Why you and your family should use Divine Noni?

Reason 8

Noni inhibits HIV replication along with inhibition of HIV integrase, HIV Vpr induced cytopathic effect and HIV IN-LEDGF prove the new evidence for antiviral activities of Noni

Scientific Reference :

Phytochemical and anti-HIV investigation of Noni fruit *(Morinda citrifolia* L.)

Dr. P. Selvam & Dr. S. Mahalingam
Devaki Amma Memorial College of Pharmacy, Chelembra, Pulliparamba, Kerala
Department of Biotechnology, Indian Institute of Technology Madras, Chennai, Tamil Nadu

1. Introduction

Acquired immunodeficiency syndrome (AIDS) is a fatal pathogenic disease caused by a retrovirus *viz*., Human Immunodeficiency Virus (HIV). Recently much attention has been devoted to the development of vaccine and chemotherapeutic agents for the eradication of HIV/AIDS. Despite so much effort in the field of HIV, no effective vaccine is available till now to combat HIV/AIDS. The only available option is chemotherapy that can reduce the viral load and improve the quality of life of HIV/AIDS patients. Present therapeutic agents are suffering with emergence of resistance and thus demanding large proportion of the potent molecules and novel targets to sustain the treatment and enhance the life span of the infected population. At this juncture designing of simple and novel molecules with broad-spectrum antiretroviral activity against HIV which is cheaper and affordable by the patients is essential. HIV integrase is one of the attractive therapeutic targets for inhibition of virus infection, thus a potential inhibitor of HIV-Integrase (HIV-IN) is provide additional candidate for control of HIV infection. Hence, the present investigation is aimed at identification of novel compounds from Noni followed by screening for their inhibitory activity against HIV-Integrase activity. Further, to carryout Hepatoprotective activity and cytotoxicity studies against hepatocellular cancer cell for evaluation of safety profile and anticancer activity of Noni, respectively.

Recently much attention has been devoted for searching the effective anti-HIV agents from medicinal plant and *Morinda citrifolia* L Noni reported to possess the board

spectrum pharmacological activities (Pawlus AD and Kinghorn D. 2007). The Polynesians utilized the whole Noni plant (*Morinda citrifolia*) in various combinations for herbal remedies and reported to possess wide spectrum biological activities such as arthritis, diabetes, high blood pressure, muscle aches and pains, menstrual difficulties, headaches, heart disease, AIDS, cancers, gastric ulcers, sprains, mental depression, senility, poor digestion, atherosclerosis, blood vessel problems and drug addiction (Wang *et al.,* 2002). Leaf and stem extracts of *Morinda citrifolia* tested for anti-HIV activity and cytotoxicity in MT-4 cells, exhibited significant cytotoxicity in MT-4 cells (Selvam *et al.,* 2008;2009). Noni fruit extracts demonstrated for potent inhibitory activity against HIV integrase enzymatic activity (Selvam *et al.,* 2010) Present work is to study the anti-HIV activity and cytotoxicity, possible mechanism of action of the *Morinda citrifolia* L Noni fruit. Recently much attention has been devoted for searching potential antimicrobial agents from natural products and anti-HIV activity of *Morinda citrifolia* is relatively less explored. The present work is also to study the inhibitory activity of HIV integrase, HIV IN/LEDGF, HIV Vpr induced cell apoptosis of various extracts and their isolated compounds of the fruit powder of *Morinda citrifolia* L Noni.

2. State of knowledge

Acquired immunodeficiency syndrome (AIDS) is a life threatening and debilitating disease state caused by retrovirus HIV. Three different classes of chemotherapeutic agents are generally combined to block the replication of human immunodeficiency virus type 1 (HIV-1) responsible for AIDS and to prevent the occurrence of resistance: reverse transcriptase inhibitors (RTI), protease inhibitors (PRI), and fusion inhibitors. This widespread triple combination therapy is referred to as HAART [highly active antiretroviral therapy] (Richman, 2001). HAART effectively inhibits HIV replication to such an extent that the virus becomes undetectable in the blood. However, it fails to eradicate viruses that are integrated in the host genome or that persist in cellular and anatomical "reservoirs". In addition, prolonged drug exposure led to HIV drug resistance, thus reducing patients' therapeutically available options (Cohen, 2002). The above considerations and the toxicity of a number of antiretroviral agents have fueled the discovery of drugs against additional targets. Recently much attention has been devoted for searching of potential anti-HIV activity with novel mechanism of action from medicinal plants.

