yamai et al., 2003). The crude seed extract of *Quercus lusitanica* plant also has a good inhibitory effect on the replication of dengue virus type 2 (Muliawan et al., 2006). The purified Egyptian pea (*Pisum sativum*) lectin which was isolated from its seed showed a high inhibitory effect on HCV replication (Al-Sohaimy et al., 2007). In addition, the black cumin seed (*Nigella sativa*) exhibited antiviral activity against infectious Laryngotracheitis virus (Zaher et al., 2008).

Inhibitory action of *D. caryophyllus* L. and *L. termes* L. extracts comparing with Antiviral drugs (Acyclovir and Amentadine) against HSV-1 and HAV-MBB viruses. Effectness of *D. caryophyllus* extract and anti HSV-1 therapeutic agent (Acyclovir) was compared. Using similar concentrations of acyclovir and extract starting from 10 to 50 µg /100 µL were tested against the same virus (HSV-1). In similar manner, *L. termes extract* and anti HAV-MBB control (Amentadine) was also compared. Similar concentrations of amentadine and each extract (10 to 50 µg /100 µl) were individually tested against the

Table 2. Inhibitory activity of seed extracts (n = 16) using plaque reduction assay against HSV-1 and HAV-27.

Seed extract	Conc. (µg)	Antiviral effect					
		HSV- 1			HAV- 27		
		Initial viral count ×10ʻ	Viral count (PFU/ml) × 10ʻ	% of virucidal effect	Initial viral count ×10ʻ	Viral count (PFU/ml) × 10ʻ	% of virucidal effect
A. precatorius L.	10	2.6	High toxicity	0	3.5	High toxicity	0
	20	2.6	2	0	3.5	1.6	0
A. cepa L.	10	2.6	2.6	0	3.5	1.76	49.7
	20	2.6	2.8	0	3.5	1.6	54.3
A. nobilis L.	10	2.6	3	0	3.5	2.8	20
	20	2.6	2.54	2.3	3.5	2.6	25.7
C. frutescens L.	10	2.6	2.8	0	3.5	2.8	20
	20	2.6	2.88	0	3.5	2	42.9
D. caryophyllus L.	10	2.6	0.3	88.5	3.5	0.42	88
	20	2.6	0.2	92.3	3.5	0.26	92.6
E. sativa Mill.	10	2.6	2.4	7.7	3.5	3.3	5.7
	20	2.6	1.96	24.6	3.5	2.8	20
G. hispida L.	10	2.6	2.7	0	3.5	2.6	25.7
	20	2.6	2.65	0	3.5	2	42.9
L. usitatissimum L.	10	2.6	3.2	0	3.5	1.7	51.4
	20	2.6	1.7	34.6	3.5	1.2	65.7
L. termes L.	10	2.6	2.6	0	3.5	0.28	92
	20	2.6	2.64	0	3.5	0.22	93.7
N. sativa L.	10	2.6	2.6	0	3.5	2.4	31.43
	20	2.6	1.8	30.77	3.5	2	42.6
P. harmala L.	10	2.6	2.5	3.9	3.5	1.9	45.7
	20	2.6	2.4	7.7	3.5	1.2	65.7
P. vulgaris L.	10	2.6	3.2	0	3.5	1.2	65.7
	20	2.6	2.52	3.1	3.5	1	71.4
P. sativum L.	10	2.6	1.8	30.77	3.5	1.8	48.6
	20	2.6	1.4	46.15	3.5	1.7	51.4
P. armenia Marshell	10	2.6	2.7	0	3.5	2	42.6
	20	2.6	2.8	0	3.5	1.8	48.6
Sinapis alba L.	10	2.6	2.3	11.5	3.5	2.4	31.4
	20	2.6	2	23	3.5	1.6	54.3
Trigonella f. graecum	10	2.6	2.8	0	3.5	3.6	0
	20	2.6	2.3	11.5	3.5	2.6	25.7

same virus (HAV-27). The results in Figures 1 and 2 show that the inhibitory activity of all applied concentrations of the natural seed extracts were higher than that shown by Acyclovir and Amentadine at the same concentrations. Treatment of viral infection either using herbal extracts or combination between natural extracts and therapeutic agents has been reported in many investigations. Soybean oil showed significantly higher activity *in vitro* against both Herpes simplex virus and Para-influenza–3 virus as compared to acyclovir and Oseltamivir (Orhan et al., 2007). The synergistic effect of betulin, a pentacyclic triterpenoid, isolated from the bark of *Betula papyrifera* with acyclovir against herpes simplex viruses (Yunhao et al., 2004). A combined application of flowers of *Verbascum thapsiforme* and three amentadine derivatives resulted in a marked enhancement of the inhibitory effect of the natural extract on the reproduction of influenza virus (Serkedjieva, 2000).

