Antiviral Activity of leaves extract of lantana camara against the replication of A virus A/Puerto Rico/8/34(PR8)

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Abstract

Influenza A virus is considered as of the main causative agents of mortality due to the pandemic rate, among those pandemic infection was 1918 in which; the human population lost about 140 million worldwide. In this study, we identified anew anti-viral activity of different concentrations of *Lantana camara* against the replication of influenza A virus A/Puerto Rico/8/34 (PR8) after the extraction of active compound from the plant leaves using hexane extraction method and testing different concentration on A549, MDCK II cell lines extract on and we checked the effect of this extract on some cytokines like: IFNB and IRF3 using different techniques represented by Luciferase assay. Also, we investigated the effect of this extract on some viral protein expression like NS1 protein using western blotting and real time PCR techniques and we found that the plant extract played important role in restriction of viral replication when we got a low ability of virus to replicate in different cell lines efficiently like the control sample and that was due to the enhancement of activation of cytokine expression like INFB and IRF3 in the cells that were treated with this extract and in the future we can use this extract for such enhancement to resist any viral infection because of its role in regulation of cellular immunity against any viral infection.

Keywords: lantana camara, virus A/Puerto Rico/8/34(PR8)
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الفعالية المضادة للفايروسات لأوراق نبات Lantana camara ضد تضاعف فيروس Influenza A/Puerto Rico/8/34(PR8)

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الخلاصة

تعتبر الأنفلونزا فايروس السبب الرئيسي للوفيات في العالم بسبب الوبائية العالية ومن بينها الوبائية التي حدثت سنه 1918 والي راح ضحيتها اكثر من 140 مليون إنسان في العالم. تهدف هذا الدراسة إلى استكشاف فعالية مضادة لهذا الفايروس باستخدام تراكيز مختلفة من مستخلص أوراق نبات Lantana camara . فبعد استخلاص المواد الفعالة لهذا النبات بطريقة الاستخلاص الكحولي تم معاملة بعض خطوط الخلايا A549, MDCK II بتراكيز مختلفة من النبات. وأوجدت النتائج قابلية هذا المستخلص على تحسين قدرة الخلايا على زيادة إنتاج الانترفيرون بيتا(IFNB) والاي ار اف 3 (IRF3) والتي تعتبر احد أنواع الجينات التعبيرية الخاصة بإنتاج السايتوكاينات (cytokines) وهذا بدوره قد اثر على قابلية الفايروس بالتضاعف داخل هذا الخلايا المعاملة بالمستخلص إضافة إلى التعبير البروتيني لاحد البروتينات المهمة في حياة الفايروس وهو NS1 والذي يلعب دور مهم في تثبيط المناعة ضد أي فايروس. فعملية زيادة إنتاج الانترفيرون بيتا (IFNB) و(IRF3) أدت إلى حدوث تحسن ملحوظ في قدرة الخلايا على تقييد قابلية الفايروس على التضاعف بعد إصابتها مقارنة مع الخلايا الغير معاملة بالمستخلص والمصابة بالفايروس وبالتالي يمكن استخدام هذا المستخلص لتحسين قدرة الخلايا المناعية على مقاومة أي إصابة فايروسية من هذا النوع في المستقبل.

الكلمات المفتاحية: لانتانا كمارا، فايروس A/Puerto Rico/8/34(PR8).