HIV integrase (IN) is a crucial enzyme for HIV virus replication and no cellular counterpart. Integration occurs via a sequence of reactions, which start with the IN-mediated cleavage of terminal dinucleotide from the 3'- end of the viral cDNA (termed "3'-processing", 3'-P) shortly after reverse transcriptions in the cytoplasm. Following transfer of the resulting processed viral cDNA into the nucleus, IN

catalyzes the insertion of both ends into target cellular host DNA. That second reaction is referred as"strand transfer" (ST). In the past 15 years, a range of natural and synthetic compounds have been identified as inhibitors of recombinant IN enzyme in biochemical assays (Fesen *et al.,* 1993; Pommier *et al.,* 2005; Dayam *et al.,* 2008). Interestingly, polyhydroxylated aromatics and diketo compounds were among the first inhibitors identified. (Fesen *et al.,* 1993; 1994). IN has recently been fully validated as a therapeutic target with the first FDA approved IN inhibitor raltegravir (Evering *et al.,* 2007). To overcome HIV-1 resistance, medications with novel mechanisms of action should be developed.

Human cellular cofactors play key roles in HIV-1 Integrase performing function (Van Maele *et al*., 2006). Among them, lens epithelial-cell derived growth factor (LEDGF, also referred to as p75) was identified in complex with HIV-1 IN and plays an essential role in the distribution of Integrase in the nucleus, which is the key procedure for viral replication (Llano *et al.,* 2006). Experiments confirmed that p75 bound to HIV-1 IN via a small, approximate 80- residue IN-binding domain (IBD) within its C-terminal region. IBD of p75 was mapped to residues 347–429 and interacted specifically with the IN core domain (Vanegas *et al*., 2005). p75-mediated chromatin tethering depended on specific interactions between the p75 IBD and the IN core domain. Therefore, it was speculated that disturbing or blocking p75-IN interaction would prevent the replication of the virus (Al-Mawsawi *et al.,* 2007; Poeschla, 2008; De Rijck *et al.,*2006). Small molecule inhibitors of HIV IN/LEDGF have emerged as promising new class of antiviral agents for the treatment of HIV/AIDS. We have identified novel inhibitor of HIV IN/LEDGF along with significant anti-HIV activity (Selvam *et al.,* 2012).

As normally for any replication competent retrovirus, the human immunodeficiency virus type 1 (HIV-1) encodes the precursors to the major structural proteins, enzymes and envelope glycoproteins of the viral particle. In addition, HIV-1 codes for essential regulatory factors, notably Tat, Rev and Vpr. Over the past decade, Vpr has been the subject of many studies because it was suspected to play a direct role in the physiopathology of the viral infection. In fact, Vpr was found to interact with the C-terminus of Gag, causing its virion incorporation (Kondo *et al*., 1995; Lavallee *et al* 1994; Paxton *et al.,* 1993; Bachand *et al.,* 1999) and with cellular proteins in infected cells. Due to these interactions Vpr promotes the transactivation of HIV-1 long terminal repeat (LTR) and can cause a G2/M arrest and apoptosis of cells, but the relationship between these two roles of Vpr is still a matter of debate (Le Rouzic *et al.,*2005; Andersen *et al.,* 2005; Li *et al.,* 2007). Vpr also appears to contribute to the nuclear import of the preintegration complex (PIC) and thus of viral DNA (Popov *et al.,* 1998; Vodicka *et al.,* 1998). This last function is supported by the nuclear envelope (NE) localization of Vpr which is mediated by interaction with components of the

nuclear pore complex (NPC) (Le Rouzic *et al.,* 2002; Fouchier *et al.,*1998; Waldhuber *et al.,*2003). Vpr is a 96 amino acid protein with an N-terminal domain required for virion incorporation, nuclear localization and oligomerization (Yao *et al*., 1995; Zhao *et al*., 1994). Its C-terminal domain is involved in the G2/M cell cycle arrest (Di Marzio *et al*., 1995), apoptosis (Jacotot *et al.,* 2001) and interaction with the viral nucleocapsid protein and nucleic acids (De Rocquigny *et al.,* 2000; Li *et al.,* 1996). Moreover, Vpr-Vpr interaction was shown to be required for nuclear localization but not for cell cycle blockade (Bolton *et al.,* 2007).

Recently much attention been devoted for searching potential safe herbal medicines from natural products for the treatment of various diseases. *Morinda citrifolia* used for the treatment of a variety of diseases in Indian system of medicine and demonstrated for safe herbal drug (West *et al.,* 2006). Review of literature revealed that only two studies were available for anti-HIV activity of Noni. 1) The compound isolated form Noni Roots named 1-methoxy-2-formyl-3-hydroanthraquinone suppressed the cytopathic effect of HIV infected MT-4 cells, without inhibiting cell growth (Umezawa *et al.,* 1992). 2) Viral protein R (Vpr), one of the human immunodeficiency virus type 1 (HIV-1) accessory proteins, contributes to multiple cytopathic effect, G2 cell cycle arrest and apoptosis. The mechanisms of Vpr were intensely studied because it was believed that they under lied HIV-1 pathogenesis. Damnacanthal (Dam), a component of Noni was an inhibitor of Vpr induced cell death (Masakazu *et al.,* 2006; Bina *et al.,* 2007). Vpr induces apoptosis when expressed alone in mammalian cells. Apoptosis is the major cause of cell death which is attributed to presence of high concentration of vpr protein present in blood serum of HIV infected patients. Present work is to investigate the effect of *Morinda citrifolia* extracts and isolated compounds on HIV replication, HIV Integrase enzyme, HIV IN/LEDGF interaction, Vpr localization in HeLa cell (Human cervical cancer cells) and Vpr induced cell apoptosis (programmed cell death) in HeLa Cell.

