

Amaryllidaceae Alkaloids Exhibit Anti-Influenza Activity in MDCK Cells, an Investigation of Amaryllidaceae Alkaloids and MDCK Cells Insight

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Abstract: Influenza A viruses occasionally cause large epidemics and kill thousands every year. While little is known about the mechanism of cell fusion in diseases, especially influenza virus infected and protection from Amaryllidaceae Alkaloids. The two compounds lycorine (AA1) and haemanthamine (AA3) were obtained from bulbs of *L. radiate*, exhibited anti-influenza activity after influenza virus entry cells. The two compounds were investigated for *in vitro* displaying different levels of resistance to pro-apoptotic stimuli. Seven influenza viruses were used, A/CK/GD/178/04 (H₅N, 178), A/DK/GD/212/04 (H₅N₁, 212), A/swine/GD/166/06 (H₃N₂, 166), A/CK/HN/170/03 (H₁N₁, 170), A/Puerto Rico/8/34 (H₁N₁, PR8), A/Ck/GD/400/07 (H₅N₂, 400), A/Ck/GD/228/04 (H₅N₂, 228), the two compounds exhibited potency in the single-digit micromolar range. The studies also showed that AA1 and AA3 exerted their *in vitro* anti-influenza activity through cytostatic rather than cytotoxic effects. Many viruses interact with the host cells to change their own growth which they favor the speed. The cells infected with virus, growth of MDCK cells was slowed down by arresting cell cycle at G1/S phase. With the compound treated, the growth cycle was decreased in S phase. With H₅N₁ influenza virus treated, the cytoskeleton of cells was changed while with the compound treated the protection of cytoskeleton was obviously protected. The data showed differences between drug treated cells and virus infected cells, provided a basis to further explore cell fusion and anti-influenza mechanism of the two compounds.

Key words: Amaryllidaceae alkaloid, influenza A virus, cytoskeleton, apoptosis, epidemics, lycorine

INTRODUCTION

The bulbs of *Lycoris radiata* have long been used as a traditional Chinese medicine in the treatment of laryngeal complications, furuncles and carbuncles (Jin, 2003). Lycorine is a pyrrolo phenanthridine ring-type alkaloid and its structure was firstly determined by Nakagawa *et al.* (1956). Various biological properties of lycorine and haemanthamine including inhibition of growth and cell division in higher plants, animal and algae (Jimenez *et al.*, 1976; De Leo *et al.*, 1973) inhibition of ascorbic acid biosynthesis (Arrigoni *et al.*, 1975, 1997; Evidente *et al.*, 1983, 1986) and antimalarial activity (Sener *et al.*, 2003). Furthermore, lycorine, haemanthamine and other Amaryllidaceae small molecule constituents have been investigated for their potent anti-tumor effects and anti-leukemia effects, both *in vitro* and *in vivo* (Berkov *et al.*, 2011; Van Goietsenoven *et al.*, 2010; Liu *et al.*, 2007, 2009). Previous studies have been reported lycorine and haemanthamine displayed

significantly higher anti-proliferative activity in cancer than in normal cells and killed cancer cells by induction of apoptosis pathway (Liua *et al.*, 2004; McLachlan *et al.*, 2005; Griffin *et al.*, 2007; Dumont *et al.*, 2007; Ingrassia *et al.*, 2009; Lefranc *et al.*, 2009).

