

Effect of Hippophae Rhamnoides Leaf Extract against Dengue Virus Infection in U937 Cells

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Abstract

Hippophae rhamnoides (Seabuckthorn) (Family: *Elaeagnaceae*), a medicinal plant from Himalayas, traditionally used for health promotion. The study has been designed to investigate the effect of Sea buckthorn leaf alcoholic extract and other well-known medicinal plants, in Dengue infected U-937 cells (Human Monocytic cell line), as monocytes are the host cells for Dengue virus. Significantly high anti-dengue activity of Seabuckthorn was observed indicating it as a potential candidate for management of Dengue infection.

Keywords: Seabuckthorn leaf alcoholic extract; Dengue virus; Cytotoxicity; Plaque number

Abbreviations

ROS: Reactive Oxygen Species; DHF: Dengue Hemorrhagic Fever; DSS: Dengue Shock Syndrome; HSP: Heat Shock Protein; SBT: Seabuckthorn Leaf Alcoholic Extract; SBT: Seabuckthorn; FBS: Fetal Bovine Serum; PBS: Phosphate Buffer Saline; HCL: Hydrochloric Acid; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-Diphenyl Tetrazolium Bromide; ELISA: Enzyme Linked Immunosorbent Assay; TNF – α : Tumor Necrosis Factor – alpha; INF – γ : Interferon – gamma; MEM: Modified Eagles Medium; BHK-21: Baby Hamster Kidney Cell; NaCl: Sodium Chloride; HPLC: High Pressure Liquid Chromatography; OD: Optical Density; AZT: Azidothymidine; HIV: Human Immunodeficiency Virus; RNA: Ribonucleic Acid

Introduction

The use of herbal extracts either as alternative or complimentary medicine to the conventional chemotherapy is well documented in Ayurveda. Number of medicinal plants having significant immunomodulatory properties have been identified [1]. There are well known and widely used plants possessing antiviral properties e.g., *Mimosa scabrella* and *Leucaena leucocephala* against Yellow fever virus and Dengue 1 virus [2], Olive leaf extract against HIV-1 [3], *Hippophae rhamnoides* and *Rhodiola* are documented to have antiviral properties against Dengue virus [4,5]. Several plants, approximately 50 different species have been reported to contain antiviral components such as terpenoids, coumarins, polyphenols, tannins, proteins, alkaloids, and biflavonoids which can inhibit HIV life cycle at different stages [6], *Aristolochia indica* *Cassia occidentalis*, *Phyllanthus niruri* *Withania somnifera* *Tinospora cordifolia* against HIV [7], *Rhus chinensis* and *Rhus javanica* against HSV [8].

Seabuckthorn (*Hippophae rhamnoides*), Family- Elaeagnaceae, a traditional popular medicinal plant, also called a wonder plant, is used in various parts of the world. Medicinal properties of Seabuckthorn are documented by the name “Amlavetas” in the ancient Indian Medical Classic “Ayurveda” where it is described as a plant with various

medicinal properties, briefly as a health protection plant in Himalaya. It is a shrub growing in the drier ranges of the north-western Himalayas at an altitude of 2,100-3,600 meters. It is well reported for its enormous medicinal properties and well documented for immunomodulatory as well as anti-inflammatory potential [9-12]. It is widely used for preparation of vitamins and nutrient products [13,14].

All parts of this medicinal plant are rich source of various biologically active compounds. Its fruits are used for commercial scale production of medicinally important fatty oil [15-17]. In the leaves of seabuckthorn [18]. Diwaker et al. [4] have found isorhamnetin, and flavonoid glycosides isorhamnetin-3-O- β -D-glucopyranoside, isorhamnetin-7-O- α -L-rhamnopyranoside, isorhamnetin-3-[O- β -D-glucopyranosyl]-7-O- α -L-rhamnopyranoside, and kaempferol- β -glucopyranoside (astragalins). Leaf extract has been reported to have marked anti-bacterial, anti-viral, anti-tumor, and wound healing activity by decreasing cytotoxicity and ROS generation and adaptogenic activity under multiple stresses respectively [5,18-23], besides having significant anti-oxidant, anti-inflammatory and immunomodulatory properties [11,12,24]. Leaf extract based drugs, containing flavanoids, have been demonstrated to increase the wound healing after chemical burns and plain wounds [25,26]. The total flavonoid extracted from Seabuckthorn leaves had considerable anti-hyperlipidemia, anti-myocardial and anti-fatty liver effects [27,28].