Liver is one of the vital organs in human body and the chief site for extreme metabolism and excretion. It is involved with almost all the biochemical pathways to increase, fight against disease, nutrient supply, energy provision and reproduction. The major functions of the liver are to metabolize, detoxification of carbohydrates, proteins and vitamins. Liver diseases are a serious health problem. In the absence of reliable liver protective drugs in allopathic medical practices, herbs play a role in the management of various liver disorders. Numerous medicinal plants and their formulations are used for liver disorders in ethanomedical practices and in traditional system of medicine in India. However, we do not have satisfactory remedy for serious liver disease, most of the herbal drugs speed up the natural healing process of liver. So the search for effective hepatoprotective drug continues. The present work is to study the hepatoprotective activity of acetone and methanol extracts of *Morinda*

citrifolia against CCl_4 induced toxicity using Chang liver cells. The present work is to also study the cytotoxicity activity of ethanol, methanol extracts and isolated compounds prepared from *Morinda citrifolia* fruit against HepG2 (Human liver cancer) cells.

Liver injuries induced by CCl_4 are the best characterized system of xenobiotic-induced hepatotoxicity and commonly used models for the screening of anti-hepatotoxicity and or hepatoprotective activities of drugs (Lin *et al*., 2002; Clawson, 1998). Since the changes associated with CCl_4 induced liver damage are similar to that of acute viral hepatitis (Rubinstein, 1962), CCl_4 mediated hepatotoxicity was chosen as the experimental model. It has been established that CCl_4 is accumulated in hepatic parenchyma cells and metabolically activated by cytochrome P450 dependent monooxygenases to form a trichloromethyl radical (CCl_3). The CCl_3 radical alkylates cellular proteins and other macromolecules with simultaneous attack on polyunsaturated fatty acids, in presence of oxygen, to produce lipid peroxides, leading to liver damage (Bishayee *et al.,* 1995). Thus, antioxidant or free radical generation inhibition is important in protection against CCl_4 induced liver lesions (Castro *et al*., 1974). Hepatotoxic compounds such as CCl_4 are known to cause marked elevation in serum enzymes and bilirubin levels. It causes marked decrease in TP levels. Silymarin is used as standard hepatoprotective compound since it is reported to have a protective effect on the plasma membrane of hepatocytes (Ramellini *et al.,* 1976).

3. Objectives

- Preparation of various extracts from *Morinda citrifolia* fruit.
- Isolation of active constituents from various extracts.
- Studies of Anti-viral activity against HIV -1 and -2 in MT-4 cells.
- Cytotoxicity activity of crude extracts and isolated compound.
- Investigation of HIV-Integrase inhibitory activity and HIV-Vpr induced cytopathic effect of crude extracts and isolated compound

4. Experimental details

Phytochemical Investigation: *Morinda citrifolia* fruits (5kg) were collected from Noni firm, Vadacancherry, Thirussur dist, Kerala. The fruits were subjected to hot continuous extraction in Soxhlet apparatus to using different solvents like ethanol, methanol and acetone and to get different crude extracts. These crude extracts were concentrated and dried for further studies reduced temperature and kept in desicator.

Isolation of active constituents of ethanolic extract of *Morinda citrifolia* L.

The crude ethanolic extract (14gm) of *Morinda citrifolia* L fruit was subjected to column chromatography (Silica gel Acme 60-120 mesh 500gm) using Chloroform: Methanol (100:0, 95:05, 90:10, 85:15……0:100) is a solvent system to afford 320 fractions (150 ml each, average 3 fraction/day). As a result 320 fractions were obtained and combined on the basis of TLC (Thin Layer Chromatography, Silica gel G, mesh size 200, Acme) using chloroform: Methanol (3:1) solvent system to ultimately afford nineteen fractions (1……19). The same R_f value fractions are combined together and labeled. The visualization of spots was carried out by sprayed with 5% methanolic KOH solution or iodine chamber. Overall 320 fractions are collected from ethanolic extract and obtain some purified compounds and in this process are completed. The dried fruit powder of *Morinda citrifolia* L. were extracted with methanol and distilled out the solvent concentrate the extract and kept in a desicator. The concentrated crude methanolic extract (18gm) of *Morinda citrifolia* L was subjected to column chromatography (Silica gel Acme 60-120 mesh 500gm) using Pet. Ether, Pet. Ether : ethyl acetate, chloroform, chloroform : methanol (100:0, 95:05, 90:10, 100:0, 90:10, 85:15……0:100) is a solvent system and to increase the gradient elution (150 ml each, average 3 fraction/day). Still now 32 fractions are collected from methanolic extract and side by side monitored by TLC further isolation is in the proces. The *Morinda citrifolia* L fruit were collected from Noni Farm located in Thirussur Dist. Kerala. The fruits were shade dried and coarsely powdered. The coarsely powdered fruit material was subjected to hot continuous extraction in Soxhlet apparatus with 1.5 liter Acetone for 72 hrs.