Actin was first observed and isolated by Halliburton (1987). The cutoskeletal components of host cells were changed during virus infection (DNA viruses, retroviruses and RNA viruses) involved in protein transport and RNA sorting (Cudmore *et al.*, 1985; Rutter and Mamweiler, 1977; Tashiro *et al.*, 1993). For cytoskeletal components of host cells were involved in protein transport and RNA sorting (Heskett and Pryme, 1991; Lafont *et al.*, 1995; Luna and Hitt, 1992; Ojakian and Schwimor, 1988; Sundell and Singer, 1991). Influenza virus could disrupt the stabilization of the actin cytoskeleton (Sun and Whittaker, 2007; Gottlieb *et al.*, 1993). In the study, modifications in actin cytoskeleton organization were observed by the two compounds with computer-assisted fluorescence microscopy. Apoptosis

was induced by influenza virus infected and apoptosis was contributed to the release of the progeny influenza virus. However with the drug cultivated, apoptotic rates were decreased that may be the mechanisms of drug resistance to influenza virus. WST-1 assay was utilized to determine EC₅₀ and growth inhibitory activity of each compound under study. Lycorine (AA1) and haemanthamine (AA3) induced changes in cell proliferation and cell cycle were monitored by means of microscopy and flow cytometry.

MATERIALS AND METHODS

Two compounds of amaryllidaceae alkaloids: The two compounds lycorine (AA1), haemanthamine (AA3) (Fig. 1, 4.67 μmol L⁻¹ and 4.90 μmol L⁻¹) were obtained from institute of Traditional Chinese Medicine and Natural Products, Jinan University, Guangzhou 510632, P.R. China. The isolation procedure for alkaloids from *L. radiata* was previously described (Wang *et al.*, 2009). The purity of the two compounds was identified >95%. Plants were collected from Lanxi City, Zhejiang province of China and were identified by Min-jian Qin of the Department of Pharmacognosy, China Pharmaceutical University, Nanjing, P.R. China. A voucher specimen (No.: 2006101201) was deposited in the herbarium of China Pharmaceutical University. The two compounds dissolved in DMSO and Amaryllidaceae alkaloids (each concentration of Amaryllidaceae alkaloids contained DMSO <0.5%).

Virus and cells line: Avian influenza virus A/CK/GD/178/04 (H₅N₁, GD178), A/DK/GD/212/04 (H₅N₁, GD212), A/swine/GD/166/06 (H₃N₂, GD166), A/CK/HN/170/03 (H₁N₁, HN170), A/Puerto Rico/8/34 (H₁N₁, PR8), A/Ck/GD/400/07 (H₃N₂, GD400), A/Ck/GD/228/04 (H₃N₂, GD228) were obtained by the MOA Key Laboratory for Animal Vaccine Development, P.R. China (Table 1). MDCK cell lines were obtained from Shanghai Veterinary Research Institute (CAAS, China) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, USA) with 10% (v/v) Fetal Bovine Serum (FBS, Cell Culture Bioscience, Austria). All experiments involving influenza virus were carried out in BSL-3 facilities.

Plaque-forming assay: The titers of infectious virus in culture supernatants harvested at the indicated time points were determined by plaque-forming assays. MDCK cells in a 6 well plate were infected with serial 10 fold dilutions of the virus in a serum-free medium. After washing twice with PBS, cells were overlaid with DMEM

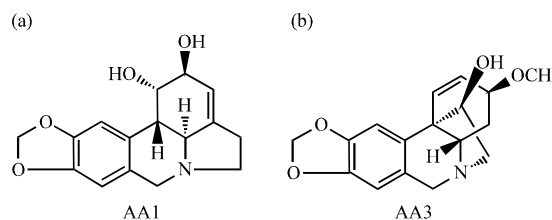


Fig. 1: Chemical structure of alkaloids; a) AA1 and b) AA3

Table 1: Antiviral activity of compounds of Amaryllidaceae Alkaloids

Virus	AA1		AA3	
	EC50 μmol L ⁻¹	SI	EC50 μmol L ⁻¹	SI
H ₅ N ₁ GD178	<0.46	>45	1.48±0.003	34.0
H ₅ N ₁ GD212	1.40±0.12	15	1.61±0.050	31.0
H ₃ N ₂ GD166	3.37±0.90	6.2	3.41±0.900	14.6
H ₁ N ₁ HN170	2.05±0.24	11	2.05±0.200	25.0
H ₁ N ₁ PR8	6.21±2.60	3.4	-	-
H ₃ N ₂ GD400	-	-	-	-
H ₃ N ₂ GD228	-	-	-	-

--: Not be detected

containing 0.8% (w/v) low-melting agarose, 0.2% (w/v) BSA, 2 μg mL⁻¹ TPCK trypsin and 0.01% DEAE. After 2 days of incubation, cells were fixed with crystal violet for 1 h at room temperature. The plaques were counted by visual examination and the titer expressed as Plaque-Forming Units (PFU) mL⁻¹. Means and standard deviations were calculated from three independent experiments.