Dengue virus is an arthropod born flavivirus that causes serious human disease, principally in the tropical areas of Asia, Oceania, Africa and America [29]. There are four serotypes of Dengue virus (Den-1, 2, 3 and 4) and infection by any one of them causes a spectrum of clinical disease ranging from an acute debilitating self-limited febrile illness- Dengue Fever (DF) to a life threatening syndrome Dengue hemorrhagic fever/Dengue shock syndrome (DHF/ DSS). No specific therapeutic agents are available for the treatment of Dengue fever. Lack of an appropriate animal model for Dengue disease [30-32], and the presence of four distinct serotypes of Dengue virus have compounded the task of developing an effective Dengue treatment [33]. Even present treatment strategies for Dengue are more supportive than curative. At best prescription is bed rest, plenty of fluids and medications to reduce fever and most of all mosquito control, is best prevention.

Immunopathological studies suggest that many tissues may be involved during Dengue infection e.g., liver, lymph node, spleen and bone marrow [34,35]. In Autopsy samples, macrophages are among cell types reported to display Dengue virus antigens [36,37]. Blood constitutes heterogeneous population of monocytes due to differentiation stages which takes place in between 36-104 hrs [38], during maturation of cells. Earlier studies suggest that Dengue virus after attaching itself to the envelope protein E, enters the target cells, through an uncharacterized receptor that may display highly sulfated glycosaminoglycans. Binding moieties present on the cell-surface membrane may vary between cell types and species origin, as well as for the different serotypes [39-42]. The present study was undertaken to determine the effect of Seabuckthorn Leaf Alcoholic Extract in *in-vitro* Dengue virus infection using human macrophage cell line U-937 (Promonocytic cell stage). Different medicinal plant extracts like *Emblica officinalis* (*Amla*), *Terminalia bellerica* (*Bahera*) and *Evolvulus alsinoides* (*Shankhpushpi*) were used and compared with Seabuckthorn (SBT) and screened for their cytotoxicity followed by evaluating the effect on Dengue virus infection in U-937 cells.

Materials and Methods

Cells

U-937 and BHK-21 cell lines were obtained from ATCC, Atlanta and maintained in RPMI-1640 medium (Sigma, USA), supplemented with 10% fetal bovine serum (FBS, Sigma), 100 U/mL penicillin (Pubchem ID C₁₆H₁₈N₂O₄), and 100 µg/mL streptomycin (C₂₁H₃₉N₇O₁₂) (Sigma), at 37°C in 5% CO₂ atmosphere in an incubator (Sanyo, Japan).

Virus 1

Dengue virus type-2 (Dengue virus), New Guinea strain, was obtained from ICGEB, New Delhi, India. Virus was expanded in C6/36 cell line for 14 days at 28°C in absence of CO₂. Culture supernatant was collected and centrifuged at 2000 rpm for 15 min. Again the supernatant was collected, pooled and stored at -200°C as stock until use.

Plant materials

Medicinal plants used for the study were fruit of *Amla*, fruit of *Bahera*, whole plant of *Shankhpushpi*, and leaves of SBT. All plants collected from authentic sources, were dried under shade and powdered in the laboratory. Crude powder of *Amla*, *Bahera* and *Shankhpushpi* were extracted in water overnight, centrifuged at 3000 rpm and the supernatant was collected for further studies.

The leaves of SBT plant were collected from hilly regions of Western Himalayas, India, in the month of September. The voucher specimen (058/FRL/2002) has been identified and conserved in DIHAR, DRDO, Leh, India.

Preparation of plant extract

The shade dried leaves of SBT were powdered and extracted with 70% ethanol (Pubchem ID C₂H₆O) overnight. The supernatant was saved and the residue was subjected to re-extraction with 70% ethanol. The process was repeated four times for complete extraction. The supernatant of leaf extracts were pooled and dried under vacuum at 40°C. For experimental use the dried extract was dissolved in 70%

alcohol. The extract was diluted in RPMI medium according to the requirement for various assays. The extract was named as SBTLAE (Seabuckthorn Leaf Alcoholic Extract).

Infection of cells

U937 cells were infected with Dengue virus at a multiplicity of infection (MOI) of log ten. The virus inoculum (about 20 µL/ well of 96 wells plate), was incubated with 20 µL (0.2 × 10⁵/well) of cells in serum-free medium at 37°C for one hour to permit viral absorption. The culture plates were gently agitated for optimal virus cell contact.