Purification

The combined fractions of compounds like fraction-17, fraction-18 and fraction-19 were separated from ethanolic extract by column chromatography and further purified by using prep. TLC (Preparative Thin Layer Chromatography). Fraction 17 (300mg) on purification through prep. TLC ($CHCl_3$: MeOH 85:15) provided a dark brownish semi solid single compound (80.0mg), while fraction 18 (220.0mg) on prep. TLC ($CHCl_3$: MeOH; 90:10) furnished brownish semi solid single compound (55.0mg). Fraction 19 (150.0mg) was also subjected to prep. TLC ($CHCl_3$: MeOH; 85:15) furnished yellowish semi solid compound (40.0mg). The further spectral studies are in the process. The combined fractions of compounds like fraction-1, fraction-2, fraction-3, fraction-4, and fraction-5 were separated from methanolic extract by column chromatography and further purified by using Prep.TLC (Preparative Thin Layer Chromatography). Fraction-1 (400mg) on purification through Prep.TLC ($CHCl_3$: MeOH 19:1) provided a white color waxy compound (MCF-Me-C, A-1 60mg), while Fraction-2 (350 mg) on prep. TLC ($CHCl_3$: MeOH; 19:1) furnished brownish yellow semi solid single compound (MCF-Me-C-1, A-2 55.0mg). Fraction 3

and 4 (260.0mg) was also subjected to prep. TLC ($CHCl_3$: MeOH; 85:15) furnished brownish semi solid compound (MCF-Me-C-1, A-3 and MCF-Me-C-1, A-4 30.0mg, 20mg). While after purification of fraction-5 (MCF-Me-C-5, 50mg) showed white fluorescence spot under UV lamp. The compound fraction-2 (MCF-Me-C, A-1) and fractions-5 (MCF-Me-C-5) were characterized by Mass Spectrum.After extraction the excessive solvent was distilled out by using Rotary distillation apparatus from acetone extract and the solvent was removed under reduced pressure to give an oily crude product 12.5 g. The oily crude mixture was filtered by using a fine muslin cloth followed by filter paper (Whatman No. 1). The filtrate was placed in an oven to dry at 40°C. The clear brownish yellow residue was obtained.

Hepatoprotective effect in Chang liver cells: The screening of hepatoprotective activity was based on the protection of human liver derived Chang liver cells against CCl_4 induced damage determined by estimating mitochondrial synthesis using tetrazolium assay. Chang liver cells were routinely grown and sub-cultured as monolayer in DMEM supplemented with 10% newborn calf serum. The experiments in this investigation were conducted with cells that had been initially batch cultured for 10 days. At this stage, the cells were harvested and plated at approximately 30,000 cells/well in 96 well microtitre plates (Nunclon) and left to rest for 24 h at 37°C in a humidified atmosphere of 5% CO_2 (Thabrew *et al*., 1997). The cells were then exposed to toxicant (medium containing 15mM CCl_4) along with/without various concentrations of *Morinda citrifolia* acetone (MCF-AC) and methanol extracts (MCF-ME) or the medium alone (as normal). At the end of the period, cytotoxicity was assessed by estimating the viability of Chang liver cancer cells by MTT reduction assay. After 1 h incubation, the test solution from each well was removed by aspiration and replaced with 50µl of MTT prepared in MEM without phenol red (MEM-PR). The plates were gently shaken and incubated for 3 h at 37 °C in a humidified 5% CO_2 atmosphere. The supernatant was removed and 50µl of propanol was added and the plates were gently shaken to solubilize the formed formosan. The absorbance was measured using a microplate reader at 540nm.

***In vitro* cytotoxicity screening:** The present work is to investigate the cytotoxicity activity of ethanol, methanol extracts and isolated compounds prepared from *Morinda citrifolia* fruit against HepG2 (Human liver cancer) cells. The ability of the cells to survive a toxic insult is the basis of most cytotoxicity assays (Francis and Rita, 1986). The monolayer cell culture was trypsinized and the cell count was adjusted to $1.0x10^5$ cells/ml using medium containing 10% new born calf serum. To each well of the 96 well microtiter plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours partial monolayer was formed, the supernatant liquid was flicked off washed the monolayer once and 100ml of different drug concentrations

were added to the cells in microtiter plates. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations recorded every 24 hours. After 72 hours, the drug solutions in the wells were discarded and MTT assay performed.