Cytotoxicity assay, antiviral assay and Selectivity Index (SI):

The cytotoxic effect of Amaryllidaceae alkaloids on MDCK cells was determined with WST-1 assay (Ishiyama *et al.*, 1996). MDCK cells in 96 well plates (Corning, NY) were incubated for overnight and then with serial 2 fold dilutions of Amaryllidaceae alkaloids (each concentration of Amaryllidaceae alkaloids contained DMSO <0.5%) in DMEM (100 μL). After incubation for 72 h, the medium was removed and CCK-8 (Cell Counting Kit-8, Dojindo Chemicals, Kumamoto, Japan) 10 μL was added and incubated for 4 h at 37°C, measured by scanning at 450 and 630 nm reference wavelengths in a Diotek microplate reader (Molecular Devices, USA). Three independent experiments were carried out and each experiment was performed in triplicate (Table 1). The percentage of viable cells was compared with untreated controls and plotted against the concentration of the two compounds and the 50% cell cytotoxic concentration was calculated according to the Reed-Muench Method.

MDCK cells were incubated in 96 well plates (Corning, NY) for 24 h. The culture was removed and incubated the 0.01 MOI (100 μL) virus 37°C 1 h and then

removed and PBS washed three times followed by the addition of serial 2 fold dilutions of Amaryllidaceae alkaloids (each concentration was lower than CC_{50} and the cell activity was according to negative control cells) (100 μ L) per well. After incubation for 72 h, The HA titer was used to test 50% Effective Concentration (EC_{50}). The Selectivity Index (SI) for each Amaryllidaceae alkaloid was calculated by dividing the CC_{50} by the EC_{50} (CC_{50}/EC_{50}).

Cell morphology assay and cell cycle analysis: MDCK cells were synchronized in G_0 phase by serum deprivation and approximately 5×10^5 /well in a 6 well plate for 24 h. Then, cells were infected with 178 influenza virus (0.1 MOI). After 1 h adsorption period at 37°C , cells were washed and AA1 or AA3 was added containing 10% FBS until 48 h. Cell morphology was observed under the microscope (Leica, Germany). Cell cycle analysis was measured used cell cycle staining buffer (MultiSciences Biotech Co., Ltd.) by flow cytometry. The operation was followed by protocol. MDCK cells were fixed in 1 mL of cold 70% ethanol overnight at 4°C , cells were washed and collected by treatment with trypsin then washed with PBS. Nuclear DNA content was measured using Propidium Iodide (PI) staining for 30 min at room temperature. PI-stained cells were then analyzed by flow cytometry.

Time of addition assay and multicycle growth inhibition assay: Pre-treatment, simultaneous treatment and post-treatment of MDCK cells were used to determine which stages of the viral life cycle were affected by the compounds. All of these influenza viruses were added at 0.01 MOI (178 virus) to cells for 1 h adsorption. The supernatant was collected and tested HA titer after 72 h.

