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Original Article

Screening of Medicinal Plants: *Rheum emodi* Induces Interferon Stimulated Gene 56 (IFIT1), a Antiviral Protein

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ABSTRACT

Medicinal plants are important sources of medicines for healthcare. Several plants could offer a rich resource for agonistic molecules to cure various infectious diseases. Viruses are one of the major factors of human infectious diseases. Due to the increasing resistance to antiviral drug there is an urgent need to search for alternative agonistic compounds from plants. Plants are vital sources to induce innate immune system against viral disease. Interferon plays important role in innate immunity and mediates antiviral activity by inducing many interferon stimulating genes (ISGs) also called IFITs. ISGs block different stages of the virus replication, viral protein synthesis, and assembly of new virus particles. Among ISGs, ISG56, a component of innate immunity plays a critical role in mounting immune response against viruses. In this study, we screened plant extracts for their anti-viral properties by exploring their potential to induce ISG56 in cell culture rather than inhibition of viral protein function. Five medicinal plants were selected from NeMedPlant database. Out of the 5 ethnically known medicinal plants, *Rheum emodi* (RE) root extract was identified to induce ISG56 expression specifically. Therefore, the current study could provide an important insight into the development of a novel natural plant based anti-viral therapeutic from the RE extract without compromising on viral resistance.

Key words : Medicinal plant, Innate immunity, ISG56, Antiviral protein.

1. INTRODUCTION

Human population is challenged by a number of emerging infectious diseases. Among several ailments, viral infections have threatened mankind survival[1]. During viral infection among vertebrate species, there are two major protection systems. First, innate immunity which is the first line of

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protection but is not specific to a particular pathogen and second is adaptive immunity which is highly specific with amazing property of memory[2]. The key elements of innate immune responses are primitive pattern recognition receptors (PPRRs) or pattern recognition receptors (PRRs)[3] in the immune cells. PPRRs recognize components such as RNA, DNA and proteins called pathogen associated molecular patterns (PAMPS) produced by pathogens. PAMPs binding to PPRRs induce induction of type I IFNs and other pro-inflammatory cytokines in the infected cells and surrounding cells[4].

IFNs are potent cytokines which specifically block virus replication[5] and also effect cellular physiology, mainly cells of the immune system. By large, these effects are due to induction of cellular genes by IFNs treatment to cells[6] which are called IFN stimulated genes (ISGs)[7]. Type I IFNs binds to IFN receptors (IFNAR), leads to the induction of nuclear translocation factor ISGF3 which further induces the transcription of hundreds of different ISGs[8]. ISGs also have immune modulatory functions like priming of adaptive immunity[9].

ISG56 is highly induced gene among all ISGs[10]. ISG56 is a 56 KDa cytoplasmic protein with no enzymatic activity. The expression of this protein normally remains undetectable but strongly induced by IFNs, viral infections and various molecular patterns such as dsRNA and lipopolysaccharides [11]. ISG56 protein has a consensus sequence of 34 amino acids which forms a basic helix-turn-helix fold. ISG56 protein have 10 TPR motifs arranged adjacently to form anti-parallel helices sheet that curves into a unique super-helix fold, forming concave and convex curved surfaces that help diverse ligands to bind[12]. ISG56 protein inhibits replication and translation of varied families of viruses through divergent mechanism. Few examples include (i) inhibition of replication of Human Papillomavirus by binding to its helicase E1[13], (ii) inhibition of replication of the Japanese encephalitis virus by binding to 5' capped 2'-O unmethylated RNA[14], (iii) inhibition of Hepatitis C virus translation initiation by binding to elongation initiation factor3[15], (iv) inhibition of viral mRNAs translation which lacks N7- and 2′-O-methyltransferases[16], (v) inhibition of the translation of PIV5 mRNA[17] and also exhibits antiviral activity against influenza A virus and vesicular stomatitis virus (5′PPP-RNA generating viruses)[18]. Hence, ISG56 protein indeed has broad-spectrum antiviral activity. Modulation of ISG56 expression may offer novel approaches in the treatment of a variety of viral diseases.

Bioactive molecules have been extensively used to treat a number of non-infectious and infectious diseases[19]. Several plants could offer a rich resource for the drug discovery against viral infections. Many plant-derived compounds have been identified to inhibit viral proteins[20]. There is a growing need for new antiviral compounds to treat viral infections as the current antiviral drugs are either inefficient due to increasing viral resistance or ineffective due to recurrent infection and viral latency in immune compromised patients[21]. Hence, plants were screened to identify a new bioactive compound that can stimulate innate immune system against viral disease and has led to the discovery of potent stimulators[22].

