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EVALUATION OF *IN VITRO* ANTI - INFLUENZA ACTIVITY OF *TYLOPHORA INDICA* (ASCLEPEADACEAE) LEAVES

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Influenza viruses are ubiquitous, they have been around for hundreds of years, and are likely to remain with us for a long time. They produce significant annual morbidity and mortality throughout the world, the current annual vaccines and effective antiviral drugs are not available sufficiently, due to antigenic shifts and drifts of influenza virus, long-lasting vaccine has not been developed so far. The use of herbal products is one of the best alternatives against influenza virus. Cytotoxicity assay was carried out on Madin Darby Canine Kidney (MDCK) cell line by using MTT assay prior to anti influenza examination. *In vitro* anti-influenza assay was performed. The anti influenza activity was confirmed by Hemagglutination assay using 0.75 % guinea pig RBC's. *Tylophora indica* extract of 20mg/ml showed 3 fold reduction of the HA titre (1:8) as compared to the virus control titre of 1:64, which indicates that the extract has an anti-influenza activity. This is the preliminary study in which the *Tylophora indica* extract showed anti-influenza activity at 20mg/ml in post exposure treatment.This report is not well documented till date.

Keywords: Anti influenza activity; Cytotoxicity; *Tylophora indica* plant extract.

Introduction

Influenza viruses are ubiquitous, they have been around for hundreds of years, and are likely to remain with us for a long time[1]. They produce significant annual morbidity and mortality throughout the world, and the occasional pandemic with potentially devastating consequences for human and animal health and the global economy. Some innovative molecular approaches have been suggested, based on cellular signaling pathways utilized by the virus for its replication[2,3]. Herbal remedies, including traditional Chinese Medicine (TCM) have also been suggested as alternatives[4]. These generally may be safer than chemical drugs, and are less likely to encounter resistant viruses, because of their multivalent functions. Several hundred plant and herb species that have potential as novel antiviral agents have been studied, with surprisingly little overlap. A wide variety of active phytochemicals, including flavonoids, terpenoids, lignans, sulphides, polyphenolics, coumarins, saponins, compounds, alkaloids, polyamines, thiophenes, proteins and peptides have been identified. Some volatile essential oils of commonly used culinary herbs, spices and herbal teas have also exhibited a high level of antiviral activity. Several of these phytochemicals have complementary and overlapping mechanisms of action, including antiviral effects by either inhibiting the formation of viral DNA or RNA or inhibiting the activity of viral reproduction. If viral enzymes could be neutralized, viral replication would not take place. The proteolytic processing of viral poly protein precursors by a viral proteinase is essential for maturation of the virus. Designing specific inhibitors for each of viral protease is thus a desirable objective, so the basic requirement behind the use of herbs as an antiviral agents are to disrupt the replication cycle, to stimulate as also to support the immune system[5].

Every plant on our planet manufactures at least one anti-viral medicine in its own cells[5]. The development of viral resistance towards antiviral agents enhances the need for new effective compounds against viral infections. Medicinal plants have a variety of chemical constituents, which have the ability to inhibit the replication cycle of various types of DNA or RNA viruses. Compounds from natural sources are of interest as possible sources to control viral infection[6]. In this context various research groups in Asia, Far East, Europe and America have given particular attention to develop antiviral agents from their native traditional plant medicines. Some typical examples of such medicines and their antiviral activities are shown in Table 1[7].

Keeping all the above facts *Tylophora indica* was

Table 1.

Phytochemicals	Mode of Action	Families
Alkaloids constitute: β-carbolines, furanoquinolines, camptothecin, atropine, caffeine, indolizidines swainsonine, castanospermine, colchicines, vinblastine	DNA and other polynucleotides and virions proteins. In some interactions are enhanced by UVA	Rutaceae, *Camptotheca acuminate, Atropa belladona* (L.), *Swainsona canescens, Astragalus lentiginosus, Castanospermum australe, Aglaia roxburghiana*
Flavonoids: amentoflavone, theaflavin, iridoids, phenylpropanoid glycosides, agathisflavone, robustaflavone, rhusflavanone, succedaneflavanone, chrysosplenol C, coumarins,galangin (3,5,7-trihydroxyflavone), baicalin	Blocking RNA synthesis. Exhibited HIV-inhibitory activity	*Agastache rugosa, Euphorbia grantii, Barleria prionitis, Calophyllum cerasiferum, Camellia sinensis, Garcinia multiflora, Helichrysum aureonitens, Macluracochin chinensis, Markhamia lutea, Monotes africanus, Pterocaulon sphaecelatum*

