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QUERCETIN ALTERS PRO-INFLAMMATORY CYTOKINE CHANGES IN WILD DENGUE VIRUS CHALLENGED HEPG2 CELL LINE

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ABSTRACT

There is no specific antiviral drug available against the life threatening dengue virus till date. In search of newer antiviral agents which may act on dengue fever we observed that in alternative medicine *Eupatorium perfoliatum* extract is used frequently to treat dengue fever. We also observed that Quercetin, a plant polyphenolic flavanoid is an important constituent of *E. perfoiliatum.* Quercetin has been found to possess potent antiviral property against many viral diseases including Flaviviruses; but there is no detailed study of its cytokine modifying activities on dengue virus infected HepG2 cell line. In this study, we explored its effect and role on pro-inflammatory cytokines in HepG2 cells infected with wild virulent dengue viruses of NS1 antigen positive patients' sera, and compared their regulation with respect to the housekeeping gene Beta-Actin. With quercetin cell aggregates were less, Although there were no statistically significant change of TNF- α and IFN-γ, but there was significant down regulation of IL-1β in quercetin treated virus challenged HepG2 cells.

KEYWORDS: Quercetin, Dengue virus, cytokines.

INTRODUCTION

The dengue fever epidemics started in countries like Asia, Africa and North America. At present, dengue fever has become the leading cause of mosquito borne diseases with high morbidity and mortality particularly in children, in many countries of the tropics and subtropics. Dengue fever is caused by one of the four viral serotypes - DENV-1, DENV-2, DENV-3 and DENV-4. In a report published in 2015, a fifth serotype has also been suggested but still it is not widely accepted in scientific community. The serotype classification is based on the type of antigens present on the surface of the virus.[1]

Dengue virus is an enveloped virus with 50 nm of dimension, belonging to the family of Flaviviridae. The virus has a positive single stranded RNA as its genome. The virulence factor is the glycoprotein, which mainly helps in the fusion of the envelope with the host cell membrane. After binding with the receptor, the virus undergoes endocytosis followed by fusion of the envelope and release of the virion into the cytoplasm. Upon internalization, the uncoating and release of the genome occurs once the nucleocapsid is exocytosed into the cytoplasm. Translation of the positive ssRNA occurs using host machinery, forming a polypeptide which is further cleaved into further structural and non-structural proteins. The structural proteins involve capsid, the envelope glycoprotein and the membrane protein.[2]

NS1 antigen is a secreted monomeric glycoprotein, which is not present in the virus particles itself yet it is an essential protein. It has some role as a cofactor for viral RNA replication. During infection it is released into the blood stream as a hexamer from the infected cells.[3] It adheres directly to different types of epithelial and mesenchymal cells but has shown poor attachment to peripheral blood cells. It has been shown that soluble NS1 antigen adheres to the uninfected cells by primary interaction with glycosaminoglycans, heparan sulphate and chondroitin sulphate.[4] Hence, it acts as a method to detect dengue virus infection in human beings through ELISA test of the serum, before dengue virus IgM antibody appears in the blood (within 5 days of fever). This test was first introduced in 2006.

The life cycle of dengue virus starts with mosquito. The most common vectors are *Aedes aegypti* and *A. albopictus* - the Asian tiger mosquito. On biting a human host, the infected mosquito allows the virus to enter through the bite wound and infect the keratinocytes and epidermal cells. Dengue viruses can spread by multiplying inside the macrophages. When a

person gets infected with the virus of different serotype from that of the first one, he is more likely to develop dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) which is the consequence of the phenomenon called "antibody dependent enhancement". DHF and DSS are more serious forms of the disease and can be life threatening. Recently, in a report, it has been observed that about 2.5 million people live in dengue transmission prone areas. Still there has not been any proper medicine or treatment available till now. The patients are prescribed an analgesic and are given oral rehydration therapy.[5]

Cytokines are a group of proteins secreted by cells of the immune system that act as chemical messengers. They actively participate in cell signalling pathways by acting as the link between the humoral and cell mediated immune system. They are produced form a wide range of cells such as macrophages, B and T lymphocytes, endothelial cells etc. From a recent study, it has been observed that in case of dengue haemorrhage fever, there is a significant increase in the serum levels of IL-4, IL-6 and IL-10, whereas the levels of IFN-γ and IL-2 are highest in dengue fever. TNF-α did not show any significant changes.[6] There are few cytokines, which have shown some beneficial role and others some harmful activities, against dengue virus. For example, IFN-γ is actively involved in controlling the replication of the virus hence provide resistance against it, whereas TNF-α is linked with the dengue fever manifestations in humans, hence can cause severe disease.