Thereafter, the unabsorbed virus was removed by washing the cells with phosphate buffer saline (PBS). The Dengue virus infected and non-infected cells were replenished with fresh medium with 2% FBS and further incubated for three days. At the end of the incubation, cells were harvested and the cell free supernatant was stored in aliquots at -70°C until assayed for infectious-virus production. Ribavirin, (Pubchem ID C₈H₁₂N₄O₅), a commercially available anti-viral drug, was used at a concentration of 200 µg/mL as positive standard.

Cytopathic effect

The cytopathic effect in infected U937 cells was studied after causing infection with Dengue virus for three days as described earlier [43].

Cytotoxicity of plant extracts

Different plant extracts like *Amla*, *Shankhpushpi*, *Bahera* and SBTLAE were screened for cytotoxicity by measuring cell viability using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Pubchem ID C₁₈H₁₆BrN₅S). Briefly, plant extracts of various concentrations (i.e., 10, 50 and 100 µg/mL) were added to U-937 cells (0.2 × 10⁵/20 µL) grown in 96 well plates in 0.2 mL of culture medium containing 2% FBS. Cells were incubated at 37°C in 5% CO₂ atmosphere. Three days later, 10 µL of MTT solution (5 mg/mL) in PBS was added to each well and incubated in dark for four hrs, following which the plates were centrifuged at 1500 rpm for 10 min at RT.

The supernatant was removed carefully and 100 µL of Dimethyl sulfoxide (Pubchem ID C₂H₆O₅) was added to each well to dissolve the formazan crystals while shaking the plates for five min. The absorbance was measured in ELISA reader at 570 nm. Cytotoxicity of plant extracts was calculated as absorbance value of test/absorbance value of control × 100 [44].

Effect of SBTLAE on cell viability of dengue infected U-937 cells

To study the effect of SBTLAE on cell viability of infected U-937 cells, 20 µL of Dengue virus at 1:100 dilution was added to the cells for causing the infection. SBTLAE was added to infected and non-infected cells in different concentrations as 100, 50 and 10 µg/mL. Cell viability of cells was measured by MTT assay. Ribavirin obtained from Lupin, India was used as a positive control.

Cytokine estimation

Proinflammatory cytokines like TNF-α and IFN-γ were measured in cell free supernatant by ELISA using BD Pharmingen kits. The assay was performed following the manufacturer's instructions. Briefly, cells

were incubated with 100 μ L of Dengue virus at 1:100 dilution in presence or absence of extract. Cells were incubated for 72 hours, supernatant was collected and used for measuring cytokine levels.

Plaque assay

In 24-well plate, BHK-21 cells were seeded in triplicate until confluent and maintained in MEM with 10% FBS at 37°C. Supernatants collected from Dengue virus infected U-937 cells, and treated with SBTLAE and Ribavirin (as positive control), were added to the BHK-21 and incubated for 2 hrs at 37°C.

After 2 hrs MEM medium containing 1% agarose with 2% FBS was added to each well and again incubated at 37°C for 7 days. For plaques counting 3.7% formaldehyde was added to each well for fixing and followed by removal of agarose plug. Plaques were stained with 1% crystal violet solution in 20% methanol and counted as plaque forming units (PFU) per milliliter.

Hemolytic activity

Heparinized blood samples obtained from balb/c mice (n=7) were separately collected in a 15 ml falcon tube. Aliquots of blood were washed three times with sterile saline solution (SS) (0.9 % w/v NaCl,) by centrifugation at 1500 rpm for 5 min. The cell suspension was prepared by diluting the pellet in 0.5% SS. A volume of 0.5 ml of the cell suspension was mixed with 0.5 ml diluent containing different doses of SBTLAE (6.25-100 mg) in SS. The mixtures were incubated at 37°C for 30 min, and centrifuged at 2000 rpm for 10 minutes. Free hemoglobin content in the supernatants was measured spectrophotometrically at 412 nm. Saline and distilled water were used as minimal and maximal hemolytic controls respectively. The hemolytic percentage was calculated as:

$$\% \text{ Hemolysis} = \left\{ \frac{(\text{O.D. test} - \text{O.D. control min})}{(\text{O.D. control max} - \text{O.D. control min})} \right\} \times 100$$

HPLC analysis of the extract

HPLC analysis of SBTLE was performed previously [5]. The analysis of data obtained using HPLC confirms the presence of Quercetin, a potent flavonoid with strong immunomodulatory activity, in extract of SBT leaves used in the present study.