Previously we have compiled medicinal plant information to a database NeMedplant. In present study five plants were selected to analyze their potential to induce ISG56 and in turn to act as antiviral. This study is first of its own kind to screen plants to ascertain the property and will have potential implication to explore medicinal plants for their antiviral properties.

2. MATERIAL AND METHODS

Chemicals

Dulbecco's modified Eagle's minimum essential medium (DMEM), pen-strep solution and fetal bovine serum (FBS), were purchased from Gibco-Thermo fisher scientific. Anti-ISG56 (Sc-134948), β-actin (Sc-47778) monoclonal antibodies, and peroxidase-conjugated secondary antibody (Sc-2004) were obtained from Santa Cruz Biotechnology, Inc. MTT purchased from Sigma–Aldrich. ECL plus detection kit purchased from GE Healthcare Biosciences. Two-Step RT-PCR kit was obtained from Affymetrix, USB. ISG56 and β-actin primers were purchased from Integrated DNA Technologies. Chloroform, Hexane and Methanol were obtained from Merk.

Cell culture

Human embryonic kidney HEK-Blue™ hTLR8 cell line was obtained from Invivo Gen. HEK-Blue™ hTLR8 cells were cultured in DMEM media added with FBS (10%) and pen-strep solution (100 IU/ml), at 37°C in an atmosphere of CO_2(5%).

Plant extract

Plant extracts were made from *Hybanthusenneaspermus L,Jatrophagassypifolia L,Rheumemodi Wall,Vitexnegundo L* and *Wedeliachinensis Merr*. Each plant was air-dried and extracted with chloroform using a soxhlet apparatus. These crude extracts accurately weighed (100mg), dissolved in 50% (v/v) DMSO. The dissolved solution was filtered through a 0.22um syringe filter aseptically and stored at -20°C.

MTT assay to measure the maximum-non toxic dose of extract.

The *in vitro* cytotoxicity assay was carried with the plant crude extracts to find out the maximum-non toxic dose (MNTD) of HEK-Blue™ hTLR8 cells. Briefly 10,000 cells cultured in 96-well microtitre plate. After 24h different concentration of the crude extracts prepared in complete media were added to the cells. Untreated cells were incubated with 0.1% DMSO. The microtitre 96well plates were incubated at 37^0C with 5% CO_2 in an incubator for a period of 24h.After the crude extracts treatment, the media was replaced with100µl new complete media. 20µl of MTT (5mg/ml) solution were added to eachmicrotitre-96 well and

further incubated at 37°C for 4h. After 4h incubation media was removed carefully and added 150µl of DMSO. MTT formazan product absorbance was determined by ELISA plate reader at 590nm with the reference filter set to 620nm.

Western blotting for ISG56

To test the effect of each plant extract to induce ISG56 expression in HEK-Blue™ hTLR8, treated and untreated cells were collected by centrifugation. The cell pellets were resuspended in RIPA lysis buffer and incubated for 30 min on ice. Pierce BCA Protein assay kit was used for determine the protein concentration. 50µg of treated and untreated cellular protein was electro blotted onto a nitrocellulose membrane, further separated on a 10% SDS–PAGE. Membrane was blocked by 1X TBST contain 5% nonfat *dry milk* at room temperature for 1h. Membrane was incubated with primary antibody (ISG56 1:4000; β-actin 1:5000) at room temperature for 2h. Later membrane was washed three times with TBST fallowed by incubation with secondary antibodies linked horseradish peroxidase at room temperature for 1h. Blots were developed by ECL plus Western blot detection solutions. Fiji Image analysis software was used to determine the band intensities.

RT-PCR for ISG56

Total treated and untreated cellular RNA was isolated using Trizol method. cDNA was prepared from the isolated total RNA using RT-PCR kit from Affymetrix, USB.PCR analyses were performed to detect ISG56 mRNA and β-actin mRNA as an internal control. The volume of PCR reaction was 25µl containing; TaqDNA polymerase (1.25 unit), dNTP (0.2 mM), reaction buffer (1X), and forward and reveres primers (0.4 µmol). The PCR amplification cycles were performed for ISG56 and βactin (initial denaturation at 95 °C for 3min,denaturation 95 °C for 30sec, annealing at 56 °C for 1min, and extension at 72 °C for 30sec).The ISG56 and βactin primers used in this present study are listed in Table 1.The PCR reaction mixture were electrophoresed on agarose gel (1%). The bands visualized by ethidium bromide (EtBr) staining under UV gel doc**.**