Flow cytometric analysis for apoptosis: To determine the influence by AA1 and AA3, the extent of early apoptosis MDCK cells were tested by flow cytometry. MDCK cells were incubated in 6 well plates for 24 h. The culture was removed and incubated 178 0.1 MOI (1000 μ L) virus 37°C 1 h and then removed and PBS washed three times, AA1 and AA3 were added until 12 hpi, the operation was followed by protocol. Cell death was determined by staining cells with annexin-V and PI (Invitrogen). Flow-cytometric analysis was used and positioning of quadrants on annexin-V and PI dot plots were performed and living cells (annexin-V-/PI-), early apoptotic cells (annexin-V+/PI-), late apoptotic cells (annexin-V+/PI+) and necrotic cells (annexin-V-/PI+) were distinguished (Vermes *et al.*, 1995), three independent experiments were performed by Beckman Coulter FC 500. The data was analyzed on FACSCalibur (BD Bioscience).

G-actin and F-actin assays: To test the protection of cytoskeleton by AA1 and AA3, actin polymerization was measured by G-actin and F-actin assays. MDCK cells were grown at 6 well, cells (1×10^6) in 1000 μ L were exposed to virus strain 178 at an MOI of 2 and incubated at 4°C for 1 h to allow virus binding. AA1 and AA3 (1000 μ L) were added and cells was collected at 12 and 24 hpi. The cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.25% triton for 15 min, washed with PBS three times and methanol once incubated with DAPI dilution (DAPI Dyeing kit, KeyGEN, China) for 15 min in the dark to stain nuclei.

For F-actin assay, Phalloidin-Tetramethylrhodamine B isothiocyanate (cat, P1951, sigma) was added to cells to stain cytoplasm for 20 min at 37°C for G-actin assay, deoxyribonuclease I, Alexa Fluor[®] 488 conjugate (cat, D12371, molecular probes) was added to cells to stain nucleus for 20 min at 37°C , removed the culture washed three times with PBS. The cells were observed using a confocal laser scanning microscope (Olympus, Japan).

Statistical analysis: All experimental groups were matched and carried out at least 2 times. Data were analyzed using Excel for determination of mean, SD and the statistical difference between the groups was determined by AVOVA.

RESULTS AND DISCUSSION

Antiviral efficacy assay: The result demonstrated that AA1 and AA3 exerted their effect at a step after virus entry into cells. AA1 and AA3 were effective against GD178, GD212, GD166, HN170 and PR8, a little inhibition on GD400 and GD228 (Fig. 2a, b). The CC_{50} of AA1, AA3 was $20.9 \pm 0.07 \mu\text{mol L}^{-1}$, $50 \pm 0.12 \mu\text{mol L}^{-1}$ for 72 h treatment by WST-1 assay. Amaryllidaceae alkaloids was demonstrated to have similar potency against these strains, interestingly, inhibition activity of H5 strain was higher than other strains (Table 1).

Cytopathic Effect (CPE) protection assay: To test the protection of the drug was added to the cells at post viral adsorption, the cells were evaluated morphologically at 48 h pi. After influenza virus infected, MDCK cells exhibited a typical tear-like cytopathic effect. This CPE was apparently inhibited when a final concentration of $4.67 \mu\text{mol L}^{-1}$ (AA1) or $4.90 \mu\text{mol L}^{-1}$ (AA3) was added. Meanwhile, there was no obvious change in cell morphology observed the drug treated containing the same concentration of AA1 or AA3, implying that AA1 or AA3 alone at this concentration did not have any

cytotoxic effect but density of cells the drug treated alone was fewer than mock cells indicated that AA1 and AA3 may affect growth of MDCK cells (Fig. 3a-f).

The inhibition of apoptosis and cell cycle: Apoptosis is an important part of the release of progeny virus of influenza virus. To check whether the drug affected