Introduction

Influenza A virus is considered as one of the most pathogenic microorganisms, this virus has the ability to infect the epithelial cells of upper respiratory tract of human and animals, annually this virus can infect more than 10% of human population resulting in the death of more than 250000 patient (1). This virus is categorized under the *Orthomyxoviredae* family which is considered a single stranded RNA virus (ssRNA) divided into Influenza A, B and C. both of Influenza A and B contain 8 genomic segment while influenza C contains seven segments onlyThe main symptoms that accompanied viral infection are; inflammation of upper respiratory trachea, high fever, headache, cough and malaise. Influenza viruses cause seasonal, endemic infections and periodic, unpredictable pandemics. In 1918, approximately 50 million people worldwide died due to infection with Spanish flu (2). The genetic material of this virus is divided into eight segment coded for one or more than one protein, the largest three segments are coded for polymerase protein Basic2 (PB2), polymerase protein Basic1 (PB1) and polymerase protein acidic (PA) (3). While the intermediate segment are coded for nucleoprotein (NP), nuraminidase (NA) and hemagglutinin (HA) (4) and the smallest segment are coded for two proteins, the largest one is coded for matrix protein (M) and M2 ion channel and the smallest one is coded for non-structural protein (NS1 and NS2) (5). Each one of those proteins has its role during viral replication cycle. *Lantana camara* is considered as one of the medical plant which are used as a strong anti-bacterial plant due to its ability to inhibit the growth of many species like; *Bacillus subtilis*, *Staphylococcus aureus*..etc. (6). Lantana camara is a flowering ornamental plant which is related to *Verbenaceae* family. This plant is known as Wild Sage, Surinam Tea Plant, Spanish flag. Recent studies had shown that, *L. camara* is used traditional medicinal system and due to the large advantages that came from its activity in the treatment against many bacterial species(7). In this study, we tried to investigate a new activity for this plant which will serve to improve the ability of the immune system to restrict and antagonize the viral replication there by reducing its pathogenicity.

Materials and Methods

Experimental procedures

- **Cell lines, viruses:** In this work, different sets of cell line were used to achieve this research like: The human lung adenocarcinoma cells (A549) and The Mardin-Darby canine kidney cells (MDCKII). Those cells were cultivated in minimum essential medium (Sigma Aldrich) and Dulbecco's modified Eagle's medium and minimum essential medium were supplemented with 10% fetal calf serum (Merck-Millipore) then, they were incubated at 37°C and 5% CO2 under constantly humidified conditions. Regarding the virus, we used human influenza virus A/Puerto Rico/8/34 (PR8; H1N1. The infection of the virus were performed in those cell lines at a standard multiplicity of infection (MOI) for the indicated times as described previously(8).
- **Plant extraction:** *L. camara* leaves were extracted using hexane, ethanol. First of all, Leaves were infused in hexane (1:7 w/v) for 24 hours and filtered using What man filter paper. Finally, the extracts were filtered and concentrated at 50oC in rotary evaporator with a final volume of 20 mL. The dilution of plant extract was done through preparing of different concentrations (0.05 and 0.1 mg/ml) from stock solution of 1mg/ ml.
- **Protein analysis by western blot:** The protein expression was done by Western blotting. At specific times after infection, the infected monolayer was washed with PBS (Sigma) and cells were lysed in RIPA buffer (137 mMNaCl, 10 % glycerol, 25 mMTris-

HCl [pH 8], 0.1% sodium dodecylsulfate, 1% Igepal, 2 mM EDTA [pH 8],0.5 % sodium deoxycholate) and this lyses buffer is supported with protease inhibitors to inhibit protein lyses. Lysates were cleared by centrifugation (20000 × g, 4°C, 10 min). The sample buffer were added to lysed samples and clear supernatant was then boiled for 5 min at 95°C. Equal amounts of total protein were used for separation in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Finally, proteins were blotted onto nitrocellulose membranes (GE Healthcare). The staining of protein were done by incubation with the primary mouse anti-NP (clone AA5H, AbDSerotec), mouse anti-NS1 (clone NS1-23-1) or rabbit anti-ERK2 (Santa Cruz) which were diluted in TBST buffer (150 mMNaCl, 0.2% Triton X-100,50 mMTris-HCl [pH 7.5]) was performed over night at 4°C. Horseradish peroxidase labeled secondary antibodies anti-goat (Cellular Microbiology ImmunoResearch Laboratories or Santa-Cruz), anti-rabbit (Bio-Rad or Cell Signalling) or anti-mouse (Jackson Immuno Research Laboratories or Cell Signalling) were diluted in TBST and incubation was performed for 0.5-1 h at room temperature. Protein detection was performed on Stella system.