Determination of total phenol content

Total Phenol content of extract/extract fractions were determined by the Folin-Ciocalteu method as described earlier by [45]. Briefly, 150 μ L of extract, 2400 μ L of ultrapure water and 150 μ L of 0.25 N Folin-Ciocalteu reagent were combined and then mixed well. The mixture was allowed to react for 3 min then 300 μ L of 1 N Na₂CO₃ solution was added and mixed well.

The solution was incubated at room temperature in the dark for 2 h. The absorbance was measured at 725 nm using a spectrophotometer and the results were expressed in milligram of gallic acid equivalents (GAE) per gram extract/extract fractions.

Statistical analysis

All the experiments were conducted six times and results were determined as mean \pm SEM and statistical comparisons were carried out using Analysis of Variance (ANOVA). Wherever required, post

hoc, Newman Keul's and Dunnett's test were applied. Significance level was set at p<0.05.

Results

Cytopathic effect

Non-infected and infected cells with Dengue virus were stained with haematoxylin and eosin. After three days of infection, marked difference between the control and infected cells was observed.

Crenation of cells was observed with clear features of cellular damage at a dose of 1:100 TCID₅₀ in infected cells. No such change in the morphological pattern was observed in non-infected control cells [43].

Cytotoxicity of plant extracts

The cytotoxicity of different concentrations of four plant extracts in U-937 cells is indicated in (Figure 1).

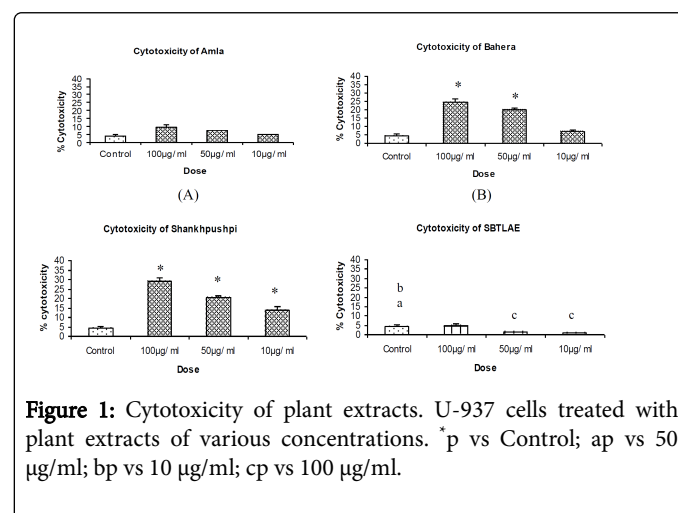


Figure 1: Cytotoxicity of plant extracts. U-937 cells treated with plant extracts of various concentrations. *p vs Control; ap vs 50 μ g/ml; bp vs 10 μ g/ml; cp vs 100 μ g/ml.

A dose response study was performed using Amla, Bahera, Shankpushpi and SBTLAE extracts ranging from 10, 50 and 100 μ g/mL concentrations. Amla at maximum concentration i.e., 100 μ g/mL showed significant (p<0.05) level of cytotoxicity whereas 50 and 10 μ g/mL did not show any cytotoxicity as compared to control cells (Figure 1a).

Bahera showed significant (p<0.01) toxicity at 100 and 50 μ g/mL concentrations but not at 10 μ g/mL (Figure 1b), Shankpushpi showed significant (p<0.01) toxicity at all the concentrations (Figure 1c). While SBTLAE did not show any cytotoxicity at any concentration as compared to control (Figure 1d). On the basis of these results SBTLAE was selected for further treatment analysis of infected cells.

Effect of SBTLAE treatment on cell viability of dengue infected U-937 cells

The viability of Dengue virus infected U-937 cells after treatment with SBTLAE is illustrated in (Figure 2).