selected for anti influenza evaluation.*Tylophora indica* (Asclepediacaeae) is a creeper plant and is used as an anti asthamatic medicine in indian pharmacopeia, Plant is having tylophorin as a main constituent, which is reported as an anti asthamatic and anti-inflammatory agent. Secondary metabolites from many plant species have been reported for the anti infuenza activity[7-11]. Some scientist have worked on the synthesis and development of anti TMV drugs, which is a derivative of phenanthrene nucleous like tylophorin, ,the main constituent of *Tylophora indica.* No work has been documented on anti influenza activity of *Tylophora indica* till date.

Material and Methods

The plant material (leaves) of *Tylophora indica* was collected from the Kelkar farmhouse, Mulund,. The plant material was dried in vacuo, powdered and subjected for the methanolic cold extraction. After subsequent extraction with methanol the extract was dried and subjected for the experiment. Cytotoxicity assay was carried out prior to anti influenza examination.

Cyto-toxicity assay: MDCK cell line was trypsinised and suspended in Minimum Essential Medium (MEM) and were then seeded in 96 well tissue culture plate. Plates were incubated overnight at 37°C in humidified 5% CO_2. Hundred microliters of dilution (100mg/ml, 50mg/ml, 10 mg/ml, 5mg/ml, 1mg/ml) were added to the wells and again incubated for 10 to 16hrs at 37°C in incubator. Cell control and positive control containing cells and Dimethyl Sulphoxide (DMSO) was also maintained along with the tests samples. Subsequent to this incubation, 10µl of 5mg/ml of MTT (3-4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) reagent was added in each well and incubated at 37°C for 4hrs in CO_2 incubator. After the incubation 100µl of DMSO was then added to each well. The well plates were then kept on shaker for 1min and readings were taken on ELISA reader at 550nm[12].

Maintaince of cell line: Madin Darby Canine Kidney (MDCK) cell lines was maintained in Modified Eagles Medium, 100 mg/ml penicillin, 100 mg/ml streptomycin , 2mM L-glutamine and 1.5g/L sodium bicarbonate, supplemented with 10% fetal bovine serum. The flasks were incubated at 37°C, in 5% CO_2 incubator for 2-3 days until a confluent monolayer is obtained[13].

In vitro anti influenza assay (post exposure method): MDCK cell line was trypsinised and suspended in Minimum Essential Medium (MEM) and were then seeded in 24 well tissue culture plate. After the monolayer is formed then the medium is discarded and the MDCK cell line was washed twice with virus growth medium (MEM containing 2µg/ml of TPCK trypsin and 50units/ml Nystatin) and Influenza virus was added to confluent monolayer of the MDCK cells and allow it to get adsorbed for 45 minutes in 37°C, 5% CO_2 incubator. Then the remaining virus growth medium containing extracts of *Tylophora indica* was added and incubated at 37°C,5% CO_2 for 3-4 days. The cytopathic effect was observed and the activity was confirmed by Hemagglutination assay using 0.75 % guinea pig RBC's[14-16].

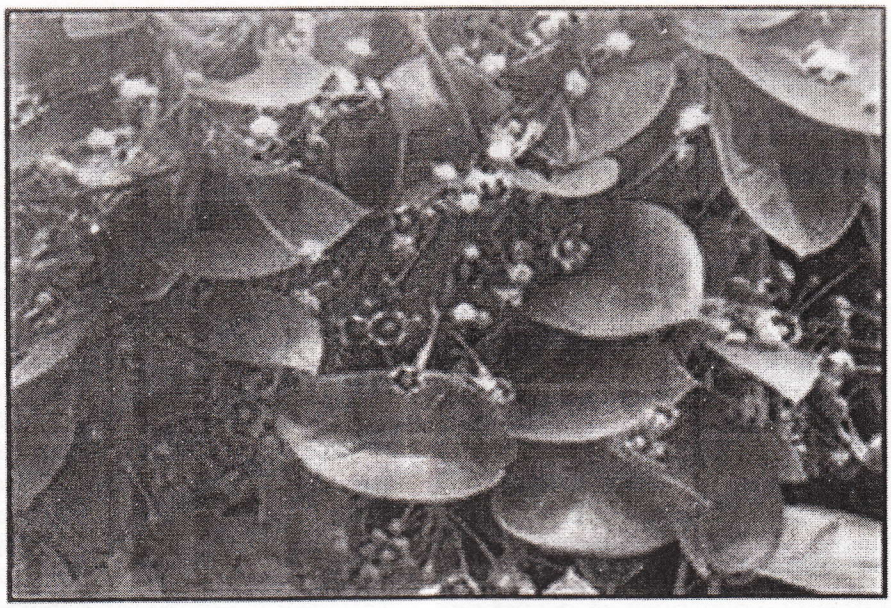


Fig.1. Showing plant of *Tylophora indica* collected from Kelkar farmhouse, Mulund.