- **Standard plague assay:** This assay were used for estimating the viral replication on MDCK II cells which were grown to 90% confluence in six-well dishes then, they were washed and infected with 0.01 MOI of PR8 diluted in PBS/BA for 30 min at 37°C and 5% CO2. The inoculum was aspirated, and cells were covered with 1.5 ml MEM/BA supported with 0.6% agar (Oxoid, Hampshire, United Kingdom), 1.5% NaHCO3 (Gibco Invitrogen, Karlsruhe, Germany) and 0.3% DEAE-dextran (Amersham Pharmacia Biotech, Germany). The incubation was performed at 37°C and 5% CO2 for 2 to 3 days and finally, virus plaques were visualized by staining with neutral red (Sigma-Aldrich, Germany).
- **RNA isolation, cDNA synthesis and rt PCR:** RNA isolation was from infected cells was implemented using the RNeasy Mini Kit or RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol. Concentration of purified total RNA was measured by a Nanodrop ND-1000 spectrophotometer (Peqlab). Then, equal amounts of RNA were used to synthesize cDNA using Thermo Fisher Scientific kit according to the manufacturer's protocol. The quantification of cDNA was performed by quantitative real-time polymerase chain reaction (qRT-PCR) and the following primers: GAPDH_fwd 5′-gcaaattccatggcaccgt-3′, GAPDH_rev 5′- gccccacttgattttggagg-3′, NS1_fwd 5′- tgccttctcttccaggacat-3′, NS1_rev 5′-ccattcaagtcctccgatg-3′, IFNB_fwd5′-tctggcacaacaggtagtaggc,INFB_rev 3′-gagaagcacaacaggagagcaa.The qRT-PCR reaction mix (Brilliant III SYBR Green QPCR Master Mix) was purchased from Agilent Technologies. Analysis was performed as described earlier, the GAPDH primers used as a control to calculate the CT values (8).
- **Reporter Gene Assay:** A549 cells were transfectedwith 0.5 μg luciferase reporter gene plasmids having the complete IFN promoter (pTATA-luc-IFN-β) using Trans ITLT1 transfection reagent (Mirus Bio) according to the manufacturer protocol. 24 h post transfection cells were treated with plant extract as mentioned previously then infected with PR8 for 8 hours with 5 MOI. Finally, luciferase activity was performed as mentioned in (5).

Results

- **Viral replication titer had been reduced due to the treatment with plant extract:** To study the effect of the plant extract on vial ability to replicate in MDCK cells, $1*10^6$ cell/ ml of MDCK cell were treated with 0.05 and 0.1 mg/ml of the plant extract respectively then, cells were infected with 0.01 MOI of Influenza A virus PR8 and the viral replication cycle were determined at different time points using standard plague assay. In figure 1A, we can see significant reduction in viral replication titer which was about one log or more between the cells that were infected with the virus after the treatment than those untreated one after 8 hours post infection which represents one cycle for viral replication. The same thing happened when we repeated the experiment using the same condition but after multiple viral replication cycles as in figure1B and C so, we can definitely conclude that the viral replication had been negatively reduced due to the treatment with this plant.