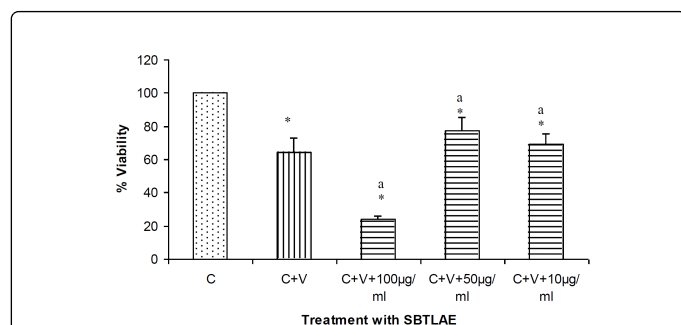


Figure 2: Cell viability of infected U-937 cells after treatment with SBTLAE. Viability was measured in terms of percent viability. C = Control cells; C+V = Cells infected with virus; C+V+100 µg/mL = Infected cells treated with 100µg/ml of SBTLAE; C+V+50 µg/ml = Infected cells treated with 50 µg/ml of SBTLAE; C+V+10 µg/mL = Infected cells treated with 10 µg/ml of SBTLAE; * Vs Control; a Vs C+V.

Cell viability of Dengue virus infected cells was significantly ($p < 0.01$) reduced as compared to control cells. Infected cells treated with 50 and 10 µg/mL of SBTLAE showed significantly ($p < 0.01$) higher cell viability than infected cells but less than control cells. Whereas 100 µg/mL concentration of SBTLAE showed significantly ($p < 0.01$) lower viability than control and infected as well as 50 and 10 µg/mL of SBTLAE treated cells. Between 50 and 10 µg/mL concentrations of SBTLAE, 50 µg/mL showed better viability than 10 µg/mL. Therefore, 50 µg/mL concentration of SBTLAE was used for further analysis as an optimum dose for treatment of infected cells. Viability of control cells was considered to be 100%.

Cell viability of U-937 cells after treatment with 50 µg/mL SBTLAE was compared with Ribavirin in Dengue virus infected cells (Figure 3).

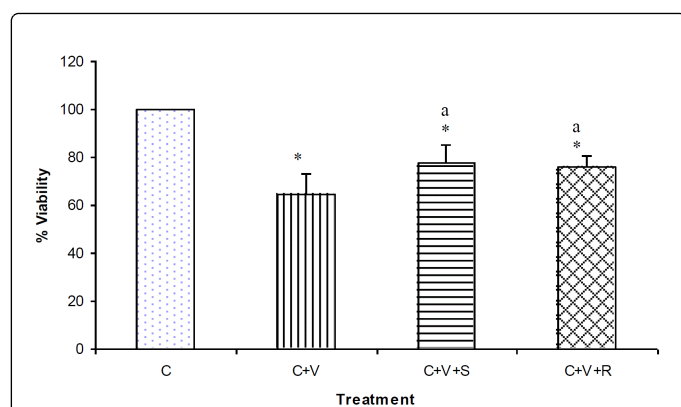


Figure 3: Comparison of 50 µg/mL SBTLAE with Ribavirin during Dengue virus infection. Viability was measured in terms of percent viability. C = Control cells; C+V = Cells infected with virus; C+V+S = Infected cells treated with 50 µg/ml of SBTLAE; C+V+R = Infected cells treated with Ribavirin; * Vs Control; a Vs C+V.

Cell viability of Dengue infected cells treated with SBTLAE and Ribavirin was significantly ($p < 0.01$) reduced as compared to control cells. Whereas viability of infected cells treated with SBTLAE and Ribavirin were significantly ($p < 0.05$) higher than infected, non-treated cells. There was no difference between the percentage of cell viability of

SBTLAE and Ribavirin treatments. Thus, the anti-viral activity of SBTLAE was comparable to Ribavirin.

Cytokine estimation

Production of TNF- α , a pro-inflammatory cytokine, in Dengue virus infected cells was significantly ($p < 0.05$) higher in infected cells as compared to either control or SBTLAE treated infected or SBTLAE treated non infected cells (Figure 4a).

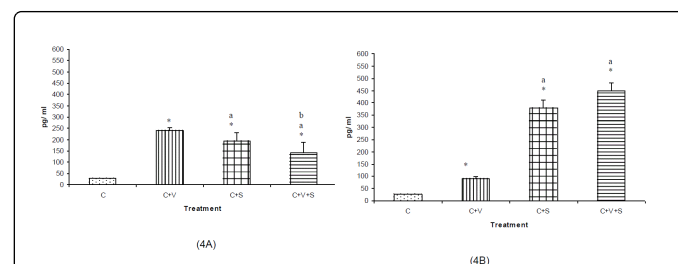


Figure 4: Cytokine profile of Dengue virus infected U-937 cells. Figure 4a: Profile of TNF- α . Figure 4b: Profile of IFN- γ . C=Control cells; C+V = Cells infected with virus; C+S = Non-infected cells treated with 50 µg/mL of SBTLAE; C+V+S = Infected cells treated with 50 µg/mL of SBTLAE; * Vs Control; a Vs C+V; b Vs C+S.

