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Research Article

Immunological exploration of virally infected and non-infected leaves of Tobacco plant on human whole blood and peripheral blood mononuclear cells

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ABSTRACT

Introduction- Virally infected plants are of great concern especially for farmers including researchers because of enormous loss and cause disease in different crops e.g. vegetables, fruits, cereals etc. In this study, our group focused on those plant viruses which are utilized and showed some significance as an effective tool for vaccine development against various infectious diseases including cancer.

Objective-The objective of our study is to compare the effect of aqueous leaves extract of virally infected i.e. TMV (tobacco mosaic virus) and non-infected tobacco plant (control) on human whole blood and peripheral blood mononuclear cells (PBMC).

Methods- In this study, we evaluated the samples of tobacco plant (control, non-infected and virally infected, TMV) were tested on human whole blood pertaining to determine its population i.e. lymphocytes, monocytes and granulocytes count and also estimated nitric oxide production from human PBMC. In addition, control and virally infected tobacco plant also determined its protein content (SDS PAGE).

Results- The results showed that protein content is more in case of infected TMV plant as compared to non-infected leaves of Tobacco plant. In addition, TMV increased monocytes and granulocytes count including nitric oxide production at lower doses as compared to control. On the other hand, non-infected leaves of tobacco plant showed no enhancement or decline in monocytes and granulocytes count. Overall, virally infected (TMV) leaves sample in the form of aqueous extract showed immunostimulatory effect at lower doses.

Conclusion- Overall, TMV (virally infected; tobacco plant) should be a better candidate for vaccine antigen.

Key words: Tobacco mosaic virus; protein; monocytes; granulocytes; vaccine

Introduction

Tobacco mosaic virus (TMV), belongs to the family *Virgaviridae* widespread plant pathogen, is normally found in tobacco plant i.e. *Nicotiana tabacum* as well as in many other plants [1]. The first plant virus i.e. TMV (single stranded RNA) is presently worldwide and is generally known to infect more than 150 different plants including tobacco, tomatoes, peppers and cucumbers [1, 2]. Because of its stability at high temperatures which is also reported and already mentioned in the literature as well. In other words, TMV resists tobacco manufacturing processes and can be present in cigarettes, chewing tobacco and cigars for many years [3-5]. Till date there are no efficient chemical treatments that protect various medicinal plants from virus infection. In comparison with animal viruses, plant viruses cannot be able to replicate in human blood/tissue samples or other animals, largely due to the lack of specific receptors for recognition and entry into host cells [5, 6].However, it has been demonstrated that cowpea mosaic virus enters the bloodstream in mice from the intestine when administered in cowpea leaves and induces the production of antibodies without replicating. More recently, a case-control study showed that pepper mild mottle virus may be found in human feces and is associated with clinical immune responses [7, 8]. These studies suggest that plant viruses

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may play a role in human health and disease. Until now, the possible effects of consumption or exposure of infected (TMV) and non-infected (control) plant of tobacco on human whole blood have not been investigated.

Tobacco smoking has been shown to cause cancers, heart disease and chronic obstructive lung disease. It also increases the risk for development of multiple autoimmune disorders such as rheumatoid arthritis and multiple sclerosis. Although the health risks of tobacco smoking are well documented in the literature e.g., smoking is reported to reduce human autoimmune responses in *systemic lupus erythematosus* and ulcerative colitis [9-11]. With reference to neurodegenerative disorders, epidemiological studies being conducted and consistently showed smokers to have a lower risk of developing Parkinson's disease which is associated with a long duration of smoking rather than smoking intensity. Such an inverse association is also observed in people who use chewing tobacco [12]. The main ingredient of tobacco plant i.e. nicotine which is reported as immunosuppressant and also showed neuroprotective effect. More than 47000 chemicals compounds are already identified and mentioned in the literature and most of them are responsible to modulate the immune function (humoral and cell-mediated immune responses) [9-12]. Tobacco mosaic virus can survive for years in cigars and cigarettes made from infected tobacco leaves. The objective of our study is to investigate the aqueous extract of virally infected tobacco plant i.e. TMV induces immune responses and compared the immunogenicity profile of non-infected leaves of tobacco plant.

MATERIALS AND METHODS

Sample Collection

Tobacco plants were collected from Akkol, (Nipani) Karnataka (India). Actively growing leaves of mature plant of infected and control Tobacco were used to initiate the experiment.