Corina et al. (1999) examined the effect of extracts of Romanian medicinal plants in combination with acyclovir in the treatment of 52 patients suffering herpetic keratitis. Better results and faster healing of ulceration were obtained using *Actium lappa*, *Calendula officinalis* and *Geranium robertianum* extracts than with the usual acyclovir treatment only. These herbal extracts may have different mechanisms of anti-HSV-1 action from Acyclovir thus the combination of Acyclovir with herbal extracts might have worked synergistically. It is also observed that patients in the Far East are incorporating orthodox medical drugs into herbal medicinal preparations for alleviating their illnesses (Chan and Cheung, 2000). The rationale for doing so is to reduce the side effects of orthodox medical drugs, and to produce synergistic effects for better treatment outcome.

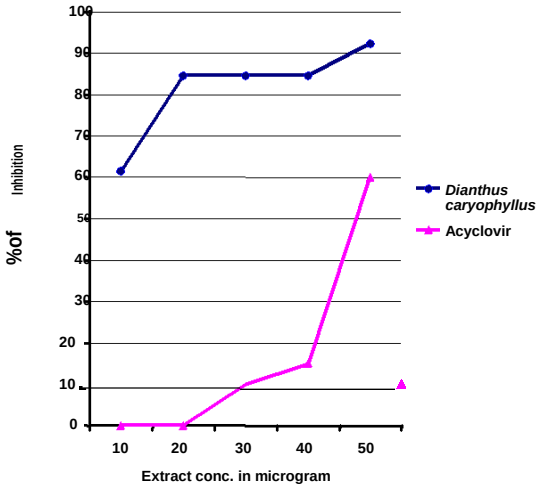

Figure 1. Comparison between *Dianthus caryophyllus* seed extract and Acyclovir for HSV-1 inhibition.

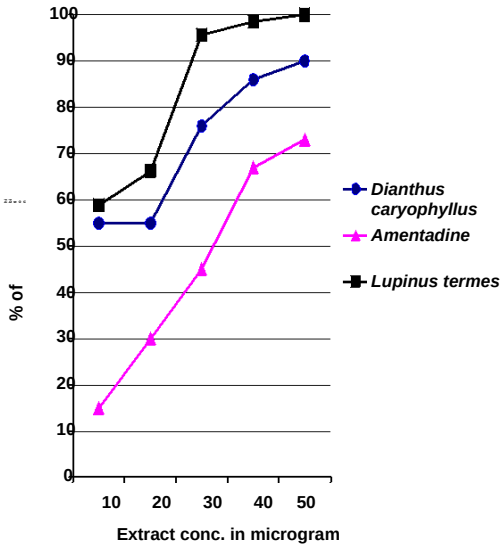

Figure 2. Comparison between *Dianthus caryophyllu*, Lupinus *termes* seed extracts and Amentadine for HAV-27 inhibition.

Mechanism of action of *D. caryophyllus* and *L. termes* extracts against HSV-1 and HAV-MBB

The results obtained by plaque infectivity count assay when the seed extracts A and B (D*. caryophyllus)* and C (*L. termes)* (Figure 3) were applied with pre and post viral treatment revealed that both seed extracts have strong virucidal activity (97.1, 88.7 and 96.9% at 60 g) either by their effect on the virus or forming a complex with the virus preventing it from being adsorbed to its binding sites on Vero or HepG2 cells. However, no effects were shown either on the early adsorption or on the replication of HSV-1 and HAV-27. These results agreed with the study on Peppermint oil which has antiviral activity against an acyclovir resistant strain of HSV-1 (HSV-1-Acv). This essential oil is capable to exert a direct virucidal effect on HSV (Schuhmacher et al., 2003). While, the mannose-specific plant lectins showed strong antiviral activity *in vitro* against the two corona viruses severe acute respiratory syndrome (SARS) and the feline infectious peritonitis virus (FIPV) at 50 - 100 µg/mL by interfering with two targets in the viral replication cycle. The first target is located early in the replication cycle, most probably viral attachment, and the second target is located at the end of the infectious virus cycle (Keyaerts et al., 2007).Generally, there are many antiviral compounds can be found in botanical sources which have the ability to inhibit human DNA and RNA viruses which causing serious diseases to humans without damaging or affecting the host cells. From this investigation, we hope to open the way for several studies in this field on these promising effectiveness natural seed extracts to be used with or without commercial therapeutic agents against human viral infections.