Immunofluorescence of ISG56 and sub-cellular localization

HEK-Blue™ hTLR8 cells were grown on glass cover slips and treated with plant extract (25µg /ml) for 12h. The cells were washed with phosphate buffered saline (PBS). The cells were fixed with ice cold paraformaldehyde (4%) and blocked in 3% BSA dissolved in TBST at RT for 1hr. Cells were stained with 1:800 dilution of ISG56 antibody in TBST contain3% BSA for 2h. Further cells were washed three times with TBST. FITC-conjugated secondary antibody (goat anti-rabbit)staining was done with 1:1000 dilutions for 1h. After cells were washed three times with TBST, the glass cover slips were mounted and cells nuclei stained with DAPI (4', 6-Diamidino-2-phenylindole). Samples were analyzed with a Carl Zeiss axioscope.A1 fluorescence microscope. Images were processed using OpenLab software.

3. RESULTS AND DISCUSSION

Selection of medicinal plants for potential antiviral property:

Five medicinal plants used in this study were short listed based on the information collected from NeMedPlant Database[23], literature and existing knowledge of plants being used traditionally to treat viral fever, hepatitis using herbal medicine[24],[25],[26],[27],[28]. All information regarding plant species, code, plant part selection, vernacular name, and chemical composition of the five medicinal plants were shown in Table 2. These plants were collected from North East and Telangana region of India. The voucher specimens were stored at Kakatiya University herbarium, Warangal, Telangana, India (KUW) with accession number as fallows 1329, 1330,1331,1332,1900 for *Vitex negundo* L, *Wedelia chinensis* (Osbeck) Merr, *Hybanthus enneaspermus* (L) F. Muell, *Jatropha gassypifolia L, Rheum emodi Wall,* respectively.

Maximum nontoxic dose (MNTD) of extracts:

The maximum non-toxic dose of extracts in cell line was determined prior to ISG56 expression analysis. As shown Fig. 1 relative cell proliferation of HEK-Blue™ hTLR8 cells is unaffected up to a concentration of 25µg/ml. But when exceeded 25µg/ml toxic effects were observed.

Effects of plant extracts on ISG56 expression:

Western blot analysis was performed to check the effect of plant crude extracts on HEK-Blue™ hTLR8 cells for ISG56 protein expression. Interestingly, out of five medicinal plants *Rheum emodi (RE)* plant extract significantly expressed ISG56 in cells. As shown in Fig. 2, there was a significant increase in the induction of ISG56 protein with RE plant extract while the remaining extracts did not show any significant induction of ISG56 Protein.

Effect of *Rheum emodi* extract on ISG56 mRNA expression:

Subsequently, the effect of RE extract on ISG56 mRNA expression was analyzed. To do that cells were pre-treated with RE (25µg/ml), poly (I:C) (50µg/ml) and 0.1%DMSO (vehicle control). After 10h incubation, cellular RNA was extracted, cDNA was amplified by PCR using ISG56 specific primers (Table 1). β-actin mRNA was used as an internal control for amplification. Fig. 3shows that RE extract induces ISG56 RNA expression significantly as similar to that of Poly (I:C).While, RE had no effect on the expression of β-actin.

Sub-cellular localization of ISG56 in RE treated cells:

The function of ISG56 mostly depends on their sub-cellular location. Generally ISG56 proteins function in the cytoplasm. To examine whether RE extract have any effect on subcellular localization of ISG56 protein, cells were cultured and treated or left untreated with 25µg/ml of RE extract and Poly (I:C) (50µg/ml) for 18h. Then cells were fixed on glass cover slips and immune staining was performed to identify the induction ISG56 protein by using antibodies against ISG56. Cells nuclei were

stained with DAPI. Cells were visualized by Immunofluorescence microscopy. Immunofluorescence assays results indicates ISG56 protein induced and located in the cytoplasm upon the treatment with RE extract and Poly (I:C) in cells (Fig. 4).

Discussion

Innate immune pathway plays a significant role in regulating host defense against viral or bacterial infection. Activation of inflammatory pathway not only directly inhibits pathogen entry or their replication but also helps shaping the specific adaptive immune for a particular pathogen. Plant extracts from various ethnically known plants have been shown to help in developing as well as protecting viral diseases. Here we have discovered that root extract of *Rheum emoid* has potential antiviral property. Using a novel approach of determining the potential to induce antiviral genes, we have shown that RE root extract not only induce ISG56 expression but also promote nuclear translocation of ISG56. This observation is very interesting as many viruses replicate specifically in the nucleus of the infected cells. By regulating the ISG56 subcellular localization, root extract of the RE plant might inhibit viruses which replicate in the nucleus. Thus, these studies identified root extract of RE plant which has potential to inhibit virus growth.