Whereas amongst infected and non-infected cells, SBTLAE treated infected cells showed significantly ($p < 0.01$) lower TNF- α production. This indicates that treatment with SBTLAE reduced the TNF- α production in Dengue virus infected cells.

Production of IFN- γ was significantly ($p < 0.001$) higher in both SBTLAE treated non-infected and infected cells when compared to either control or infected cells (Figure 4b). Amongst SBTLAE treated infected and non-infected cells, the treated infected cells showed maximum IFN- γ production.

Plaque Assay

(Figure 5a) depicts the formation of plaques induced by Dengue virus in BHK-21 cells. Treatment of infected cells with SBTLAE and Ribavirin (50 µg/mL) decreased the viral titre significantly ($p < 0.01$) as compared to only non-treated infected cells. However, the viral titer in Ribavirin treated cells was further significantly ($p < 0.01$) reduced than SBTLAE treated cells (Figure 5b).

Hemolytic activity

Red blood cells are relevant tool for toxicity studies because of their easy availability and well known membrane properties as their lyses is easy to monitor by measuring the release of hemoglobin [46]. To determine the hemolytic effect of SBTLE mice erythrocytes were collected and treated with different concentrations of SBTLE. The extract was assessed for their hemolytic activity on RBCs by the amount of hemoglobin content released in the mixture. The extract did not show any hemolytic activity on animal blood as compared to maximal control ($p \leq 0.001$) at concentrations 6.25 to 100 mg/L (Figure 6).

Analysis of Phenolic compounds by RP-HPLC

Seabuckthorn contains natural antioxidant constituents such as phenolic compounds. SBT leaves were found to contain maximum phenolic content as already described earlier by MS Yogendra Kumar et al. [44].

Discussion

Dengue virus is transmitted to humans through the bite of infected *Aedes aegypti*. With the expansion of epidemic areas, the new viruses are emerging in more virulent form with the manifestation of DHF and DSS which is often fatal if not treated properly. The currently available synthetic anti-viral drugs viz. AZT and Ribavirin, the most effective against HIV and Herpes Simplex Virus, have limited therapeutic usefulness owing to high degree of toxicity towards target cells. Over the past few decades, researchers have focused their attention towards many natural compounds for anti-viral properties.

Currently there is no natural or synthetic drug that has been approved as an antiviral agent. Gradually, the scenario is changing, as at least many natural products and their derivatives are undergoing clinical trials against HIV and HCV [47]. The use of medicinal plants is widespread in India.

There are so many evidences which state about the plants activity against bacteria [48], fungi [49], protozoans [50], and non-infectious conditions [51], besides having anti-clastogenic, hepatoprotective, anti-tumor and anti-inflammatory properties of Amla [12,52,53] cholinergic and hypotensive activity of Bahera [54]. And anti-phlogistic, febrifuge activity and as a brain tonic properties of Shankpushpi [55]. However, there is lack of information about the anti-Dengue properties of plants.

In the present study SBTLAE was tested for its immunomodulatory and anti-dengue activity in human monocytic cell line i.e. U937. Macrophages are often the primary target for virus entry. Number of viruses can replicate in macrophages, and few viruses replicate almost exclusively in this cell type. It has been suggested that differentiation of mononuclear phagocytes play an important role in persistence of latency of many viruses like cytomegalovirus and Friend leukemia virus. This is also persistently evident in visna virus infected sheep where bone marrow precursors of macrophages carry only a few copies of viral RNA in contrast to thousands of copies of viral genome in tissue macrophages [56,57]. Monocyte differentiation of macrophages involves regulated changes of cell surface antigen expression and gene transcription which is concomitant to the development of specific macrophage functions, such as their cytokine profile, oxidative and microbial metabolism [58,59].

Many studies have shown that cell susceptibility to viral infections varies with the type of cells and is altered during *in-vitro* cultivation of cells [60,61,62,63,64,65]. Also Dengue virus has the ability to infect both myeloid dendritic cells and self-differentiated macrophages or "differentiated cells" derived from monocytic origin [66,67].