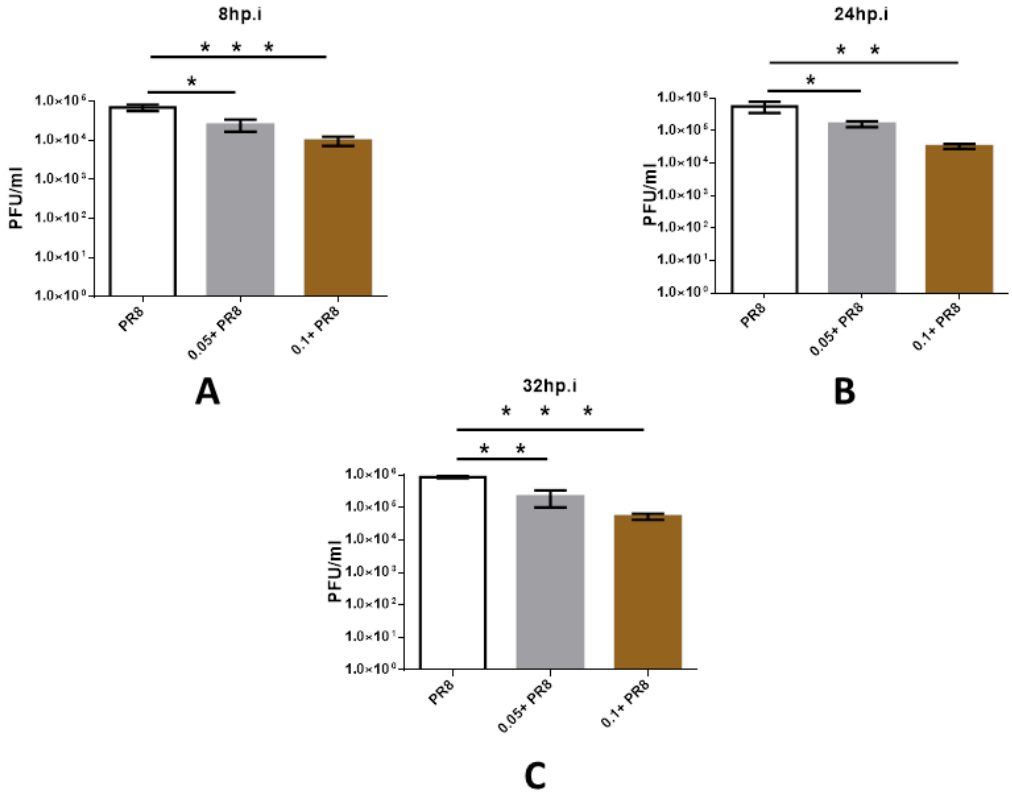

Fig. (1) showed reduced viral replication titer due to treatment with plant extract. A,B and C respectively represent MDCK cells that were either infected or treated with 0.05 and 0.1 mg/ml then infected with influenza A virus then result were analyzed by plague assay.Data represents mean ± SD of three independently repeated experiments. One-way ANOVA by Dennett's multiple comparisons test using PR8 infection as controls was used for statistical analysis of each time point separately (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

- **mRNA level and expression of viral NS1 protein expression was deceased due to the treatment with plant extract:** To check if the treatment of the cells with the plant extract affected on viral protein expression, A549 cells were either, infected with PR8 or treated with plant extract then infected with PR8, later on the cells were lysed and protein expression were checked by western blotting analyses. Figure 2 had shown NS1 protein expression of influenza A virus after 8 hours post infection. This picture is composed of 3 lanes, the first one represents influenza A virus infection only in which we can see the strongest protein expression, while in lane2 we found that the expression is going to be weaker than the control and at highest treatment with plant extract, the expression of NS1 protein was the weakest one indicating that the viral NS1 protein expression had been affected negatively due to this treatment. This finding confirms the previous one regarding the viral replication which had shown weak ability to replicate upon the treatment.

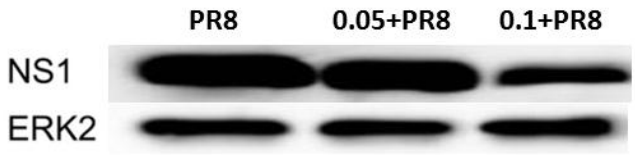

Fig. (2) Regression of viral NS1 protein expression upon treatment with plant extract. NS1 expression by Western blotting after infection of A549 cells with PR8. The detection of NS1 protein is done by mouse anti-NS1 antibody. ERK2 protein served as loading control.

The expression of viral NS1 protein were strongly supported by mRNA level of NS1 using real time PCR technique in which, we either infected A549 cells with 5 MOI of PR8 as a control or treated the cells with 0.05 and 0.1 mg/ml of plant extract then infected with 5 MOI of PR8. Later on, the cells were lysed at 4, 6 and 8 hour and results were analyzed by real time PCR. At 4 hour post infection, we can see significant decrease in mRNA level of NS1 protein with the cell that were treated with plant extract then infected with PR8 than untreated cells as in figure 3A. Interestingly, these results did not change at 6 and 8 hours post infection. The mRNA level of viral NS1 protein in untreated cells had shown higher expression than the treated samples suggesting that mRNA level of NS1 protein had been regressed after the treatment with plant extract as in figure 3B and C.