Anti-HIV activity and cytotoxicity assay: The extracts and isolated compounds were tested for anti-HIV activity against the replication of HIV-1(III_B) (Popovic *et al.,* 1984) and HIV-2(ROD) (Clavel *et al.,* 1986) in MT-4 cells (Miyoshi *et al.,* 1982). The cells were grown and maintained in RPMI 1640 medium supplemented with 10% heat–inactivated Fetal Calf Serum (FCS), 2 mM- glutamine, 0.1% Sodium bicarbonate and 20 μg/ml gentamicin(culture medium). HIV-1 (HTLV-IIIB/LAI) strain and HIV-2 ($LAV\text{-}2_{ROD}$) strain were used in the experiment. The virus strains were propagated in MT-4 cells. Titer of virus stock was determined in MT-4 cells and the virus stock was stored at - 70°C until used. Inhibitory effects of the compounds on HIV-1 and HIV-2 replication were monitored by inhibition of virus-induced cytopathic effect in MT-4 cells and were estimated by MTT assay. Briefly, 50 ml of HIV-1 and HIV-2 (100-300 $CCID_{50}$) was added to a flat-bottomed MT–4 cells ($6x10^5$ cells/ml). After 5 days of incubation, at 37°C the number of viable cells were determined by the 3 - (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) method (Pauwels *et al.,* 1987). Cytotoxicity of the compounds for mock-infected MT-4 cells was assessed by the MTT method. Anti-HIV activity and cytotoxicity of standard AZT were also performed by a similar method in MT-4 cells.

Effect of MCF VPR transfected HeLa cells: Although Vpr displays evident karyophilic properties, it does not contain any canonical basic NLS. A significant fraction of Vpr shows a nuclear rim staining coincident with the NE, indicating that it could interact with components of NPC. Interestingly, it was recently reported by de Noronha and colleagues that Vpr expression can induce transient herniations in the NE, leading to local bursting and mixing of nuclear and cytoplasmic components, especially some key cell cycle regulators. Vpr localization is closely associated with its function of G2 arrest and apoptosis. Hence it will be interesting to investigate effect of anti viral drugs on Vpr localization pattern in mammalian cells. Here we treated Vpr-GFP tagged HeLa cells with various fractions of Noni plant extract. All the compounds were dissolved in DMSO and a minimal concentration of 50 microliter/ml was taken for treatment. DMSO was taken as a negative control.

A. Drug Concentrations: 20 mg of MGF –ET were dissolved in 100μL of DMSO and made upto 1ml with DMEM. The stock was diluted appropriately to arrive at desired concentrations i.e- 10ug/ml,20μg/ml and 30 μg/ml. C17, C18 and C19 (40mg, 60mg , 20mg) were dissolved in 200μl, 300μl and 100μl of DMSO respectively to obtain the stock concentrations of 5μM each.

B. Transfection: HeLA cells were grown in 10 % FCS supplemented DMEM. Cells were trypsinized when reached 60-70 % confluency and were cultured in nine different 60mm dishes (for untreated, GFP alone, DMSO control, MGF-Et in 10,20 and 30μg/ml C-17,C-18,C-19, 5 μM each). The cultured cells were transfected with VPR-GFP using lipofectin reagent and allowed to grow for 5 hrs in serum free medium after with the medium was replaced with 10 % FCS supplemented medium. They were then treated with drugs diluted in respective concentrations and allowed to grow for 48 hrs. Cells were harvested and subjected to FACS analysis.

Method: HeLa cells were seeded in 60mm dish at $3X10^5$ cell per well cell density. Cultured cells were transfected with GFP tagged Vpr using lipofetctin reagent. After five hours media was replaced with DMEM complete medium. Drug treatment was given 12 hours post transfection and cells were allowed to grow for next 48 hours. Cells were harvested and stained with Propidium Iodide for FACS analysis.

C. Processing of cells to be used in FACS: Cells were trypsinized and neutralized with DMEM and pelleted down at 3000 rpm for 2 minutes. Supernatant was discarded and cells were washed with 500ul of PBSA twice, followed by addition of100 μl of annexin binding buffer. The pellet was gently mixed and 5ml each of annexin alexa flour 488 and propidium iodide were added. Tubes were vortexed gently and incubated for 15 minutes in dark. 400 ul of annexin binding buffer were added, vortexed gently and subjected to FACS analysis using BD-Canto FACS setup.