SBTLAE has been compared with other medicinal plant extracts like *Amla*, *Bahera* and *Shankpushpi* in U937 cell line. It was clearly evident that SBTLAE did not show any cytotoxicity at any concentration whereas *Amla*, *Bahera* and *Shankpushpi* showed significant levels of cytotoxicity at some concentrations tested. Percent cell viability of infected and SBTLAE treated cells showed highest cell viability of infected cells at concentration of 50 µg/mL. The possible explanation for the observed cytotoxic effect of other extracts may be (1) U-937 cell

line could be more sensitive to those extracts. (2) Worthy of note however, is that all the aqueous extracts (except SBTLAE) are more cytotoxic than ethanolic extract of SBTLAE.

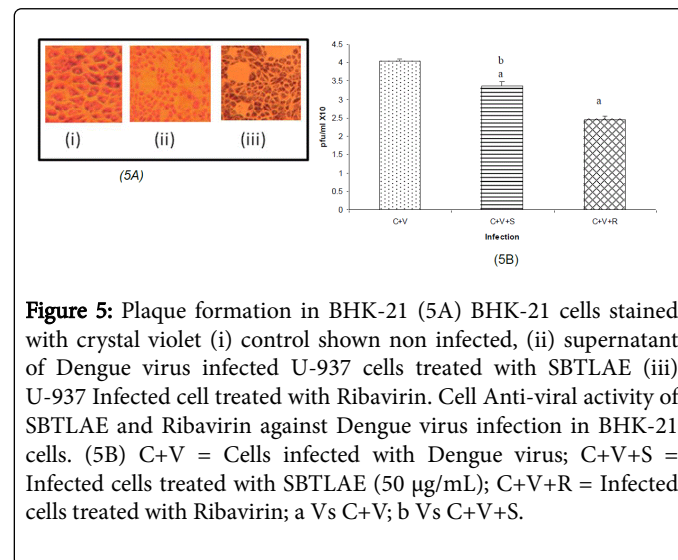


Figure 5: Plaque formation in BHK-21 (5A) BHK-21 cells stained with crystal violet (i) control shown non infected, (ii) supernatant of Dengue virus infected U-937 cells treated with SBTLAE (iii) U-937 Infected cell treated with Ribavirin. Cell Anti-viral activity of SBTLAE and Ribavirin against Dengue virus infection in BHK-21 cells. (5B) C+V = Cells infected with Dengue virus; C+V+S = Infected cells treated with SBTLAE (50 µg/mL); C+V+R = Infected cells treated with Ribavirin; a Vs C+V; b Vs C+V+S.

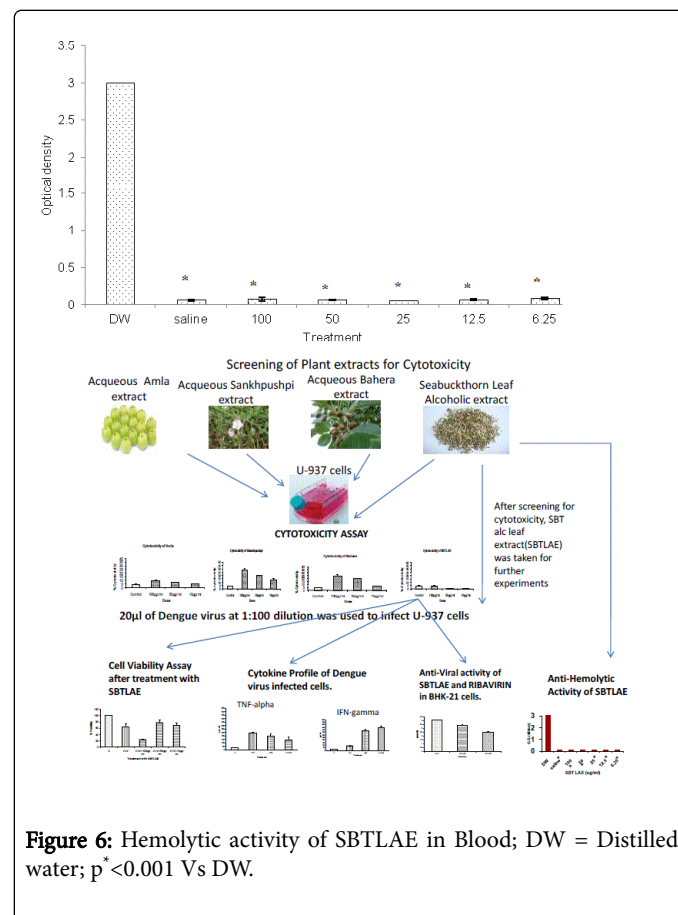


Figure 6: Hemolytic activity of SBTLAE in Blood; DW = Distilled water; $p^* < 0.001$ Vs DW.