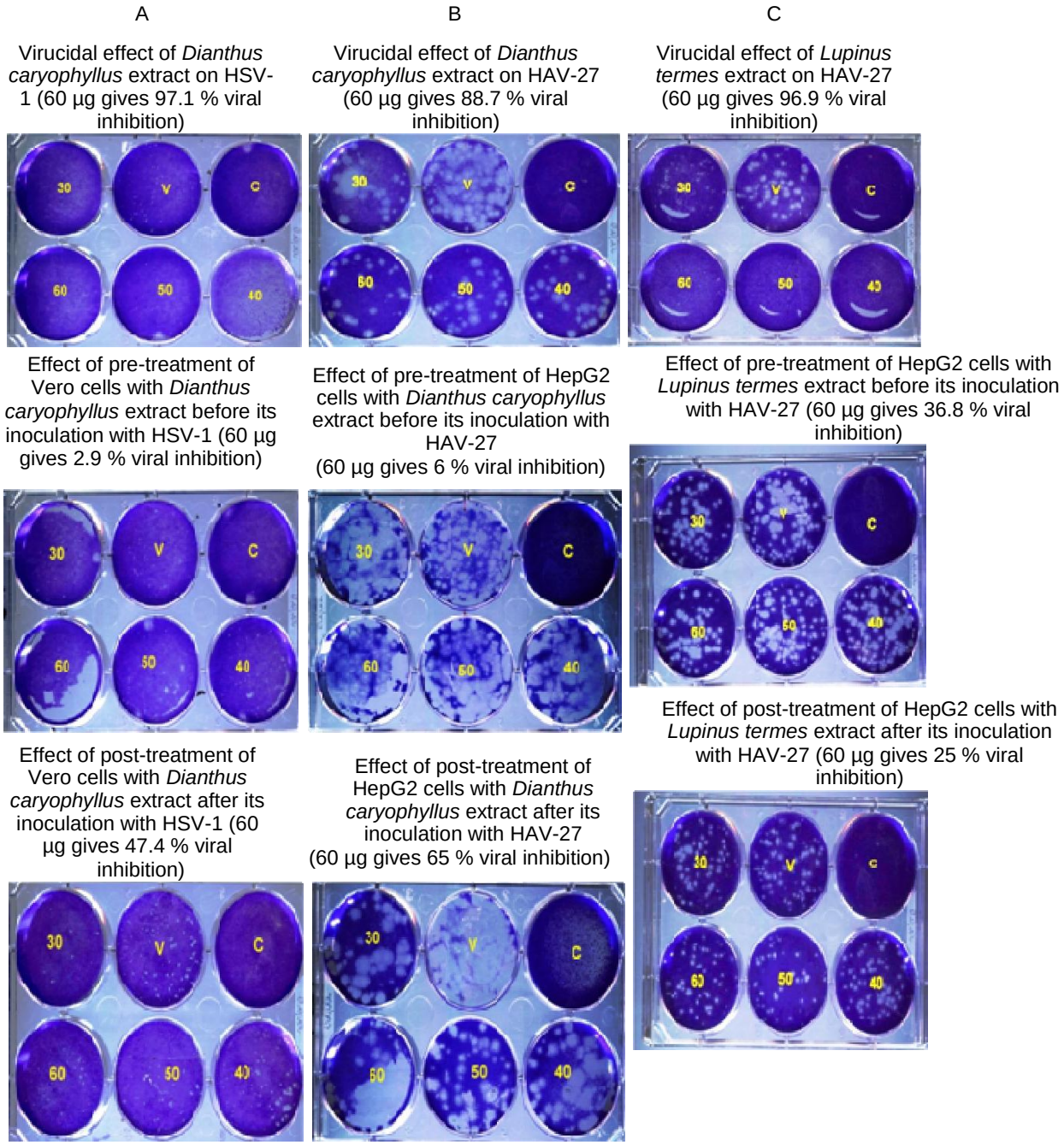

Figure 3. Studying the mechanism of action of *Dianthus caryophyllus* L. and *Lupinus termes* L. seed extracts against human viruses (HSV-1 and HAV-27).

C: Cell control; V: Virus control; 30, 40, 50, and 60: concentration / µg of seed extract used in treating each well. Color wells: no viral growth; dotted wells: obvious virus growth.

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