HIV Integrase and HIV Integrase/LEDGF inhibitory activity of MCF

Integrase Catalytic Assay (Marchand *et al.,* 2004): Integrase reactions were carried out by mixing 20 nM DNA with 400 nM IN in a buffer containing 50 mM MOPS, pH 7.2, 7.5 mM $MgCl_2$, 14 mM 2-mercaptoethanol, and the drug of interest or 10% DMSO. Reactions were incubated at 37 °C for 1 h and quenched by addition of an equal volume of gel loading dye (formamide containing 1% SDS, 0.25% bromophenol blue, and xylene cyanol). Reaction products were separated in 20% polyacrylamide denaturing sequencing gels. Dried gels were visualized using the Typhoon 8600 (GE Healthcare, Piscataway, NJ). Densitometry analyses were performed using ImageQuant software from GE Healthcare

Alpha Screen assay : The HIV IN-LEDGF/p75 Alpha Screen was developed as a 3-step procedure for high-throughput screening (Yan *et al.,* 2008; Selvam, 2012). For the assay, 2.5-μl aliquots of test compounds, resuspended in 10% DMSO, were first predispensed on a 384-well ProxiPlate. Then 5 μl of IN CCD, in 1.25μl assay buffer (25 mM HEPES, pH 7.3, 150 mM NaCl, 1 mM DTT, 2 mM MgCl2, 0.1% bovine serum albumin [BSA]) was added to a final concentration of 10 nM, after which the assay plates were incubated for 30 min at room temperature. The remaining

components in 1.25 x assay buffer were then added in 5 µl and included p75 IBD protein (final 10 nM), glutathione donor beads (final 2.5 µg/ml), and Ni-chelate acceptor beads (final 2.5 µg/ml). Following a 60-min room temperature incubation under subdued light conditions (less than 100 Lux), the assay plates were analyzed with an EnVision multilabel plate reader (PerkinElmer, Boston MA).

5. Experimental findings

Extraction and Isolation

Morinda citrifolia fruits were collected from Noni Research cum demonstration centre, Vadakkancherry, Thirussur District, Kerala, India, fresh fruits were cut into small pieces, shade dried at room temperature and then powdered. Dried fruit powder (400gm) was subjected to hot continuous extraction in Soxhlet extractor with ethanol for 72 hours. After extraction the solvent was distilled out and syrupy extract was concentrate under the vaccuo and kept in a desicator. Active constituents were separated from dried ethanolic extract (MCF-ET) by using column chromatography a solvent system of $CHCl_3$-MeOH (95:5, 90:10 and 85:15) and increase the polarity by gradient elution. All fractions were monitored by TLC (Thin Layer Chromatography) and spots are visualized by iodine chamber. Compound MCF-C-10 was identified as anthroquinone glycoside by Borntrager's test. Physical data of the isolated compounds are given Table 1.

Table 1: Physical Data of Isolated Compounds from Noni Extracts

Samples Code	Fractions	Solvent System (TLC)	R_f Value	Character of Isolated Compound
MCF-C-17-ET	257-276	Chloroform: methanol1.1:0.9	0.63	Dark brownish semi solid Single compound
MCF-C-18-ET	277-299	Chloroform: methanol1:1	0.52	Dark brownish semi solid Single compound
MCF-C-19-ET	300-322	Chloroform: methanol1.5:0.5	0.72	Dark yellowish brown semi solid Single compound
MCF-Me-C-1	33-69	Chloroform: methanol1.9:0.1	0.62	Dark brownish yellow single compound
MCF-Me-C-5	70-109	Chloroform: methanol1.8:0.2	0.67	Dark yellowish single compound

Cytotoxicity of methanolic extract (MCF-Me) and Acetone extract (MCF-AC)

The methanol (MCF-ME) and acetone (MCF-AC) extracts investigated for cytotoxicity in human liver cell line (Chang Liver cells) to assess hepatotoxicity. The methanolic and acetone extracts were exhibit cytotoxicity (Table 2) in human liver cells only at higher concentration (more than 220 ìg/ml).

Table 2 : Determination of $CTC_{50}$by using MTT assay in CTC_{50}values with Chang liver cells

Extract	CTC_{50} value (ìg/ml)
MCF-ME	223.89 ±3.24
MCF-AC	245.09±2.33

*Cytotoxic 50% concentration

Hepatoprotective

The present study had been admitted to demonstrate the role of hepatoprotective activity of methanolic and acetone extracts of *Morinda citrifolia* L (Noni). To our knowledge, this is the first study which reveals the hepatoprotective effect of *Morinda citrifolia* acetone and methanol extracts against CCl4 induced toxicity in Chang liver cells. The CCl4 exposed Chang liver cells showed a percentage viability of 42%. These exposed cells, when treated with different concentrations of *Morinda citrifolia* acetone and methanol extracts, showed a dose-dependent increase in percentage viability and the results were highly significant ($P < 0.001$, when compared to CCl4 intoxicated group). The percentage viability ranged between 72–84% at 100–150µg/ml concentration of *Morinda citrifolia* acetone (MCF-AC) and methanol (MCF-ME) extracts (Table 3). The increase in percentage viability of the chang liver cells treated with *Morinda citrifolia* acetone and methanol extracts was significant ($P < 0.01$, when compared to standard) and equally active to that produced by the standard at the same concentration. The above result proves the hepatoprotective activity of *Morinda citrifolia*. Further studies for isolation of active constituents and *in vivo* models for hepatoprotective activity have to be investigated.