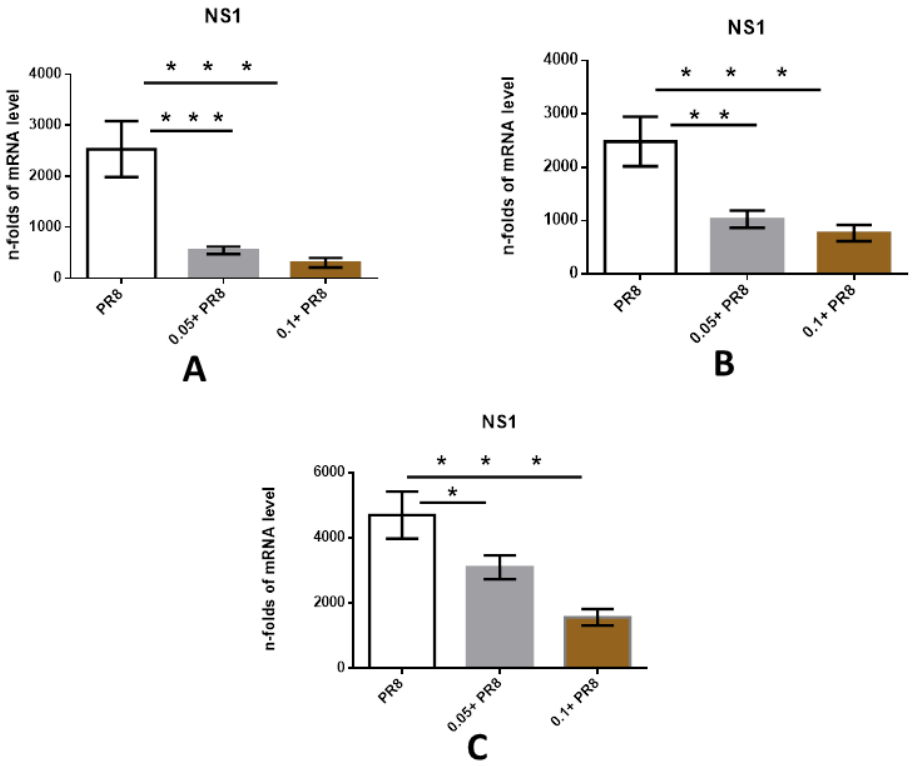

Fig. (3) mRNA levels of NS1 protein was negatively reduced by this treatment.

A,B and Crepresent expression levels of mRNA coding for NS1 proteins, after infection of untreated and treated A549 cells with plant extract . Results were analyzed using qRT-PCR at 4, 6 and 8 hours post infection. Data represents the mean ± SD of three independently repeated experiments. One-way ANOVA followed by Dennett's multiple comparisons test using untreated cells as controls was used for statistical analysis of each time point separately (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

- **The treatment of plant extract induced the production of IFNβ genes:** The IFN promoter comprises three functional transcription factor binding sites, AP-1, NF-κB and IRF3. To reveal if IFN promoter activity was influenced bythe treatment of the cells with plant extract, A549 cells were overexpressed with IFN luciferase promoter gene for 24 hours then cells were, either infected with PR8 as a control or treated with the plant extract at different concentrations then infected with PR8, but here we checked the induction of IFNB by overexpression of the cells with IFN promoter gene and treated them with plant extract as a positive control. Figure 4A explains the promoter activity that was determined by luciferase assay in which we detected a significant increase in the promoter activity of IFN with the cells that were treated with plant extract compared with the cells that were infected with the virus alone. To confirm these finding, we checked mRNA levels of IFNB by real time PCR when we infected A549 cells with 5 MOI of PR8 for 8 hours and here, we used untreated cells as a control for this experiment and we found that the induction of INF mRNA levels was enhanced significantly upon treatment with plant extract as shown in figure 4B. This explains the important role of plant extract to induce the induction of IFN stimulating gene which in turn lead to reduce the viral replication ability.