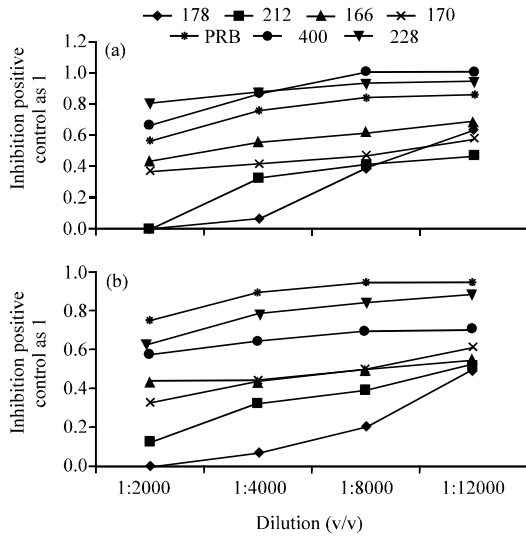


Fig. 2: Antiviral effect of AA1 or AA3 after virus entry in post assay. Seven influenza viruses at 0.01 MOI were removed after 1 h adsorption and MDCK cells were treated with AA1 or AA3 at different concentration for 72 h at 37°C under 5% CO₂ atmosphere. Each concentration was assayed by two times in triplicate; a) the inhibition of AA1 at concentration 9.34, 4.67, 2.33 and 1.55 µmol L⁻¹; b) the inhibition of AA3 at concentration 9.85, 4.92, 2.46 and 1.64 µmol L⁻¹

apoptosis rate induce by influenza virus. MDCK cells was stained with Annexin V and PI. In virus infection group (Fig. 4a), the apoptotic rate was 18.8%. In treatment of AA1, apoptotic rate was 8.4% and in AA3 treated group, apoptotic percent was 6.4%. The data indicated that the two compounds of amaryllidaceae alkaloids decreased apoptosis which was the influenza virus induced. The flow cytometry assay also showed an increase in the population of cells at the S phase in virus infected group, after cells cultivated with the compound exhibited a decrease in S phase (Fig. 4b).

Remodeling of actin cytoskeleton was inhibited by Amaryllidaceae alkaloids:

Monomeric Globular actin (G-actin) readily polymerizes to form polymeric filamentous actin (F-actin) with the concomitant hydrolysis of ATP (Taylor *et al.*, 2011). When host cells were infected virus, the mechanisms of movement ranging from diffusion within the cytosol to active transport along cytoskeletal filaments. To determine the effect of Amaryllidaceae alkaloids on the actin cytoskeleton, G-actin and F-actin were examined after virus infected. G-actin polymerization and F-actin polymerization were tested (Fig. 5a, b). With the drug treatment, G-actin and F-actin led to an increase. The F-actin and G-actin were detected by phalloidin staining. Researchers found that influenza infection of MDCK cells also affected actin fiber formation and the ratio of F-actin to G-actin. These experiments indicated that the influence of Amaryllidaceae alkaloids protected the influenza infection leads to remodeling of the actin cytoskeleton.

Different subtypes of influenza viruses causes different cellular responses. In the data, the EC₅₀ values in different subtypes of influenza viruses were so different. The stronger virulence, the better antiviral activity of the compound exhibited.

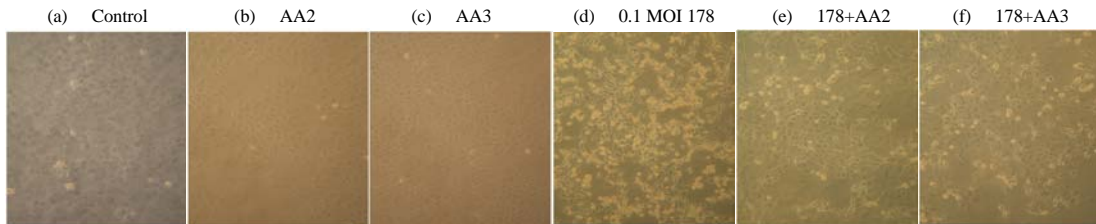


Fig. 3: Two compounds possessed antiviral activity in multiple infectious cycles. MDCK cells were adsorbed with 178 H5N1 influenza virus (MOI 0.1) for 1 h, removed and washed three times with PBS, AA1 and AA3 was added, respectively (4.67 and 4.90 µmol L⁻¹). These compounds inhibit virus-induced CPE. The antiviral effect of two compounds was subsequently evaluated morphologically 48 hpi under a light microscope. a) Mock-infected MDCK cells; b) cells incubated with AA1 only; c) cells incubated with AA3 only; d) cells infected with virus; e) cells infected with virus plus AA1; f) cells infected with virus plus AA3. The illustrations reveal cytostatic effects rather than cytotoxic ones. The concentration used here measured in WST-1 assay