Quercetin is a plant polyphenolic flavonoid which was first described by Szent Gyorgyi in 1936. It is derived from quercetum which denotes oak forests. They are chemically defined as 3,3′,4′,5,7-Pentahydroxyflavone, frequently occurring as glycosides[fig. 1]. It mainly exists as glycosides and ethers. Although it contains five hydroxyl groups, still its derivatives can be both, lipophilic as well as hydrophilic according to the type of substituent attached with the molecule.[7]

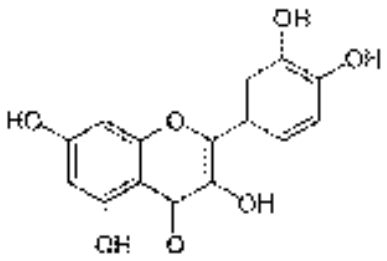

Fig.1: The structure of Quercetin.

Quercetin is widely present in plants and plant products mainly of the family Labiatae and Compositae. It is mostly found in capers (234 mg/100g), onion (32 mg/100g), cranberry (15 mg/100g). In many published papers, the potential medicinal properties of quercetin have been seen and proven, which includes antioxidant property, antiviral, anticancer, apoptotic behaviour and many more. In human breast cancer cell line, it also has been found to act as an agonist of G protein coupled estrogen receptor.

It has been shown that quercetin glucosides (found predominantly in onion or shallot flesh) has more uptake than its rutinosides (the major quercetin glycoside in tea). The glucosides are hydrolyzed in the small intestine by beta-glucosidases to form aglycone, and then absorbed. Quercetin glucuronic acid and its sulphuric acid derivatives are more easily absorbed than quercetin. Purified quercetin glucosides are capable of interacting with the sodium dependent glucose transport receptors in the mucosal epithelium and may therefore be absorbed by the small intestine *in vivo*. After absorption, quercetin becomes metabolized in various organs including the small intestine, colon, liver and kidney.[8,9]

Thus nowadays, quercetin has become popular especially in the medical field, and is undergoing continuous research with the aim of utilising the useful properties in combating the life threatening viral diseases like Japanese encephalitis, Dengue fever etc.

In an experiment performed by Kaul *et.al*[10], it was shown quercetin reduced intracellular replication of several human viruses like HSV-1, Poliovirus type 1, when cell culture monolayers were infected and subsequently cultured in medium containing quercetin. Quercetin showed concentration dependent decrease in plaque assay. In another paper, the antiviral activity of quercetin was seen against Japanese Encephalitis virus during its different stages of replication, in Vero cells. They performed foci forming unit reduction assay (FFURA) and quantitative RT-PCR. The results showed a weak but significant antiviral property against JE virus with IC_{50}=212.1 µg/mL.[11]

In an experiment performed by Zandi and his co-workers, quercetin was found to show significant inhibitory activity against DENV-2 infection in Vero cells. The half maximal inhibitory concentration (IC50) of quercetin against dengue virus was 35.7 µg mL^{-1} when it was used after virus adsorption to the cells. The IC50 decreased to 28.9 µg mL^{-1} when the cells were treated continuously for 5 h before virus infection and up to 4 days post-infection. The SI values for quercetin were 7.07 and 8.74 µg mL^{-1}, respectively.[13]

There are many *in vitro* models available for study of dengue virus, like HepG2 cell line, Vero cell line, C6/36 mosquito cell line. Among these, HepG2 cell line was chosen as it is cancerous in nature derived from human liver which is an active site of infection for dengue virus.

In this study, we determined the cytokine expressions in presence of quercetin on HepG2 cells treated with NS1 antigen positive sera of patients suffering from dengue fever, containing wild virulent dengue viruses.

MATERIALS AND METHODS

Collection of NS1 antigen positive serum and procurement of a control DENV-2 strain

Infected serum from patients having dengue fever were collected, and NS1 antigen positive, dengue IgM antibody negative (Dengue MAC ELISA negative) samples were selected for this study after permission obtained from Institutional Ethical Committee. The general pathological parameters of the three patients from which sera were collected and pooled are given in Table 1. One control Dengue virus (DENV-2) stock was procured from National Institute of Virology, Pune for standardization purposes as this study was based only on wild virulent dengue viruses present in dengue virus infected persons.

Table 1: General pathological parameters of the three dengue fever patients (Dengue MAC ELISA test negative) from which sera were collected.