Table 3 : Hepatoprotective effect of *Morinda citrifolia* methanol (MCF-ME) and Acetone (MCF-AC) extracts on CCl4 induced toxicity in Chang liver cells

Treatment	Concentration	% viability
Control	-	100
CCl_4	15μ M	$42.38 \pm 0.94^{+}$
MCF-ME	150 μg/ml	$72.54 \pm 1.09^{++}$
	100 μg/ml	$77.53 \pm 1.15^{++}$
MCF-AC	150 μg/ml	$82.46 \pm 0.94^{++}$
	100 μg/ml	$83.85 \pm 1.01^{++}$
Silymarin	150 μg/ml	$85.29 \pm 0.65^{++}$
	100 μg/ml	$86.16 \pm 0.98^{++}$

Average of six independent determinations, values are mean ± S.E.M. + = $P < 0.001$. When compared to untreated cells. ++ = $P < 0.001$, when compared to CCl_4 intoxicated cells.

Cytotoxicity

Morinda citrifolia L (Noni) plant has been enriched with flavanoids, anthroquinone, steroids, glycosides and exhibited anticancer activity (McClatchey *et al.,* 2002). The present work is to study the cytotoxic effect of ethanol, methanol extracts and isolated fraction of *Morinda citrifolia* L. fruits on HepG2 (Human Liver Cancer) cell. Ethanolic extract (MCF-ET) and isolated fraction (MCF-C-10) of Noni fruits (*Morinda citrifolia* L) showed potent cytotoxicity against HepG2 cells with CTC_{50} (cytotoxicity 50%) values of 200μg/ml, 220μg/ml and 246μg/ml 213μg/ml respectively (Table 1). MCF-ET and MCF-C-10 showed potent toxicity against human cancer cells from liver origin respectively. From the results of cytotoxicity studies of methanolic extracts and isolated compounds Noni (Table 1), Methanolic extracts (MCF-Me and MCF Me ETOAC) and isolated fractions (MCF Me C-1 ETOAC and MCF Me C-5 ETOAC) of Noni fruits (*Morinda citrifolia* L) showed significant cytotoxicity against HepG2 cells with CTC_{50} (cytotoxicity 50%) values of 220μg/ml, 232μg/ml and 218 μg/ml, 205 μg/ml, respectively (Table 4). In the present study ethanolic, methanolic extracts and isolated compounds exhibited same potency of anticancer activity. This *in vitro* study has proved the selective cytotoxicity *Morinda citrifolia* against liver cancer cells. Hence

this extract merits further investigation to screen its anti- cancer activity using *in-vivo* models.

Table 4: Determination of CTC_{50} by using MTT assay in HepG2 cells

Extracts	Method	CTC_{50}* value (in μg/ml)
MCF-ET	MTT	200.42 ± 4.31
MCF-ET	SRB	220.2 ± 11.93
MCF-C10	MTT	246 ± 5.88
MCF-C10	SRB	213 ± 7.74
MCF-ME EXT	MTT	220.96 ± 5.96
MCF-ME ETOAC	MTT	232.04 ± 4.63
MCF-ME-C1 ETOAC	MTT	218.51 ± 6.36
MCF- ME C5 ETOAC	MTT	205.83 ± 4.56
Cis Platin	MTT	11.09 ±0.59

*Cytotoxic 50% concentration, Average of six independent determinations, values are mean ± S.E.M.

Result on Anti-HIV activity of isolated compound of ethanolic extract

The ethanol (MCF-ET), methanol (MCF-Me C-9, I,II,III), acetone (MCF AC EXT) extracts, compounds isolated from ethanolic extract (MCF ET C-10,C-12,C-15,C-16,C-17,C-18), methanolic extract (MCF Me C-1,C-5), oil isolated from acetone extract of *Morinda citrifolia* fruit L noni were investigated for antiviral activity against HIV -1 (IIIB) and -2 (ROD) virus replication in MT-4 cells. The cytotoxicity also tested against uninfected MT-4 cells (C-type Adult T leukemia cells) by MTT assay for the study of toxicity profile. Acetone extract (MCF AC EXT) inhibits the replication of HIV-1 (IIIB) in MT-4 cells (Table 5) and cytotoxicity were found to be only at very high concentration in MT-4 cells (C-type Adult T leukemia cells). All the extracts and Isolated compounds shows 2-22 % maximum protection against HIV -1 and -2 replication at subtoxic concentrations (Table 6) and cytotoxicity against MT-4 cells only at a higher concentration (CC_{50} more than 125 μg/mL).