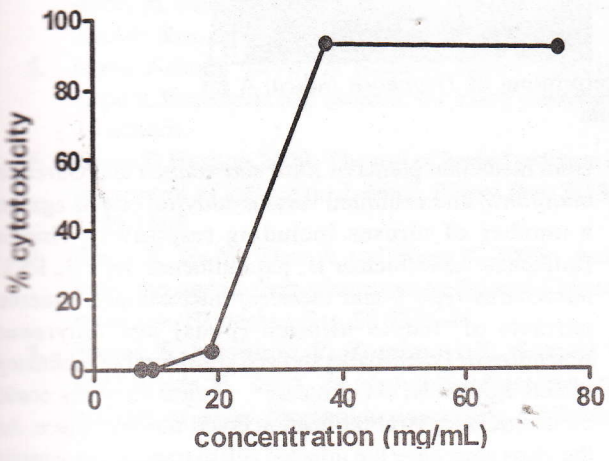
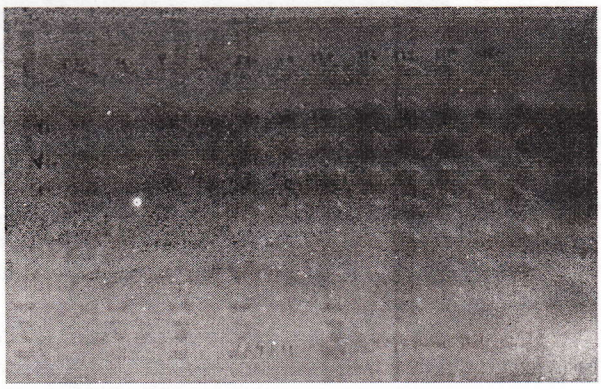


Fig.2. Graph showing the cytotoxicity of leaves (methanolic extract) of *Tylophora indica*: 30 mg/ml.



Key: ⊙ : Button formation
● : Hemagglutination

Fig.3. Showing the anti-influenza activity of methanolic leaf extract of *Tylophora indica* by Hemagglutination assay

Phytochemical analysis: The dried cold methanolic extracts of *T.indica* leaves was subjected for the HPTLC analysis for the presence of alkaloids and flavonoids using following parameters. HPTLC analysis was carried out in Anchrom Test Lab Pvt. Ltd using silica gel plates (60F254 Manufacturer E. MERCK KGaA),Sample application was carried out on CAMAG Linomat 5 instrument (CAMAG Linomat 5 "Linomat5_080222" S/N 080222).Inert gas was used as spray gas. Sample solvent type was methanol. Dosage speed was 150nl/s and syringe size was 100µl and the analysis was done at wave length 430 nm. Toluene: Ethyl acetate: Diethyl amine (14:2:2) was used as mobile phase for alkaloid and Toluene: Ethyl acetate:(14:2) for flavonoids.

Result and Discussion

Influenza is a serious threat to health in all parts of the world. The control and treatment of influenza depends mainly on chemical or biochemical agents and, to date, some anti-influenza agents have been isolated from plants as a result of chemical and pharmacological studies. Here we have described the *in vitro* anti-influenza activity of *Tylophora indica* methanolic leaves extract. *In vitro*