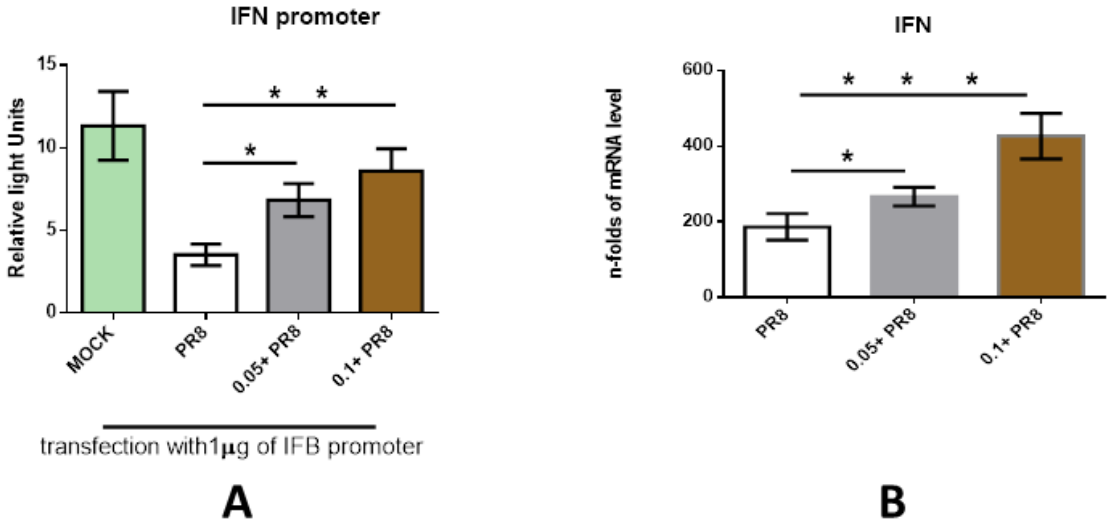

Fig. (4) Enhancement of IFN promoter due to the treatment with plant extract.

(A) A549 cells were transfected with luciferase reporter gene plasmids harboring the IFNβ promoter. After 24 h then cells were infected with PR8 as a control or treated with plant extract and infected with PR8 and luciferase activity in cell lysates was measured.(B) IFNβ mRNA expression in A549 cells infected with PR8 viruses after the treatment with plant extract was measured by qRT-PCR. Values represent n-fold expression from infected, non-treated cells Results represent means ± SD of three independently repeated experiments. Statistical significance was analyzed by one-way ANOVA followed by Dunnett´s multiple comparisons test(** $p \leq 0.01$, *** $p \leq 0.001$).

Discussion

It is well known that immune system try to defend against any viral infection via the activation of cellar signaling, the activation of these signaling cascades lead to initiate the expression of type I interferon (9). But influenza A virus is considered as one of the most pathogenic microorganisms that restrict the induction of interferon due to the ability block this type of activation and this ability to do that, came from NS1 protein which is considered as a strong interferon antagonistic factor. NS1 protein has many ways to do such work, its able to block RIG I inducible gene which is considered as a strong activator for INF expression genes (10, 11, 12, 13). In our work, we identified a new anti-viral activity for this plant beside other anti-microbial activities because upon the cellular treatment; the viral ability to replicate is weakened in all time points used in our survey if we compare them with the cells which were infected with PR8 only. Interestingly, the results of western blotting confirmed reduction in viral replication by the significant impairment in the intensity of NS1 protein expression with the cells that were treated and infected with PR8 compared with those untreated one. Also, the mRNA level of NS1 protein showed us significant reduction if we compared the n- fold of mRNA expression for the cells that were treated and infected with PR8 with untreated sample. From these results, we should look for an explanation for phenomena, in another word; what is the main reason that led to reduce all of those factors mentioned above? The best answer for this question is by checking the level of IFN in the cells which is considered as the main reason behind this reduction, the promising results that we got from promoter activity and mRNA levels of INFB genes explain the reasons behind that reduction, the cells that were treated with plant extract proved the ability of this plant to enhance the induction of INF but with viral infection only; we can see the weak expression of INFB promoter and with both of treated and infected samples, we detected the enhancement of INFB induction even with viral

infection at such level. It is well known that IFNB is considered as one of the most important transcription gene that participates in activation of Immune system due to its involvement in activation of many sophisticated pathways (14).