Preparation of aqueous leaf extract

Known weight of fresh leaves of infected (TMV) and non-infected (control) plant of tobacco were collected, washed with distilled water and then shady dried. For aqueous extract preparation, sample was macerated to powder form and used for immunological studies. Aqueous extraction of both tobacco leaves were done in two different sets using phosphate buffered saline (PBS, pH 7.4; 50 ml) and crushed in a grinder and the extract was centrifuged at 10,000 rpm at 4 °C for 10 minutes. The supernatant was collected and was used within four hours for various immunological *in vitro* assays.

Extraction of protein and determined through SDS PAGE

For extraction of protein from infected and non-infected leaves of tobacco plant using Tris HCl (pH 7.2) and acetone. In this study, both tobacco samples were weighed and finally crushed in mortar and pestle (using liquid nitrogen, -196 ºC) to prepare powder form and ultimately dissolved in Tris HCl (pH 7.4). Incubate both tobacco leaves samples for 10 minutes at room temperature followed by centrifugation. Thereafter, acetone solution was added in the supernatant and incubated for 20 minutes. Finally, the extract was centrifuging and supernatant was discarded and proteins settled at the bottom and determined the protein content using Nanodrop [13].

Resolving (15 %) and stacking (4%) gels were used for determination of protein bands in infected and non-infected leaves of tobacco plant. About 20 µl of protein sample extracted from both the leaves and was loaded into the wells and required voltage (80 Volts) in order to run the gel. After separation of protein bands of both the samples (tobacco) through electrophoresis [14], staining solution was utilized to stain the gel in order to make bands visible. Afterwards the gel was placed in to a destaining solution for 24 h on shaker and was changed frequently until clear gel was obtained.

Flow Cytometric analysis in human whole blood:

For hematological analysis, blood samples were collected one hour after the meals and the

plasma collected for the estimation of blood counts (lymphocytes, monocytes and granulocytes count) using forward and side scatter through flow cytometric analysis. For these studies, variable doses of infected (TMV) and control (non-infected) leaves of tobacco plant in human whole blood using forward and side scatter gating applied for data acquisition of 10000 events of cell populations representing different phenotypes analyzed using cell quest software. In this experiment, 100 µl of human whole blood was taken in each tube. Add serial dilutions of formulation on human whole blood and then incubated the samples of infected and non-infected tobacco plant in dark for 2 h at 37°C (using carbon dioxide incubator). Subsequently, 2 ml of 1× FACS lysis solution was added at room temperature with gentle mixing followed by incubation for 10 min. The samples were centrifuging (300 – 400 × *g*) and the supernatant was aspirated and washed two times with phosphate buffered saline (PBS). After centrifugation, pellet dissolved in PBS and observed the cells through flow cytometer [15, 16].

Nitric oxide production assay

Before blood collection, informed consent letter was collected from healthy volunteers which does not show any symptoms of disease or any other illness. Briefly, human peripheral blood Mononuclear cells (PBMC, 10^5 cells/ml) were separated by means of Ficoll–Hypaque gradient centrifugation and plated in 96 well plates were pre-incubated with Concanavalin (Con) A and then treated with serial dilution of samples of infected and non-infected tobacco plant at 37ºC for 24h.The plate were centrifuged at 2500 rpm for 10 minutes and then supernatant (100 µl) was collected for estimation of nitric oxide.

In NO estimation, PBMC cell culture supernatant was mixed with same volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediaminedihydrochloride in 2.5% phosphoric acid) and incubated the flat bottom 96 well plates at room temperature for 10 minutes and absorbance at 540nm was measured by spectrophotometer. The fresh culture medium (RPMI containing 10 % fetal bovine serum) was used as a blank. The nitrite quantity was determined from a sodium nitrite standard curve [17].

Statistical analysis

Values are expressed as Mean ± S.E. The difference between control and aqueous leaves extract of virally infected and non-infected leaves of tobacco plant which is determined through Bonferroni multiple comparison test (one way ANOVA test).

RESULTS

Estimation of protein content

For measuring the quantity of protein present in tobacco leaves (infected and non-infected) using Nanodrop. The quantity of protein in infected (27.14 mg/ml) and non-infected (14.24 mg/ml) was reported.

SDS-PAGE

SDS PAGE separation of the isolated protein shows that the amount of total protein contains in infected plant leaves is higher than the control i.e. infected 25-50 KDa and controls 25-40 KDa as shown in **Fig.1**.

