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ANTI-INFLUENZA POTENTIAL OF ALKALOIDAL MOLECULES OF *JATROPHA CURCAS* LEAVES

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ABSTRACT: Background: The Influenza viruses are major etiologic agents of human respiratory infections, and inflict sizable health and economic burden. **Methods:** The present study reports the *in-vitro* antiviral effect of isolated alkaloidal compound of Jatropha curcas leaves against Influenza virus by Hemagglutination (HA) reduction assay in two different layouts of simultaneous and post-treatment assay. The alkaloidal compound was used for anti-influenza activity in the non-cytotoxic range. The separated compound (R_f 0.5) was eluted, crystallised and identified by using NMR and FTIR analysis and co-TLC with standard compound of Tetra Methyl Pyrazine(TMP). Results: The Thin Layer Chromatographic analysis showed that ethyl acetate: dichloromethane (1:1) was the best solvent for alkaloidal separation. The identified Tetra-Methyl Pyrazine showed 100% reduction in HA in the post-treatment assays at the concentration of 10mg/ml and 5mg/ml. In simultaneous assay, HA was reduced to 71% and 42% at concentration of 10mg/ml and 5mg/ml respectively. Conclusions: These results suggest that TMP has strong anti-Influenza virus activity that can inhibit viral attachment and/or viral replication and may be used as viral prophylaxis

INTRODUCTION: Influenza virus is a severe threat to human health in all parts of world. The control and treatment of Influenza Virus depends mainly on chemical or biochemical agents and, recently, some plant derived anti-Influenza agents have been isolated as a result of chemical and pharmacological studies. These agents include a variety of polyphenols, flavonoids, saponins, glucosides and alkaloids. Traditional medicine had been shown a potential in the therapy of Influenza and its symptoms.



To date, only two classes of anti-Influenza drugs have been approved: inhibitors of the M2 ion channel, such as amantadine and rimantadine, or neuraminidase inhibitors, such as oseltamivir or zanamivir. Among influenza viruses, avian influenza H5N1 isolates from Thailand and Vietnam, 95% of the strains have exhibited genetic mutations associated with resistance to the M2 ion channel-blocking amantadine and its derivative, rimantadine. Furthermore, Influenza B viruses are not sensitive to amantadine derivatives, hence it's a necessity to find out new drugs that will develop minimum resistance in virus. With the increase in resistance to antibiotics, natural products represent an interesting alternative.

Many products have been evaluated not only for direct antimicrobial activity, but also as resistance-modifying agents. ⁶ In addition, there are several

reports, which suggest that resistance in H5N1 viruses can emerge with the current recommended treatment of oseltamivir and such resistance may be associated with clinical deterioration. ⁷ Thus, it has been stated that the treatment strategy for Influenza A (H5N1) viral infections should include additional antiviral agents. All these highlight the urgent need for new and abundantly available anti-Influenza agents.

Plants have a long evolutionary history of developing resistance against viruses and have increasingly drawn attention as potential sources of antiviral drugs. ⁸⁻⁹ A wide variety of natural compounds derived from medicinal plants (herbs) have been extensively studied for their antiviral activity. Several hundred natural active compounds have been identified worldwide. ¹⁰⁻¹⁴ Many of them have complementary and overlapping mechanisms of action, either inhibiting viral replication, or synthesis of the viral genome.

These natural active compounds contain high chemical diversity and biochemical specificity, offer major opportunities for finding novel lead structures that can be active against a wide range of assay targets. In addition, natural products are small molecules that can be absorbed metabolized by the body. Hence, the development costs of producing orally active medicines are likely to be much lower than biotechnological products. Therefore, natural products, including traditional medicinal plants (herbs), offer great promise as potentially effective new antiviral.¹⁵

A number of studies have shown the efficacy of plant derived antiviral agent. An indole alkaloid from *Uncaria rhynchophylla* and the pavine alkaloid (-)-thalimonie (Th1) from *Thalictrum simplex* also exhibit potent inhibitory effects against Influenza A viruses. Many medicinal plants (herbs) including the *Bergenia ligulata*, *Nerium indicum* and *Holoptelea Integrifolia* plants also revealed considerable antiviral activities against the Influenza virus. 16-17