Patients from which sera were collected	Dengue NS1 antigen in serum (Units)	Haemoglobin level in blood (g/dL)	RBC count (Million/Cu mm)	WBC count (per Cu mm)	Platelets count (per Cu mm)
SB	54.4	14.9	4.75	3100	1,49,000
GD	71.8	15.3	4.95	4500	2,31,000
PN	60.7	13.8	4.58	6100	1,42,000

Stadardisation of HepG2 cell line

Maintenance of HepG2 cells

The HepG2 cell line was procured from National Centre for cell science, Pune. The stock of HepG2 cell line in a cryovial was taken and thawed by putting inside 37^{o}C water bath. DMEM (Dulbecco's Modified Eagle Media) supplemented with Ham's F_{12} nutrient media and antimicrobial agents were used to mix the cell suspension and were transferred to a centrifuge tube. The suspension was centrifuged at 1200x g for 5 minutes at room temperature. The cell pellets were resuspended in fresh DMEM supplemented with F_{12}

nutrient media and antimicrobial agent. The viable cells were stained with trypan blue and counted in hemocytometer. 1 x 10^6 cells/ml were seeded in a T25 flask containing DMEM. The flasks were incubated at 37°C and 5%CO_2 for 48 hrs.

Subculture of HepG2 cells

After 70% confluence was reached, the culture medium of HepG2 cells were discarded and then washed gently with 1x PBS solution. Then 1 mL of 0.1% 1x trypsin-EDTA was added and kept for 1 minute followed by gently tapping the sides of the flask for 1 minute for the detachment of the adhered cells. After 2min of incubation, 5 mL of DMEM with 10% FBS (Fetal Bovine Serum) were added which inactivated the action of trypsin-EDTA. The cells were then washed down along the walls of the flask by pipetting. The cell suspension was then transferred to a 15 mL falcon tube for centrifugation at 1200x g for 5 minutes at room temperature. Then the supernatant was discarded and the pellet was resuspended in 1mL of fresh DMEM with 10% FBS and were transferred to T25 culture flask containing 5mL of media. Then, the flasks were incubated at 37°C and 5%CO_2 for 48 hrs.

Standardisation of Quercetin

3.0224 mg of quercetin was dissolved in 0.1% DMSO, to prepare the concentration of 10 mM, which was further used to prepare different concentrations including 0.1 µM, 1 µM, 5 µM, 10 µM, 20 µM.

Cytotoxicity Assay (MTT)

MTT assay was first described by Tim Mosmann in 1983. This colorimetric assay involves reduction of a yellow tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) to measure quantitatively, cellular metabolic activity or number of viable cells. Live cells contain NAD(P)H-dependent oxidoreductase enzymes which reduces the MTT reagent and form an insoluble, deep purple crystalline product called formazan. These crystals are then dissolved using a solubilising solution such as DMSO and the absorbance is measured at 500-600 nanometers. The darker the solution, the greater the number of viable, metabolically active cells. IC_{50} dose of quercetin is equal to 7.10 µM,[15], which was used further in this study.

Main experiment

Treatment of Quercetin on HepG2 cell line challenged with dengue NS1 antigen positive serum containing wild viruses

The selected dengue NS1 antigen positive, dengue IgM antibody negative sera were mixed together and challenged on HepG2 cells in presence or absence of quercetin. The HepG2 cells were divided into five different sets in 6 well plates having only cells, cells and dengue NS1antigen positive pooled serum, cells and quercetin at concentration 0.1 µM, cell preincubated with quercetin for 1 hour and then the positive serum was added and finally the cells preincubated with the positive serum for 2 hours and then quercetin was added followed by an incubation period of 24 hrs. This whole set was performed in triplicates.

ELISA Test

The ELISA for Dengue NS1 antigen was performed at 0 hour and at 24 hours of incubation from the supernatant taken from the dengue NS1 antigen positive serum treated culture plates following the standard protocol given in the kit (J Mitra, India) manual.

RNA extraction, RT PCR and semiq-RT PCR

After 24hrs of incubation cell supernatants were homogenized with trizol (RNAiso plus) solution maintaining the standard protocol. Then the extracted RNA was dissolved in RNAse free water. The extracted RNAs were quantified using nanodrop 2000 and they were converted to cDNA using RT-PCR. The reaction mixture had 1 µg of RNA sample, 4 µl of supermix and rest amount of nuclease free water to make up the total volume up to 20 µl. The cycle was performed following the standard protocol i.e. 5 minutes at 25^{o} C, 20 minutes at 46^{o}C, 1 min at 95^{o}C.

Primers for three different cytokines, IL-1β, TNF-α and IFN-γ were used along with the cDNAs in real time PCR following the standard protocol i.e. 95^{o}C for 2 min, 95^{o}C for 30 sec, 61^{o}C for TNF-α and IFN-γ, for 1 min; 60^{o}C for IL1-β, 1 min; 72^{o}C for 30 sec, repeated for 39 cycles and extension at 72^{o}C for 5 min.