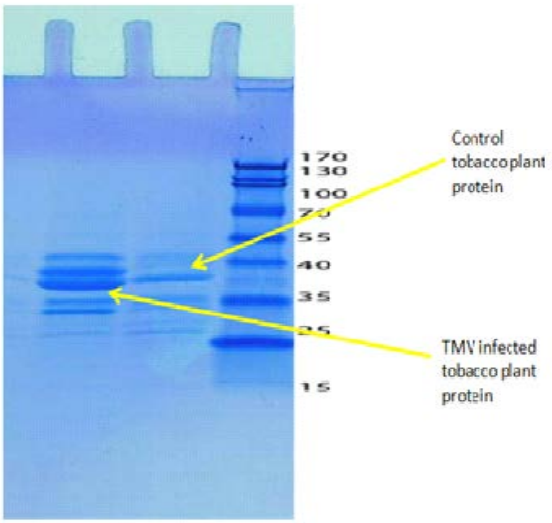
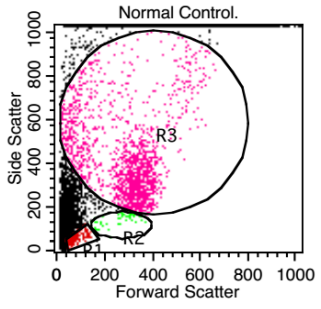


Figure 1: SDS PAGE of isolated protein (molecular weight of isolated protein for control (non-infected) and infected (TMV) tobacco plant is 25-40 KDa and 25-50 KDa respectively, comparing with 170KDa marker on SDS PAGE)

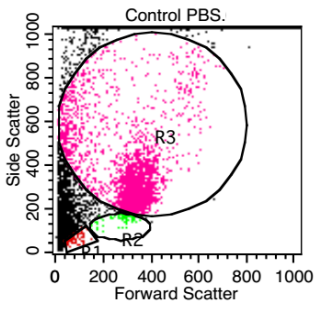
Flow cytometric analysis for the estimation Lymphocytes, monocytes and granulocytes count

Estimation of lymphocytes monocytes and granulocytes were done using FACS analysis. The result showed enhancement in cell count at the optimum dose range (i.e. 0.78 mg and 1.56 mg) and thereafter sudden decline in cell count in the form of lymphocytes (R1), monocytes (R2) and granulocyte (R3) count as shown in **Fig.2.**

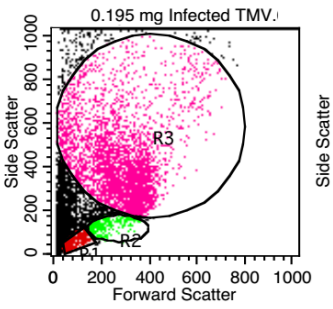
A. Infected (TMV) samples of Tobacco plant



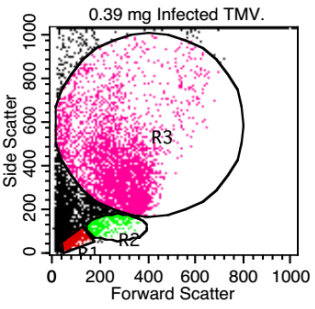
Region	Events	% Gated	% Total
R1	216	2.16	2.16
R2	50	0.50	0.50
R3	1632	16.32	16.32



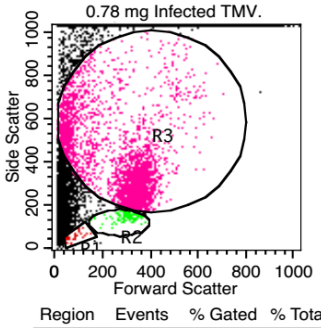
Region	Events	% Gated	% Total
R1	147	1.47	1.47
R2	87	0.87	0.87
R3	2471	24.71	24.71



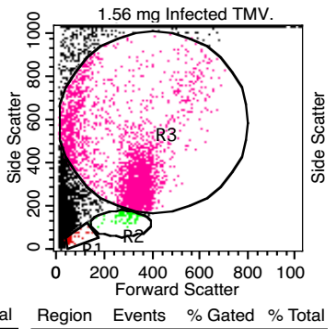
Region	Events	% Gated	% Total
R1	341	3.41	3.41
R2	298	2.98	2.98
R3	3410	34.10	34.10



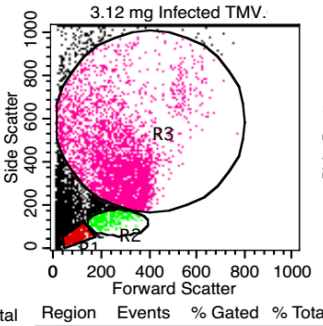
Region	Events	% Gated	% Total
R1	309	3.09	3.09
R2	295	2.95	2.95
R3	3583	35.83	35.83