Jatropha curcas is a drought resistant, perennial plant that grows even in the marginal and poor soil. In the recent years, Jatropha has become famous

primarily for the production of biodiesel. Most parts of this plant are used for the treatment of various human and veterinary ailments. In children, the white latex of Jatropha serves as disinfectant in oral infections. ¹⁸ The latex of Jatropha contains alkaloids including Jatrophine, Jatropham and curcain with anti-cancerous properties. It is also used externally against skin infections, piles and sores among the domestic livestock.

The leaves contain apigenin, vitexin and isovitexin etc, which along with other factors enable them to be used against malaria infections, rheumatic and muscular pains. The alkaloidal compound isolated from Thin Layer Chromatographic analysis of Jatropha curcas has been evaluated for its antibacterial activity against pathogenic bacteria. The Nuclear Magnetic Resonance (NMR) analysis of isolate confirmed the presence of Tetra Methyl Pyrazine alkaloid. Its anti-bacterial activity against Staphylococcus aureus, Enterobacter species, Escherichia coli and Salmonella typhie 19 showed a remarkable potency of compound, which could be used in further studies. Anti-HIV activity of methanolic extract of Jatropha curcas leaves has been established in our laboratory ²⁰. Keeping in mind, the factorial significance of Jatropha curcas as described above, the present study an extension of previous results was carried out to assess the anti-Influenza activity of the isolated alkaloid from its leaves.

MATERIALS AND METHODS:

This study was conducted in the Department of Virology and Immunology, Haffkine Institute for Training, Research and Testing, Mumbai, India and it was carried out for 9 months from October 2013 to April 2014. It was aimed to analyze the phytochemical constituent's and the antiviral effects of the isolated compound on the basis of Hemagglutination reduction assay. The study was approved by the Institutional Ethics Committee of Haffkine Institute.

Preparation of Plant extract:

The plant of *Jatropha curcas* was grown in the Haffkine Institute campus and identified by Botanist at Haffkine institute. The plant material (leaves) were collected in the month of April 2013

and kept for the further reference specimen (HITRT/ZNS/plant spec. /ref -11).

Leaves of *Jatropha curcas* were dried at 100°C for 15 minutes and then at 40°C until a constant weight were achieved. The sample was powdered, weighed and ground in mortar and transferred to a vessel. The alkaloid was extracted by using a protocol previously cited ¹⁹, it was further dried in-vacuo and crude alkaloid content was calculated.

Thin Layer Chromatography (TLC):

TLC was carried out using pre-coated silica gel plates (Merck, Germany). The dried alkaloidal crude sample of Jatropha curcas was dissolved in distilled water and was applied 1cm above the edge of the plates along with reference compounds of Tetra Methyl Pyrazine. The plates were dried and developed in various solvent system separately (nbutanol: acetone: water; 145:5:50, Iso-butanol: water; 85:15), but the best separation was seen in ethyl acetate: dichloromethane (1:1). Developed purple spot was observed under UV light and Retention factor (Rf) value was calculated (0.5).¹⁹ A developed spot corresponding with that of the standard compound was sprayed with Dragendorfs reagent that showed the characteristic brick red color and confirmed the presence of alkaloid. The fluorescent spots under the UV light were collected along the silica gel and re-extracted with distilled water.