RESULTS

Morphological changes

The dengue NS1 antigen positive serum treated HepG2 cells, were observed under inverted microscope at 400 x magnification, after 24 hours of incubation period. There was a significant decrease in formation of cell aggregates in dengue virus infected HepG2 cells in

presence of Quercetin at non cytotoxic concentration, as compared to virus infected cells (Table 2 and Fig. 2). Average rounded cells were also more in these two groups.

Table 2: Cell morphology changes in different experimental sets.

Experimental sets	Islets of cell aggregates	Average number of round apoptotic bodies (per high power microscopic field)
Control cells	"Normal"	40
With Quercetin (0.1 µM)	decreased	39
With Virus	increased	59
With Quercetin (0.1 µM) first followed by virus	Markedly decreased	58
With virus first followed by Quercetin (0.1 µM)	Markedly decreased	47

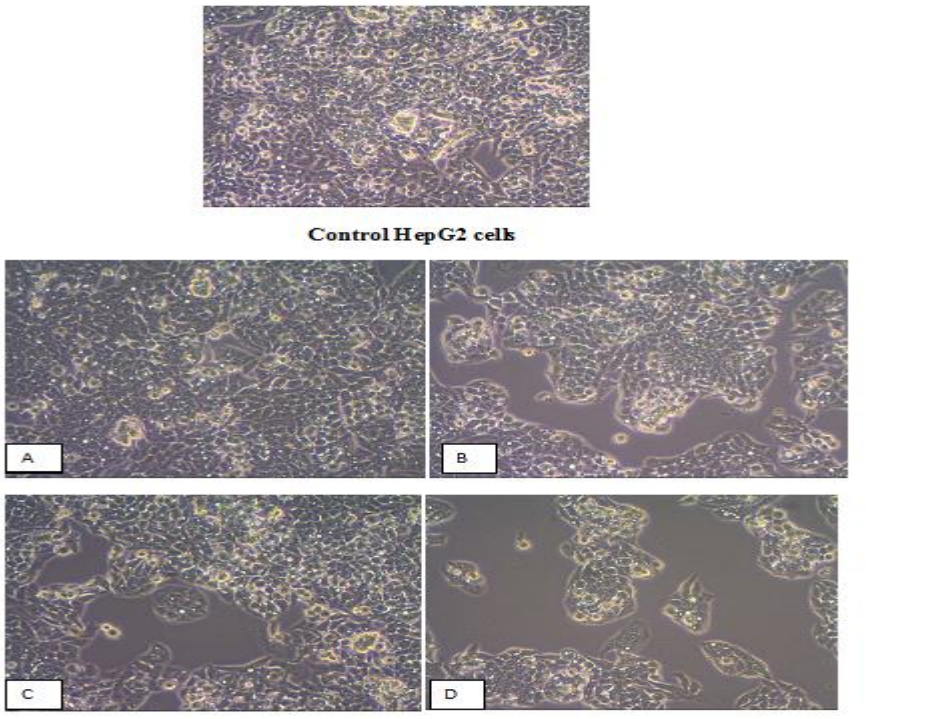

Fig. 2 : Showing morphological patterns of HepG2 cells control, with 0.1 µM quercetin (A), Virus (B), quercetin first followed by virus (C), virus first followed by quercetin(D).

ELISA Test results

There was significant decrease of dengue NS1 antigens found in HepG2 cells, when treated with Quercetin, in post treatment set (Table 3).

Table 3: Showing Dengue NS1 antigen ELISA test results (units) in experimental sets where serum was added.

ELISA test time	HepG2 cell line with dengue NS1 antigen positive serum	HepG2 cell line with dengue NS1 antigen positive serum followed by addition of quercetin	HepG2 cell line with quercetin followed by addition of dengue NS1 antigen positive serum
0 hour	271.35	101.775	208.8
24 hours	284.2	193.8	177.65

Cytokine expression observed in semi-quantitative PCR

IL1-β was somewhat decreased (Fig. 3) in both pre and post treated quercetin experimental sets (P value of differences of IL1-β values between only virus challenged cell set and quercetin followed by virus experimental set was 0.06 with a t-value of 3.8716; while these values between only virus challenged cell experimental group and virus followed by quercetin experimental set was 0.08 and 3.2053 respectively. None of the differences of TNF-α and IFN-γ in between different experimental sets were significant even at 0.10 level.