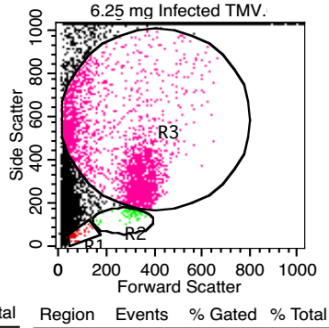
Region	Events	% Gated	% Total
R1	364	3.64	3.64
R2	303	3.03	3.03
R3	3602	36.02	36.02



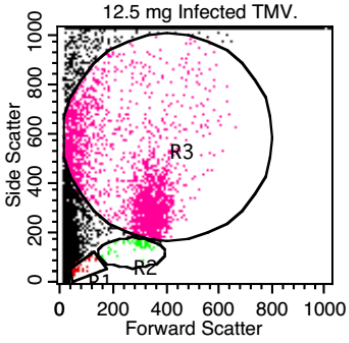
Region	Events	% Gated	% Total
R1	61	0.61	0.61
R2	120	1.20	1.20
R3	2952	29.52	29.52



Region	Events	% Gated	% Total
R1	99	0.99	0.99
R2	89	0.89	0.89
R3	2705	27.05	27.05



Region	Events	% Gated	% Total
R1	95	0.95	0.95
R2	73	0.73	0.73
R3	2484	24.84	24.84



Region	Events	% Gated	% Total
R1	83	0.83	0.83
R2	71	0.71	0.71
R3	2400	24.00	24.00

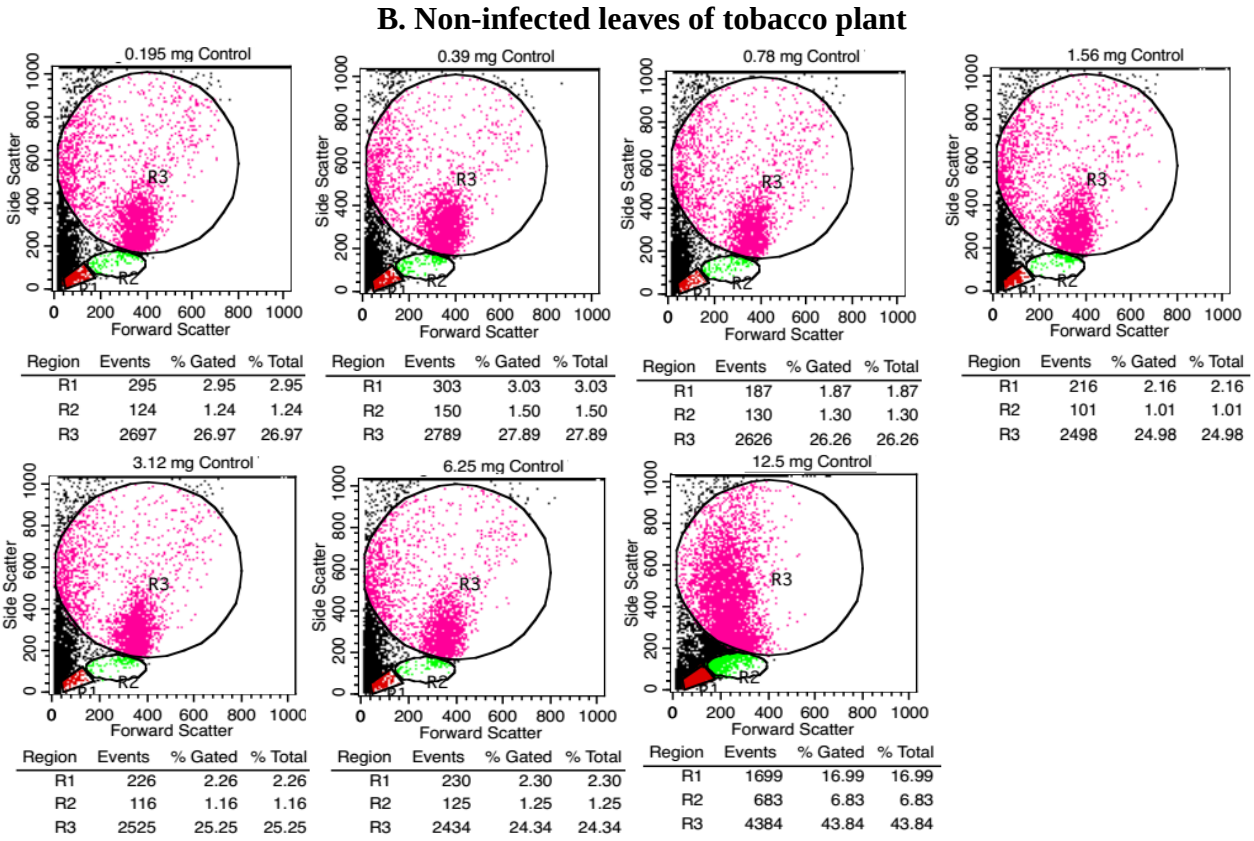


Figure 2: Flow cytometric analysis of virally infected (TMV) and non-infected leaves of tobacco plant exposed to human whole blood and determined forward (shape and size) and side scatter (granularity). Data acquisition of 10000 events and fraction or separation of cell populations representing forward and side scatter using cell quest software.