It can be assumed that Ethanolic extraction might have changed the properties of extract. This hypothesis could be related to the fact that many classes of compounds are obtained with changes in the properties when methanol/ ethanol is used as an extractant which incidentally some times lead to non-specific bioactivity. This

phenomenon has also been observed in some methanolic extracts of herbal plants [68,69]. Increase in the percent viability of infected cells at 50 µg/mL concentration states that dose of the extract is an important factor for immunomodulatory effect of extracts.

Dengue virus infection leads to production of various cytokines by infected monocytes, B lymphocytes and mast cells. In the present study we have found elevated levels of TNF-α during virus infection. The role of TNF-α in increasing the severity of the disease has also been observed by others. SBTLAE has decreased the TNF-α production in Dengue infected cells and increased the IFN-γ level in the infected cells. Anti-viral action of IFN-γ has been described by Goodman [70]. Therefore, increased levels of IFN-γ in Dengue infected cells indicated the anti-viral property of SBTLAE. The finding goes well in line with the report that SBTLAE is an IFN-γ inducer in the cell culture of normal peripheral human blood [23].

On the basis of screening, cytotoxicity, and immunomodulatory activity of infected cells, SBTLAE has been further investigated for its anti-dengue activity by plaque assay. Reduction in plaque forming units is one of the important ways for determining the anti-viral activity. Significant reduction was observed in plaque numbers after the treatment of infected cells with SBTLAE which was at par with Ribavirin used as positive standard.

Seabuckthorn leaves are rich source of flavanoids, terpenoids, alkaloids, polyphenols, and isorhamnetin. Chemical investigation of SBT leaves reveals the presence of a flavanoid; Quercetin. Quercetin possesses antiviral activity against herpes simplex virus (HSV) type-1, respiratory syncytial virus, pseudorabies virus, parainfluenza virus type 3 and Sindbis virus, a type member of genus Alphavirus. Anti-viral mechanism of Quercetin includes: increase in the interferon level and binding to the viral polymerases followed by interfering with viral nucleic acid synthesis. Consistent with present data Quercetin might be responsible for IFN-γ production [71]. Bioflavonoid quercetin exhibited significant anti-dengue virus replication properties by affecting the intracellular Dengue virus replication but not the Dengue virus attachment and entry processes. Another possible mechanism of anti-Dengue activity of Quercetin might be due to reduction in oxidative stress.

Present study shows the anti hemolytic activity of SBTLAE. Hemolysis has a long history of use in measuring free radical damage and its inhibition by plant extract which in turn describes the toxic effects and safety of the plant therapeutics. Recently few studies have reported the protective effects of plant extracts against oxidative damage in intact RBC membrane. Oxidative driven events play an important role in host defense response and viral biology. Reactive species which are produced as a result of oxidative stress presents a part of HIV pathogenesis. Hemolysis test based on the free radical induced erythrocytes in mice blood. In this study, the extract inhibited the hemolysis of animal erythrocytes in a dose dependent manner. Anti-hemolytic activity of quercetin and other flavonoid has been previously reported.

In summary, the evaluation of immunomodulatory and anti-Dengue activity of SBTLAE has proved to be nontoxic, safe and has the potential for developing as an anti-Dengue agent. Further investigations are needed to elucidate the specificity and mode of action of SBT extract for prophylactic and therapeutic approaches.

Conclusion

Hippophae rhamnoides was evaluated for its anti-Dengue activity in human macrophage cell line U-937 infected with Dengue virus type-2. The immunomodulatory activity of SBTLAE was compared with a standard Ribavirin, and extracts of other medicinal plants. The findings resulted in maximum cell viability and minimum cytotoxicity with SBTLAE in U937 cells. The anti-Dengue activity of SBTLAE was revealed by significantly reduced plaque numbers and modulated pro-inflammatory cytokines, suggesting thereby that the leaf extract of Seabuckthorn has a significant anti-dengue activity and has the potential for the treatment and management of Dengue infection.

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