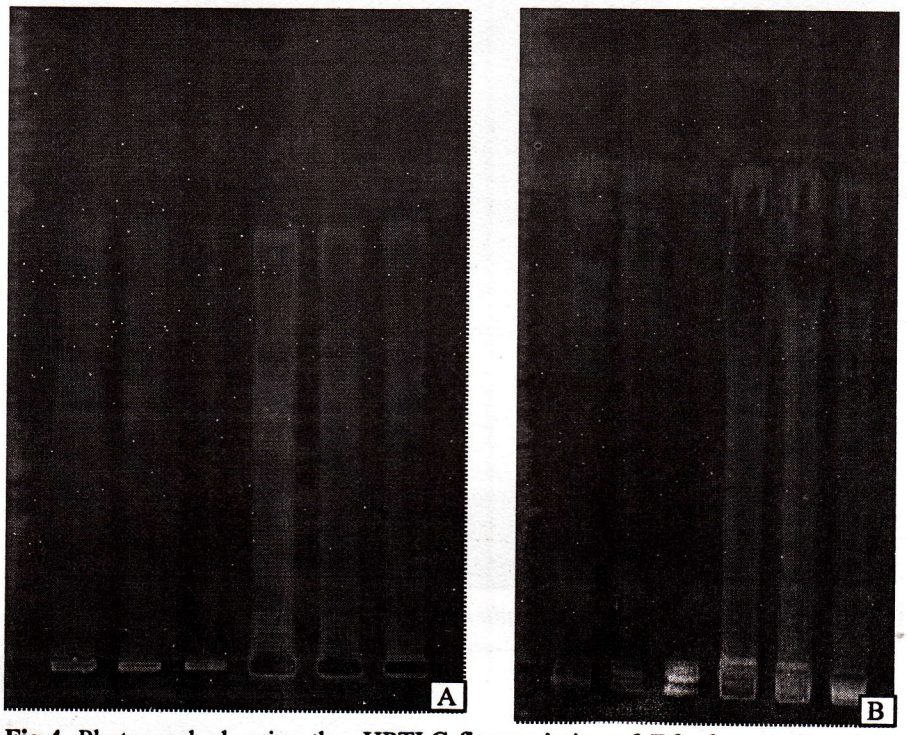


Fig.4. Photograph showing the HPTLC fingerprinting of *Tylophora indica.* A for alkaloid at 254 nm , B for flavonoids at 480 nm

cytotoxicity assay was carried out on Madin Darby Canine Kidney (MDCK) cell line by using MTT assay. The CC_{50} was calculated and found to be 30 mg/ml of methanolic extract of *Tylophora indica.* Based on the CC_{50} value, anti influenza assay was performed.

The methanolic leaf extract of *Tylophora indica* was not cyto toxic below 30mg/ml concentration. The post exposure treatment was carried out and the methanolic leaf extract of *Tylophora indica* at 20mg/ml concentration showed anti influenza activity where 3 fold reduction of the HA titre (1:8) was obtained as compared to the virus control titre of 1:64 by hemagglutination assay. HPTLC analysis depicted the presence of alkaloids and flavonoids in the methanolic extract of *Tylophora indica.*

The antimicrobial activities of plant oils and extracts have been recognized for many years. Recently, the oil of *Melaleuca alternifolia* (tea tree) has gained widespread acceptance and it is now the principal antimicrobial preservative in a range of pharmaceutical cosmetics for external use, such as face and hand washes, pimple gels, vaginal creams, foot powders, shampoos, conditioners and veterinary skin care products[17].

In another study, five groups of flavonoids (amentoflavone, agathisflavone, robustaflavone, rhusflavanone and succedaneflavanone) were isolated from medicinal plants of *Rhus succedanea* and *Garcinia multiflora,* and exhibited various antiviral effects against a number of viruses including respiratory viruses (influenza A, influenza B, parainfluenza type 3, RSV, adenovirus type 5 and measles) interestingly, aqueous extracts of *Acacia nilotica* (pods) and *Maytenus senegalensis* (stem-bark) showed considerable inhibitory effects against HIV-1 protease[18]. If viral enzymes could be neutralized, viral replication could not take place. As the virus must have the infected cell to translate its genetic information into proteins, it must be able to express mRNA in the infected cell. With negative RT activity the viral proliferation will not take place. Developing specific inhibitors for viral protease activity from medicinal plants are desirable objectives. The active principle may be alkaloid or flavonoid.It may be possible there are some antagonistic effect because of the presence of some inhibitory compounds, so it is necessary to find out the actual bioactive agent responsible for that activity.

This is the preliminary study in which the *Tylophora indica* extract showed anti-influenza activity at 20mg/ml in post exposure treatment for the first time. There is no reported work on anti influenza activity of *Tylophora indica* leaf extract till date. But the anti - influenza property of *Tylophora indica* still needs to be

investigated in depth by purifying and isolating the bioactive component responsible for anti-influenza activity and conducting toxicological experiment, animal and clinical trials, for the development of marketable therapeutic products.

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