Nitric oxide production

The effect of aqueous leaves extract of virally infected and non-infected leaves of tobacco plant on nitric oxide production in human PBMC's as shown in **Fig.3.** The results showed that virally infected leaves showed enhancement in nitric oxide at lower doses after treating with PBMC as compared to control and non-infected leaves of tobacco plant.

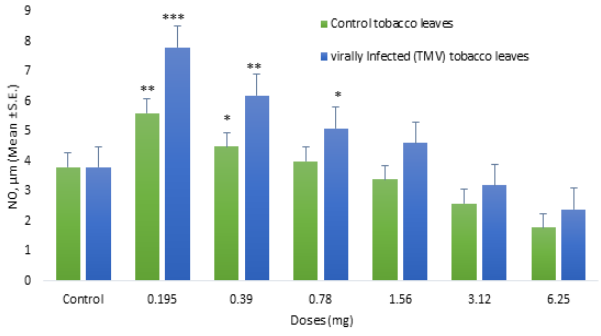


Fig.3. Nitric oxide (NO) estimation assay. PBMC (10^5 cells/ml) were plated in 96 well plates were treated with Concanavalin (Con) A, 2.5 µg/ml and then treated with serial dilution of samples of infected and non-infected tobacco plant at 37ºC for 24h. Supernatant was collected after centrifuging for estimation of nitric oxide. Values are expressed in Mean ± S.E. The difference between the control and standard is determined by one way ANOVA test

DISCUSSION

For the last several years, researchers tried to use these medicinal plant products as vaccine antigen but unfortunately none of them reached into the clinical trials. Recently, plant based human vaccines are not yet approved or commercialized till yet, although production of dozens of viral and bacterial subunit vaccines is already attempted in various transgenic plants. For plant vaccine preparation, choice of the plant species is required and technology related to vaccine administration route i.e. most medicinal plants can be consumed only when processed, whereas heat treatment may destroy the antigen. Approximately more

than 15 million deaths per year worldwide is due to infectious diseases. To reduce the burden of infectious diseases, vaccination is required and considered to be one of the most efficient and cost-effective means for health intervention to combat infectious diseases.

The identification of these cells (i.e. lymphocytes, monocytes including granulocytes) that are present in lysed human whole blood which is measured through light scatter properties in the form of forward scatter (FSC, shape and size) and side scatter (SSC, granularity of the cell) [18]. As per the data, the results claimed that aqueous leaves extract of virally infected tobacco plant showed some enhancement in monocytes and granulocytes count as compared to control. Similarly, non-infected aqueous leaves extract showed no enhancement or decline in monocytes and granulocytes count but there is surprisingly enhancement in these count at higher doses. In other words, if human may exposed to non-infected tobacco leaves at higher doses then immune system will be more activated. Overall, virally infected tobacco leaves may work like a vaccine antigen and the activity of aqueous leaves extract was affected in dose dependent fashion.

As per the literature, mammalian cells have the ability to synthesize or produce free radical nitric oxide (NO) and stimulated an extraordinary impetus for scientific research in all the fields of biology and medicine [17]. In this study, virally infected leaves of tobacco plant may showed enhancement in nitric oxide production at lower doses as compared to control whereas non-infected plant showed less enhancement in nitric oxide as compared to virally infected leaves of tobacco plant. In other words, nitric oxide production from macrophages or monocyte derived macrophages can dilate the blood vessels and improve the circulation, but at high concentrations (enhancement in enormous amount) and may cause circulatory shock and induce cell death [17]. Thus, diseases can arise in the presence of the extreme ends of the physiological concentrations of nitric oxide. So, nitric oxide signaling pathway has, in recent years, become a target for new drug development.

CONCLUSION

This is the preliminary study to report its *in vitro* toxicological property of virally infected and non-infected leaves of tobacco plant. From these studies, it showed some scientific proof for the use of these tobacco plant (virally infected) in traditional medicine used as vaccine antigen for the treatment of various infectious agents.

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