ISG56 is a cytoplasmic protein which expression is upregulated more than 10 fold during viral infection. It belongs to a group of proteins called IFIT genes family protein. Recent studies have also shown that by upregulating ISG56, interferon treatment blocks viral DNA replication. Another member of the family, called IFIT2, has shown to have pro-apoptotic activity. The functional role of the other member is not completely understood. Here we screened only ISG56 but other members may also dynamically regulated by root extract of RE plant. We have shown that root extract of RE not only regulate ISG56 expression at mRNA level but up regulate also at protein level. Further study will need to confirm whether the regulation is at the level of translation or stability of the protein.

In order to understand the cytotoxicity level of the plant extract, we carried out MTT assay for all the plants selected for this study. All the extracts have cytotoxicity above 25ug/ml and we have shown that, at this concentration, RE root extract induce the expression of ISG56 50 fold higher compared to vehicle control. We screened 5 ethnically known plant extract but only RE root extract was able to induce the expression of ISG56. This observation gave us confident that, the effect of ISG56 by plant extract is specific. In this study we have included, poly I:C treatment as positive control and observed a very strong induction of ISG56 mRNA as well as protein in the whole study.

Many of the DNA viruses replicates in the nucleus of the infected cells as they depend on the host replication machinery. ISG56 is predominantly resides in the cytoplasm, hence we ask the question whether these plant extracts have any effect on the nuclear translocation of ISG56. Immuno-staining with specific ISG56 antibody, we have shown that RE root extract specifically promotes nuclear translocation of ISG56. This suggests that RE root extract treatment impact the cyto-nulcar translocation machinery. Further experiments are needed to uncover the detail mechanism of regulation of the nuclear shuttling machinery.

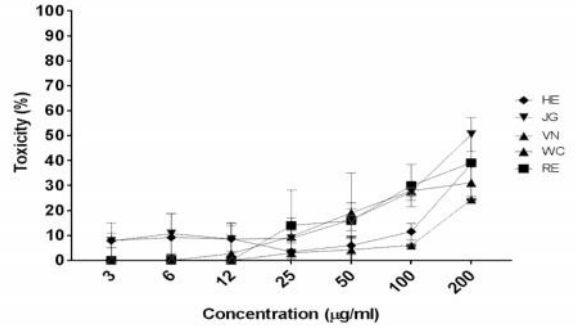

Fig 1: MTT Assay with plant extracts in HEK-Blue™ hTLR8 cells

MTT Assay representing the relative toxicity plot of plant extracts in HEK-Blue™ hTLR8cells: Cells were treated with increased concentration of chloroform extracts of plants for 24 hours and subjected to MTT assay. The experiment was performed in triplicate and data represent the percentage of toxicity cells (means ± SD).

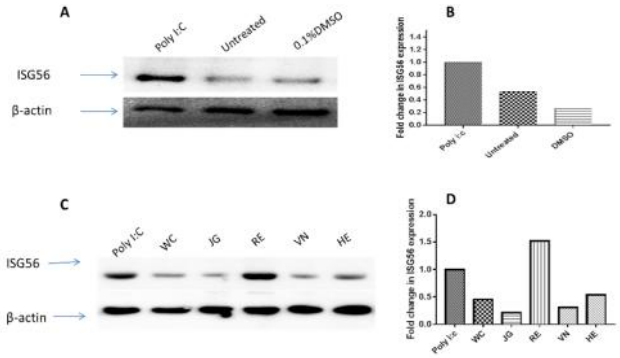

Fig 2: Effects of Plant crude extracts on ISG56 protein expression

HEK-Blue™ hTLR8 cells were treated with different plant crude extracts (25 µg/ml) and 0.1% DMSO for 18 hrs. Poly I: C (50 µg/ml) is added to culture media as positive control for induction of ISG56.Then cells were harvested, and Western blotting was performed with ISG56 antibody to detect endogenous ISG56. β-actin served as an internal control for all samples were similar. The results demonstrated RE extract (panel C) induces ISG56 protein. The expression level of panels A and C proteins blots were quantified by ImageJ processing program and the results are shown in B and D respectively.