References

1. McCaughey, C. (2010). Influenza: A virus of our times. Ulster Med. J., 79(2):46–51.
2. Watanabe, T. & Kawaoka, Y. (2011). Pathogenesis of the 1918 pandemic influenza virus. PLoS Pathog., 7(1):e1001218.
3. Nakagawa, Y.; Oda, K. & Nakada, S. (1996). The PB1 subunit alone can catalyze cRNA synthesis, and the PA subunit in addition to the PB1 subunit is required for viral RNA synthesis in replication of the influenza virus genome. J. Virol., 70(9):6390-6394.
4. Skehel, J. J. & Wiley, D. C. (2000). receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev. Biochem., 69:531-569.
5. Hillesheim, A.; Nordhoff, C.; Boergeling, Y.; Ludwig, S. & Wixler, V. (2014). β-catenin promotes the type I IFN synthesis and the IFN-dependent signaling response but is suppressed by influenza A virus-induced RIG-I/NF-κB signaling. Cell Commun. Signal, 12: 29.
6. Salada, A. T.; Balala, L. M. & Vasquez, E. A. (2015). Phytochemical and Antibacterial Studies of *Lantana camara*L. Leaf Fraction and Essential Oil. Int. J. Sci. Res. Pub., 5 (3): 1-5.
7. Kalita, S.; Kumar, G.; Karthik, L. & Rao, K. V. (2012). A Review on Medicinal Properties of Lantana camara Linn. Res. J. Pharm. Tech., 5(6): 711- 715.
8. Dudek, S. E.; Luig, C.; Pauli, E. K.; Schubert, U. & Ludwig, S. (2010). The clinically approved proteasome inhibitor PS-341 efficiently blocks influenza A virus and vesicular stomatitis virus propagation by establishing an antiviral state. J. Virol., 84: 9439-9451.
9. Goubau, D.; Deddouche, S. & Sousa, C. R. (2013). Cytosolic sensing of viruses. Immunity, 38: 855-869.
10. Guo, Z.; Chen, L. M.; Zeng, H.; Gomez, J. A.; Plowden, J.; Fujita, T.; Katz, J. M.; Donis, R. O. & Sambhara, S. (2007). NS1 protein of influenza A virus inhibits the function of intracytoplasmic pathogen sensor, RIG-I. Am. J. Respir. Cell Mol. Biol., 36: 263–269.
11. Mibayashi, M.; Martinez-Sobrido, L.; Loo, Y. M.; Cardenas, W. B.; Gale, M. Jr. & Garcia-Sastre, A. (2007). Inhibition of retinoic acidinducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. J. Virol., 81: 514-524.
12. Opitz, B.; Rejaibi, A.; Dauber, B.; Eckhard, J.; Vinzing, M.; Schmeck, B.; Hippenstiel, S.; Suttorp, N. & Wolff, T. (2007). IFNb induction by influenza A virus is mediated by RIG-I which is regulated by the viral NS1 protein. Cell Microbiol., 9: 930-938.
13. Pichlmair, A.; Schulz, O.; Tan, C. P.; Naslund, T. I.; Liljestrom, P.; Weber, F. & Reis e Sousa, C. (2006). RIG-I-mediated antiviral responses to single-stranded RNA bearing 59-phosphates. Science, 314: 997-1001.
14. Lee, M. K.; Kim, H. E.; Park, E. B.; Lee, J.; Kim, K. H.; Lim, K.; Yum, S.; Lee, Y. H.; Kang, S. J.; Lee, J. H. & Choi, B. S. (2016). Structural features of influenza A virus panhandle RNA enabling the activation of RIG-I independently of 5'-triphosphate. Nucleic Acids Res., 44(17):8407-8416.