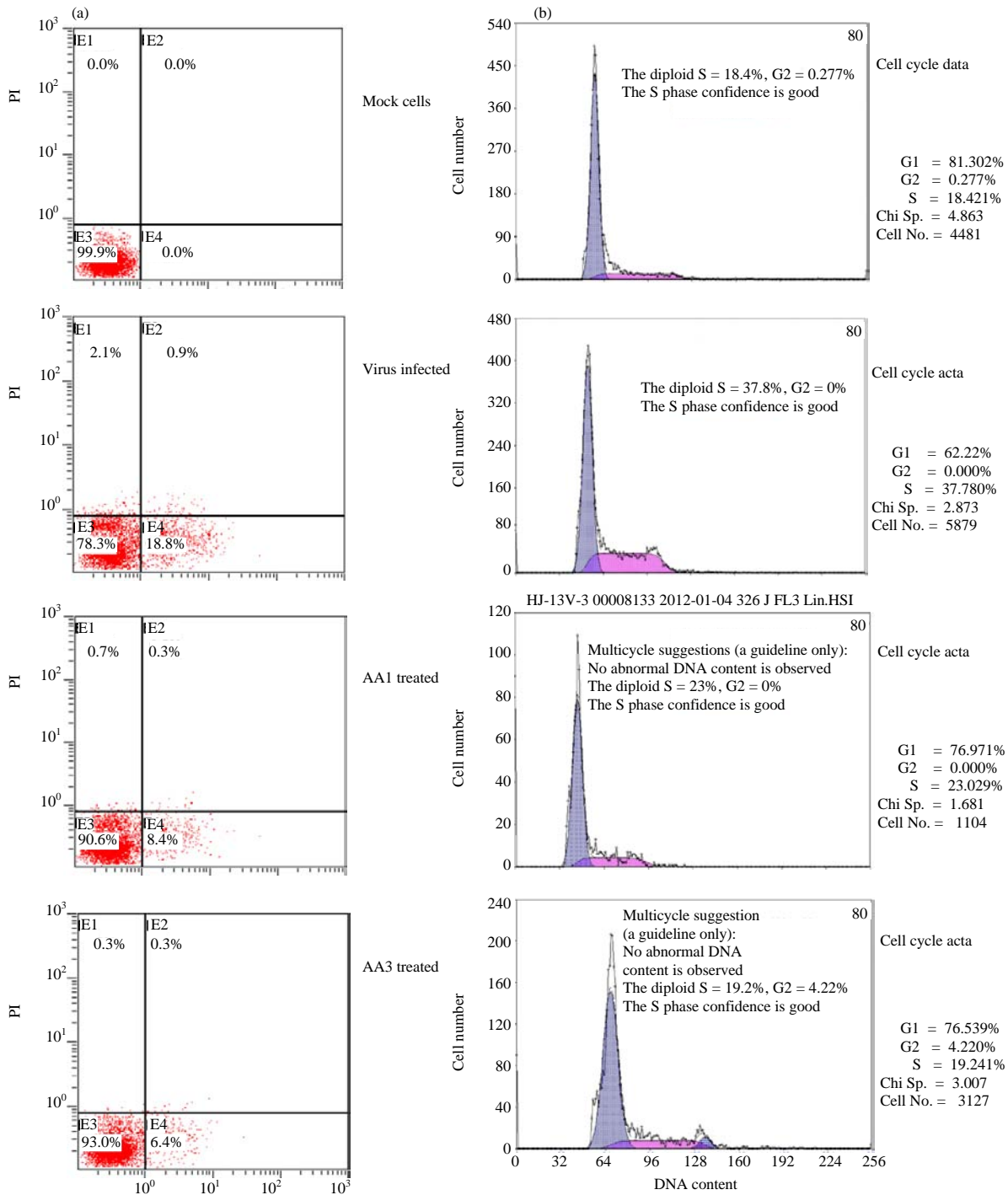


Fig. 4: AA1 ($4.67 \mu\text{mol L}^{-1}$) and AA3 ($4.90 \mu\text{mol L}^{-1}$) decreased the proportion of S phase cells and apoptotic rates (178 influenza virus, MOI 0.1); a) The ratio of apoptosis in Mock cells group, virus infected group and drug treated group for 12 h. The apoptotic percentage increased to 18.8% after infected influenza virus. The apoptotic percentage decreased to 8.4% in the treatment of AA1 group. In the treatment of AA3 group, the apoptotic percentage decreased to 6.4%; b) Cell cycle distribution was measured by flow cytometry for 48 h. The cells cultivated with the compound exhibited a decrease in S phase cells comparing with virus infected group. Three independent experiments were performed and results were consistent