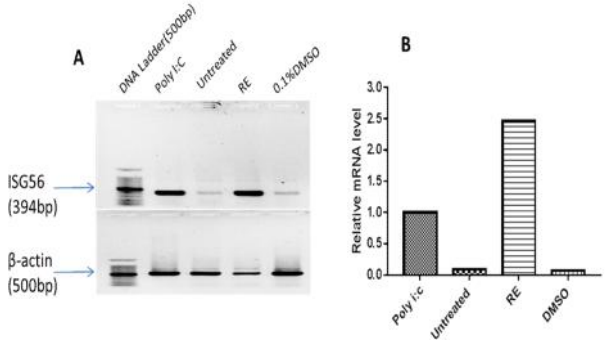

Fig 3: Effects of RE extract on ISG56 mRNA expression

HEK-Blue™ hTLR8 cells were treated with either crude plant extracts (25 µg/ml) as mentioned in the figure or Poly I: C (50 µg/ml) as positive control for 10hrs.Total RNA was extracted and cDNA was synthesized using ISG56 and βactin primers. The PCR reaction was analyzed by 1% agarose gel electrophoresis. ISG56-specific band (394 bp) was detected in Poly I:C (lane-2), and RE (lane-4). 0.1% DMSO treated cells did not induce ISG56 mRNA. β-actin

was used as a loading control. The expression levels of mRNA were quantified by ImageJ processing program and the results are shown in B.

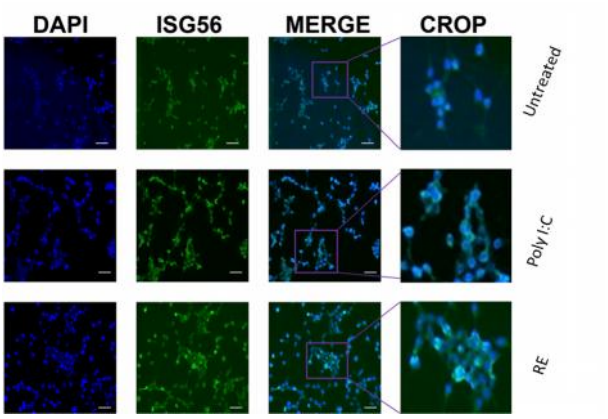

Fig 4: Subcellular localization of ISG56 after plant extract treatment

HEK-Blue™ hTLR8 cells were treated with either 50 µg/ml Poly I:C or RE (25µg/ml) for 18h. Then cells were fixed, and immunofluorescence was performed with ISG56 antibody to detect the induction and localization of ISG56 protein. ISG56 is shown in green, and nuclei are shown in blue. Zoomed images are shown in the bottom panel. Images are representative of 4 different experiments.

Table 1: Oligonucleotide primers for RT-PCR

cDNA	Primers	Annealing, ° C	Product length, bp
ISG56	F 5' – TAGCCAACATGTCCTCACAGAC-3' R 5' – TCTTCTACCACTGGTTTCATGC-3	56	394
β-Actin	F 5' –GCTCCGGCATGTGCAA-3' R 5' –AGGATCTTCATGAGGTAGT-3'	56	500

Table 2: Medicinal plants and their parts selected for ISG56 expression study

Plant species	code	Vernacular Name	Part used	Local use	Chemical composition
Vitex negundo L	VN	TellaVaavili, Pochati	Leaves	Jaundice, Asthma, Cancer, Liver disorders[28].	Flavonoid glycosides, casticin;, Agnuside, and viridiflorol[28].
Wedelia chinensis Merr.	WC	Guntagalagara	Leaves	Jaundice, couch, diphtheria, pertusis, and diarrhea[26].	Isoflavonoids, wedelolactones, Norwedelic acid, saponins, bisdesmosidicNorwedelolactone and oleanolic acid [26].
Rheum emodi Wall.	RE	Nattu-revalchini	Root	Jaundice, Diarrhea, Indigestion, Hiccough [27].	Anthraquinones and stilbenes[29].
Hybanthus ennea spermus (L.)F.Muell	HE	Ratnapurush a	Leaves	Diarrhoea, leucorrhoea, sterility, inflammation, cholera, urinary infections and dysuria [30].	Phenols, Alkaloids, Flavonoid, alcohols, Phytol and Cedran-diol, [24].
Jatropha gossypifolia L.	JG	Nalamudamu	Whole plant	Jaundice, Malaria, Syphilis, Purgative, Rheumatism, eczema and itches [30]	Flavonoids, Phenols, Alkaloids, Cardiac glycosides, Terpenoids[25].

4. CONCLUSION

We have screen five medicinal plants collected from NeMedPlant database for their potential to induce ISG56 in cell culture. Subsequently, the study resulted in a novel method for screening potential anti-viral proteins from plant extracts. Out of the 5 ethnically known medicinal plants, we have identified RE root extract which specifically induce ISG56 expression, a well-known anti-viral proteins. Therefore, the current study could provide an important insight into the development of a novel natural based anti-viral therapeutic from the RE extract without compromising viral resistance.

5. ACKNOWLEDGEMENT

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