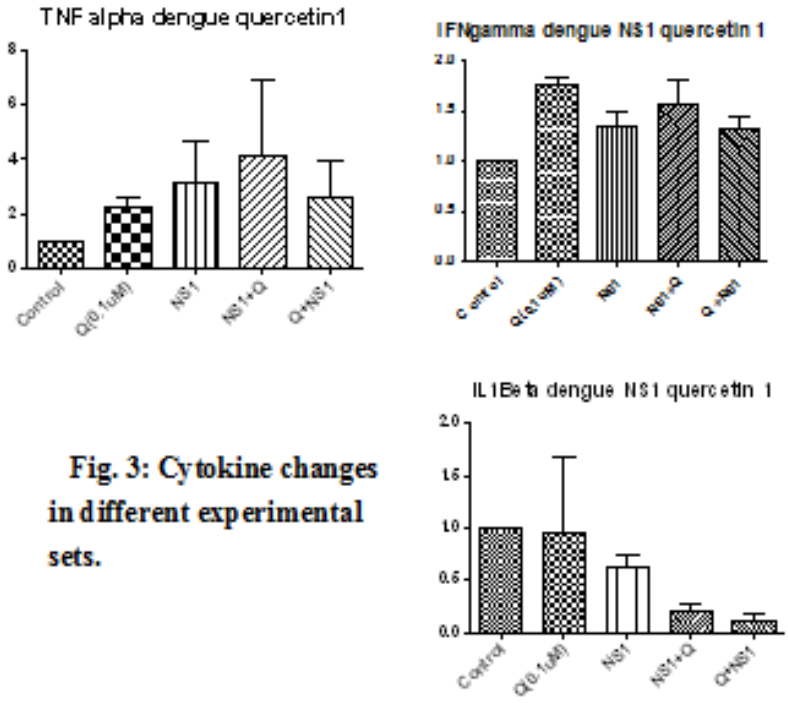

Fig. 3: Cytokine changes in different experimental sets.

DISCUSSION

Dengue fever alone contributes to 14% of acute fever cases throughout the globe[16] and liver damage in dengue fever is an important characteristic feature of severe forms of the disease.[17] In dengue fever, hepatomegaly[18], elevated SGPT (ALT)[19], presence of dengue

virus[20,21,22,23] and councilman bodies[24,25] in liver in post mortem examination- all these observations indicate that hepatic organotropism is an important phenomenon in dengue fever. Thus a study on HepG2 cell line with dengue virus may resemble the natural infection in the human body.

There are evidences that TNF- α, IL-1β and IFN-γ are all increased in dengue virus infection[26] After the viral challenge, the viruses activate signaling pathway, the cells up regulate surface activation molecules producing cytokines. TNF-α plays an important role in inducing apoptosis and among TNF family receptors, the Fas receptor is closely related to viral infection. In HepG2 cells dengue virus capsid protein induces Fas-dependent apoptosis[27] Fas signaling induces upregulation of TNF-α and IL-1β gene which modulate pro-inflammatory response by a caspase –dependent pathway.[28] The genetic pathway of programmed cell death was first demonstrated in a nematode – *Caenorhabditis elegans* and two genes namely ted-3 and ced-4 were identified as the main responsible genes for that[29] IL1 β is a mammalian homologue of ced-3.

Quercetin can provoke G2/M phase cell cycle arrest in HepG2 cells.[30] Quercetin can cause increased activity of superoxide dismutase, glutathione reductase and glutathione content of HepG2 cells[31] Again quercetin is a therapeutic agent for hepatocellular carcinoma where it acts as a competitive inhibitor of glucose transporter 1. Thus it can induce inhibition of metabolic activity and cell death by apoptosis of the cancer cells. In an important study it was found that higher doses of quercetin is not effective[32], thus in this study when we started experiments with lower (0.1µM) as well as higher (1.0 µM) concentrations of quercetin we observed inconsistent results with higher concentration of quercetin. Apoptosis induced by quercetin is by activation of caspase -3.

In this experiment we demonstrated the effect of quercetin on these cytokine expressions. It can be concluded from our study that Quercetin is able to change proinflammatory cytokine activities mainly by down regulation of IL1- β. This is important because marked up regulation of TNF leads to complications in dengue fever, quercetin not only gives a protective role preventing damaging action of TNF by further down regulating IL1- β. The TNF although is a an inflammatory cytokine activating the endothelial cells, enhances vascular permeability and plasma leakage in DHF, it is also involved in apoptosis via caspases 3 activation. IL1- β's primary receptor the IL-1 receptor is constitutively expressed

in mostly all the cells. Moreover, IFN and IL1 is known to competitively regulate each other, such that higher concentration of IFN, suppresses production of IL1 and vice versa.

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