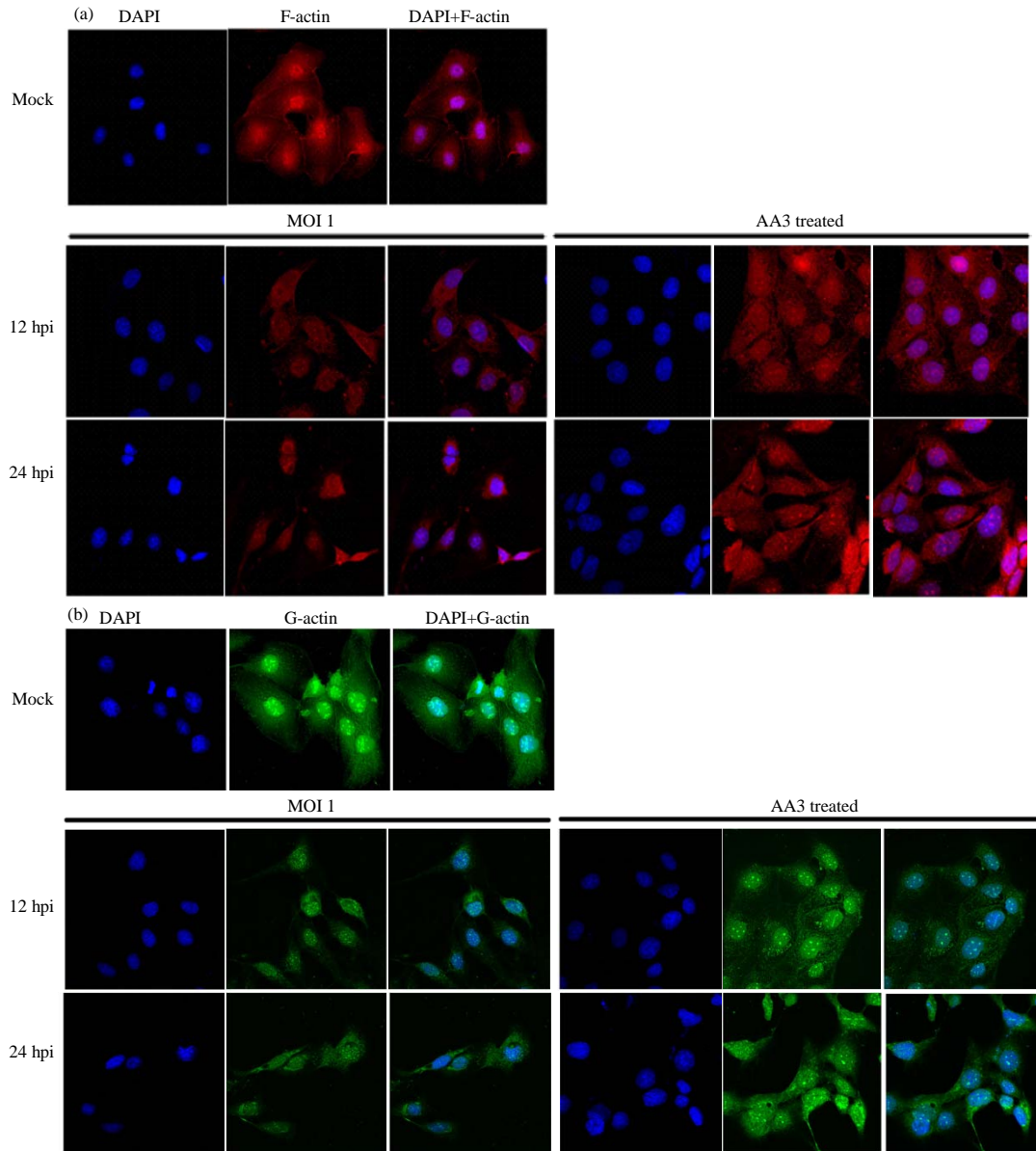


Fig. 5: Colocalization of F-actin and G-actin. Cocultured with Amaryllidaceae alkaloids during activation affected the organization of MDCK cytoskeleton. Confocal influenza infection affected actin polymerization, induced remodeling of actin cytoskeleton. MDCK cells were infected with 178 influenza virus (MOI, 2), 1 h, AA1 or AA3 was added until 12 h and 24 hpi, the F-actin (red, stained cytoplasm) and G-actin (green, stained nucleus) were observed

High pathogenicity influenza virus caused cellular changes rapidly that may be the target of the drug inhibited to exert anti-influenza activity and the anti-viral activity of two compounds was performed in the post infection. Previous reports confirmed Amaryllidaceae Alkaloids exhibited antiviral, antitumor, antimalarial and acetylcholinesterase-inhibitory activities. Some studies showed that lycorine exerted its *in vitro* anti-tumor

activity through cytostatic and induced apoptosis. Other studies showed the opposite results (Lamorral-Theys *et al.*, 2009). In the study, the inhibition of growth of cells was observed by cell morphology. The density of compounds treated MDCK cells decreased to two-thirds of the control after 2 days. These compounds suppressed the regeneration potential of MDCK cells. Researchers examined whether these compounds could

interfere with cell cycles using flow cytometry. Researchers found that after AA1 and AA3 were treated with 4.67 and 4.90 $\mu\text{mol L}^{-1}$, the percentage of cells at S phase decreased. Many anti-tumor reagents could arrest cell cycle and induced cell apoptosis (Purohit *et al.*, 2000; Kessel and Luo, 2000).

Apoptosis was very important process of the release of progeny influenza virus. With AA1 and AA3 treated, apoptosis was inhibited that may the mechanism of antiviral. Influenza virus mediated transformation included deregulation of the cell cycle and advance of apoptotic pathways. With the treatment of drugs, cells that were infected and transformed by these viruses did not produce infectious progeny virions and therefore were not lysed as a result of cytopathic effects.

The actin cytoskeleton is highly dynamic. Actin persists in the cell as two different forms: G-actin and F-actin. F-actin is composed of two parallel strands of actin monomers. One significant observation of this conversion is the reconfiguration and reorganization of cellular actin. Cytoskeleton of cells was altered when virus infected.

CONCLUSION

In this study, cytoskeleton was decreased after virus infected however with drug cultivated, the cytoskeleton was consistent with control group. Current research indicates that viral interactions with the actin cytoskeleton could be targeted for new antiviral therapies and to treat virally induced disease (Liua *et al.*, 2004). These data could provide the basis for the interaction between influenza virus and host cells that could be offered as a possible mechanism of antiviral activities.

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