Fourier Transform Infrared Spectrometry (FTIR):

The sample which was eluted using TLC was subjected to Fourier Transform Infrared Spectrometry (FTIR) (SHIMADZU) in Department of Chemotherapy, for analyzing the spectral data in a wide infrared spectral range.²¹

Reagents:

All extraction reagents such as ethanol, methanol was Analytical reagent (AR) grade purchased from Hi-media Labs (Mumbai, India). Reagents for cell culture, such as Minimum Essential Medium Eagle (MEM), Trypsin-Ethylenediaminetetraacetic acid (EDTA), and sodium bicarbonate, antibiotics and Oseltamivir phosphate were purchased from Sigma Aldrich (Catalog no – 479304; Mumbai, India). Pre-coated silica gel 60G F254 TLC aluminium

plates (10x10cm, 0.2mm thick) were obtained from E. Merck Ltd. (Mumbai, India). Analytical grade toluene, ethyl acetate, methanol, chloroform, glacial acetic acid, diethyl amine and formic acid were obtained from SD Fine Chem. Ltd (Mumbai, India).

Cell Line and Viruses:

Madin-Darby Canine Kidney (MDCK) cell lines were procured from Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGI, Lucknow) and were grown in MEM with L-glutamine (2mM), penicillin (100IU/ml), streptomycin (100µg/ml) and gentamicin (10µg/ml), and supplemented with 10% Foetal Bovine Serum (FBS). The standard strain of Influenza virus A/H1N1/2009 was obtained from the Department of Microbiology, SGPGI, Lucknow (UP). Confluent MDCK cell monolayers in 96-well tissue culture plates were washed once with serum free MEM before use. Serial 10-fold dilutions of virus in serum-free MEM containing 0.3% bovine serum albumin (BSA) and 1µg/ml L-(toslyamido 2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) were incubated in replicate wells (200µl/well) for 2 to 3 days at 37°C temperature with 5% CO₂. Wells positive for virus growth were identified by the presence of hemagglutinating activity supernatant, (HAg) in the hemagglutination units (HAU) were calculated. The virus stocks were stored at -80°C temperature for further use.

Cytotoxicity Assessment:

The evaluation of cytotoxic activity of plant extracts (CC₅₀) was carried out using MTT (3-(4,5dimethylthiazol-2-yl)-2, 5 - diphenyltetrazolium bromide) assay. MDCK cells were cultured onto 96-well plate at the density of 1.0×10^5 cells/ml. Different concentrations prepared in MEM (10mg/ml to 0.01mg/ml) of aqueous methanolic crude extract were added to each culture wells at a final volume of 100µl, in triplicate. After incubation at 37°C temperature with 5% CO₂ for 16 to 18 hours, 10% of 5mg/ml MTT (100µl) was added to each well. After 4 hours of further incubation at 37°C temperature, the formazan was solubilised by adding DMSO to each well and the absorbance was read at 550nm by an ELISA reader. 22

Antiviral Assays:

Simultaneous Treatment assay:

In simultaneous treatment assay, 50µl of virus (64 HAU) was first exposed to 50µl of different dilutions of plant extract prepared in MEM without phenol red (aqueous and methanolic extracts) and was incubated at 37°C temperature for 1 hour. Following incubation, 100µl of the above mixture was added to the 96-wells plate containing cell confluent monolayer of MDCK $(1\times10^5\text{cells/well})$. After 1 hour of incubation at 37°C temperature, the supernatants were removed and the cells were washed with MEM. After washing, the media was discarded and then 100µl of virus growth medium was added and the plate was kept at 37°C temperature in 5% CO₂ incubator.14

Post Treatment assay:

In post treatment assay, confluent monolayer of MDCK cell line was washed twice with $50\mu l$ of virus growth medium and then the medium was removed and. A $100\mu l$ of virus (64 HAU) was added to the 96-wells plate. The virus was allowed to adsorb for 1 hour at 37°C temperature in the 5% CO_2 incubator. After incubation, the virus was removed from each well by washing with MEM. The media was then removed and $100\mu l$ of different dilutions of plant extracts prepared in virus growth medium was added to the monolayer and the plate was kept at 37°C temperature in 5% CO_2 incubator. 14

Hemagglutination assay:

For carrying out hemagglutination assay, bottom' 96-well microtitre plate was used and 50µl phosphate buffer saline (pH=7.2) was added as a diluent in each well by using a multichannel auto pipette. A 50µl of sample (cell free supernatant of simultaneous and post-treatment assay) was added in the first well of each row. Two fold dilutions of the sample were made by transferring 50µl suspension from the first well of each column to the next well by using a multichannel auto pipette. This procedure was repeated till the last column of the 96-well microtitre plate. After serially diluting the sample, 50µl of 0.75% guinea pig RBCs was added to each well and the plate was incubated at 4°C temperature for 1 hour. After incubation, cell control was checked for complete settling of RBCs and results of hemagglutination assay. The resulting virus titers were recorded as hemagglutination units (HAU). ²³

Statistical Analysis:

Sampling proceeded on three independent replication (n=3) for each test. Data were subjected to Graph Pad Prism v5.04 and $v6.0^{24}$ and the HAUs were calculated by two-tailed t-test with p<0.05 as significance.

RESULTS:

Fourier Transform Infrared Spectrometry (FTIR):

TABLE 1: OBSERVED IR RANGE OF JATROPHA CURCAS EXTRACT

EMIKACI		
Functional	Observed IR Range	Standard IR
Group	(Cm ⁻¹)	Range
C=O	1859.38	1800-1600
C-O	1242.16-1303.88	1400-1200
-O- str.	1350.17	1300-1000
C=C str.	1475.12	1600-1475

The **Table 1** represents the observed and standard Infra red range of the functional groups observed in the sample.

The FTIR results of isolated alkaloidal compound are presented in C=O stretching is observed at 1859.38cm⁻¹ (Stretching Frequency) with respect to the sharp band. At 1475.12cm⁻¹ (Stretching Frequency) unsaturated C=C stretching is observed. The presence of stretching frequency of alkene and carbonyl group as shown in is similar to Tetra Methyl Pyrazine alkaloid, already reported in *Jatropha curcas*

Cytotoxicity Assay: Assessment of cell cytotoxicity was performed by MTT assay on MDCK cell line. Percent cytotoxicity was calculated using following formula. ²²

Percent Cytotoxicity = 100 – Percent Cell Survival.

Percent Cell Survival = $\{(At-Ab) / (Ac-Ab)\} \times 100$

Where,

Absorbance value of test compound - At Absorbance value of blank - Ab Absorbance value of control – Ac

Fig. 1: Log concentration vs Percent cytotoxicity

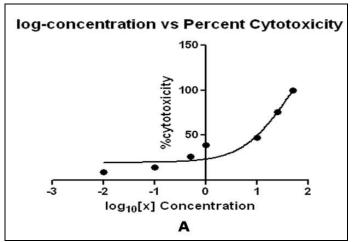


FIG. 1: LOG CONCENTRATION VS PERCENT CYTOTOXICITY OF ALKALOIDAL EXTRACT OF JATROPHA CURCAS

The **Fig. 1** presents the Log concentration vs Percent cell cytotoxicity of alkaloid of *Jatropha curcas*. The 50% cell cytotoxicity (CC_{50}) for isolated sample (TMP) was found out to be 34.7mg/ml. As this concentration indicated 50% cell cytotoxicity, concentration lower than the CC_{50} values were selected for the anti-viral assay.

Antiviral Assay: The antiviral assay was based on percent reduction in Hemagglutination activity and was calculated as follows:

The HAU was calculated using the following formula

Percent (%) log_2HAU reduction= (1 - A / B) X 100 Where,

A - log₂HAU titer of virus control

B - log₂HAU titer of sample.

Fig. 2: Hemagglutination percent reduction vs concentration.

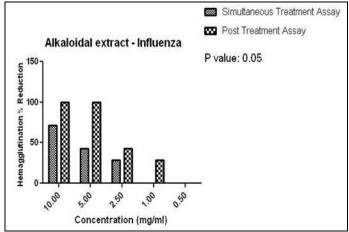


FIG. 2: PRESENTS THE HEMAGGLUTINATION PERCENT REDUCTION VS CONCENTRATION OF ALKALOID OF JATROPHA CURCAS ON THE SIMULTANEOUS AND POST TREATMENT ASSAY.

In the present study, anti-influenza activity of isolated TMP was carried out by simultaneous and post-treatment assays. Simultaneous anti-influenza treatment was used to identify whether *Jatropha curcas* compound block the viral adsorption to cells. As observed in the simultaneous assay, 71% reduction in HA was observed at the concentration

of 10mg/ml and as the concentration was further decreased, the HA reduction was decreased to 42% and 28% up to 5mg/ml and 25.5mg/ml respectively. These data suggest that isolated compound (TMP) of *Jatropha curcas* may directly interfere with the viral envelope protein and not with the SA (sialic acid) receptor at the cell surface.

We employed the post-treatment assay to evaluate the anti-influenza activity after virus infection. In this assay, the alkaloidal extract exhibited 100% HA reduction at concentrations of 10mg/ml and 5mg/ml shown in Reduction as Hemagglutination was also observed at lower concentrations from 2.5mg/ml to 1mg/ml of the extracts. We found that the alkaloidal extract of Jatropha curcas inhibited influenza virus infection suggesting the possible ways of viral inhibition by blockage of viral attachment by inhibition of viral HA protein.

DISCUSSION: Medicinal plants represent one of the significant sources of lead compounds, with up to 40% of modern drugs being derived from plant materials. Empirical knowledge based on the ethnomedical benefits of plants, coupled with bioassay-guided fractionation and isolation, has the potential to identify novel anti-virals that could be used against influenza. Even though a number of studies have been performed using purified plant chemicals, only some studies have addressed the antiviral activities of crude plant extracts. ²⁵⁻²⁶ The search for plant-based antivirals against the influenza virus is promising as several plants have been shown to possess anti-influenza activity, some which include: Thujaorientalis, Aster spathulifolius, Pinus thunbergia, ²⁸ Justica Adhatoda fistulosum, components have also been isolated from crude plant extracts employing chemical fractionation techniques.

Tetra Methyl Pyrazine (TMP), a biologically active alkaloid extracted from Ligusticum chuanxiong Hort, has been widely used in China as a drug together with other Chinese herbal medicines for the treatment of various diseases. A great deal of pharmacological research has been done on this agent, mainly focused on its cardiovascular and cerebrovascular effects. antioxidation, neuroprotection, anti-fibrosis, anti-nociception, anti-inflammatory, and anti-neoplastic activity. 30 One of the earlier studies on methanolic extract of stem of Jatropha podagrica Hook demonstrated antibacterial activity against only gram positive microorganisms. Fractionation of this extract resulted in a number of active fractions and

one of them contained the amide alkaloid Tetra Methyl Pyrazine.³¹

In the FTIR analysis, the results confirmed the presence of unsaturated carbonyl functional group, which is similar to the structure of Tetra Methyl Pyrazine already reported in *Jatropha curcas* leaves. ¹⁹

An antiviral assay previously reported in which the simultaneous exposure assays were used to identify whether the extracts blocked the viral adsorption to cells, by synergistically binding to the free virus particles or by blocking the sialic acid receptors to prevent virus entry into the cells.³² From the post exposure treatment, it was concluded that the extracts may be inhibiting the replication of influenza virus or virus budding from the infected MDCK cells.

Previous reports suggest that isolated compound (TMP) from leaf extract of *Jatropha curcas* has been shown to have *in vitro* antiviral activity. Among the viruses which are sensitive to the extracts are HIV, Influenza viruses but none of them have characterized the active principle as TMP as this report has focused on by various spectral analysis. ²⁰

CONCLUSION: To conclude, the study has evaluated that isolated alkaloidal compound (TMP) of *Jatropha curcas Linn*. has prospective antiviral activity against Influenza virus and the experimental moieties like TMP had favorable implications on the prevention or management of respiratory infections. It can therefore be deduced from the study that *Jatropha curcas Linn*. is a good candidate for anti-Influenza therapy with further clinical research.

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declare that they have no conflict of interest.

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