Table 5: Anti-HIV Activity and Cytotoxicity in MT-4 Cells

Sample	Strain	IC50[a] (μg/ml)	CC50[b] (μg/ml)	Max Protection (%)
ETMC	III_B	>125	>125	5
	ROD	>125	>125	4

MCF-ET	III_B	>125	>125	5
	ROD	>125	>125	4
MCF—AC-OIL	III_B	>534.75	534.75±226.36	22
	ROD	>534.75	534.75±229.36	22
MCF—AC-EXT	III_B	157.0±37.04	771.67±44.69	73
	ROD	>771.67±44.69	771.67±44.69	6
MCF-C9-ME-I	IIIB	>125	>125	4
	ROD	>125	>125	5
MCF-C9-ME-II	IIIB	>92.70	e"92.70	2
	ROD	>92.70	e"92.70	2
MCF-C9-ME-III	IIIB	>100.35	100.35±23.72	1
	ROD	>100.35	100.35±23.72	1
Azidothymidine	III_B	0.0015	>25.00	96
	ROD	0.0016	>25.00	72

[a]Concentrations required to inhibit the cytopathic effect of HIV-1(III_B) in MT-4 cells by 50%.

[b]Concentrations required to cause cytotoxicity to 50% of the MT-4 cells.
where as HIV-1 = (IIIB), HIV-2 = (ROD). All the value of SD of two independent experiments.

Table 6: Anti-HIV activity and Cytotoxicity of *Morinda citrifolia*

Compounds Code	Strain	IC_{50} [a] (µg/ml)	CC_{50} [b] (µg/ml)	% Protection
MCF-C-10-ET	III_B	>125.00	>125.00	1
	ROD	>125.00	>125.00	2
MCF-C-12-ET	III_B	>125.00	>125.00	2
	ROD	>125.00	>125.00	1
MCF-C-15-ET	III_B	>125.00	>125.00	0
	ROD	>125.00	>125.00	3
MCF-C-16-ET	III_B	>125.00	>125.00	2
	ROD	>125.00	>125.00	1

MCF-C-17-ET	III_B	>125.00	>125.00	4
	ROD	>125.00	>125.00	5
MCF-C-18-ET	III_B	>125.00	>125.00	3
	ROD	>125.00	>125.00	1
MCF-C-1-Me	III_B	>125.00	>125.00	4
	ROD	>125.00	>125.00	10
MCF-C-5-Me	III_B	>609.75	609.75±3.75	15
	ROD	>609.75	609.75±3.75	22
MCF-C5-ETOAC	IIIB	>61.50	61.50±2.84	2
	ROD	>61.50	61.50±2.84	2
AZT	III_B	0.0015	>25.00	96
	ROD	0.0016	>25.00	72

[a]Effective concentration of compound, achieving 50% protection of MT-4 cells against cytopathic effect of HIV. [b]Cytotoxic concentration of compounds, required to reduce the viability of mock infected MT-4 cells by 50%.where as HIV-1 = (IIIB), HIV-2 = (ROD).

All the value of SD are of two independent experiments.

Studies on inhibitory effect of MCF on HIV- Vpr induced cell apoptosis in HeLa cells

Vpr is a 14kDa protein that is conserved between HIV-1, HIV-2, and SIV. Deletion of Vpr from SIV resulted in an acute infection, but no disease was observed in Rhesus monkeys. Vpr variants have been correlated with long-term survival in HIV-1-infected individuals. HIV-1 Vpr is a virion associated protein which plays an important role in HIV-1 replication and pathogenesis. Findings from several laboratories suggest that the Vpr mediating the transport of HIV-1 genome into the nucleus in non-dividing cells by a novel pathway(s). Furthermore, Vpr induces growth arrest at the G2/M phase of the cell cycle/apoptosis during HIV-1 replication. Vpr is packaged into virus particles and plays an important role in transporting viral genome into the nucleus in conjunction with integrase (IN) and a *cis* acting element, central DNA flap. Vpr induces G2 cell cycle arrest in proliferating human cells. This effect correlates with the production of herniations in the nuclear envelope. Arrest in the G2 phase of the cell cycle enhances viral replication, in part by increasing the activity of the long terminal repeat. The prolonged G2 arrest induced by Vpr

promotes apoptosis of the infected cell, perhaps leading to increased virion release and enhanced viral burden. Present work is to investigate the effect of *Morinda citrifolia* extracts and isolated compounds on Vpr localization in HeLa cell (Human cervical cancer cells) and Vpr induced cell apoptosis (programmed cell death) in HeLa Cell. (Masakazu *et al.,* 2008)

MCF-ET at 20 and 30µg/ml and MCF ET C-17 and C-19 were found to have considerable effect on transfected cells as analyzed by Annexin Alexa flour 488 apoptosis assay (Table 7; Fig. 1). Significant decrease in cell death was observed in case of compounds isolated from methanolic extract D3 (MCF Me C-1) and D4 (MCF-Me C-5) as compared to that of cells transfected with Vpr alone (Table 8; Fig 2, 3). The treatment Vpr transfected HeLa with extracts and isolated compounds prevented the cells to undergo apoptosis.

Table 7: Effect of MCF- ET on VPR transfected HeLa Cells

COMPOUNDS	% DEAD
UT	13.1
GFP	9.8
MCF 10	17.8
MCF 20	5.2
MCF 30	4.3
C17	5.6
C18	21.3
C19	6.6
DMSO	9.3

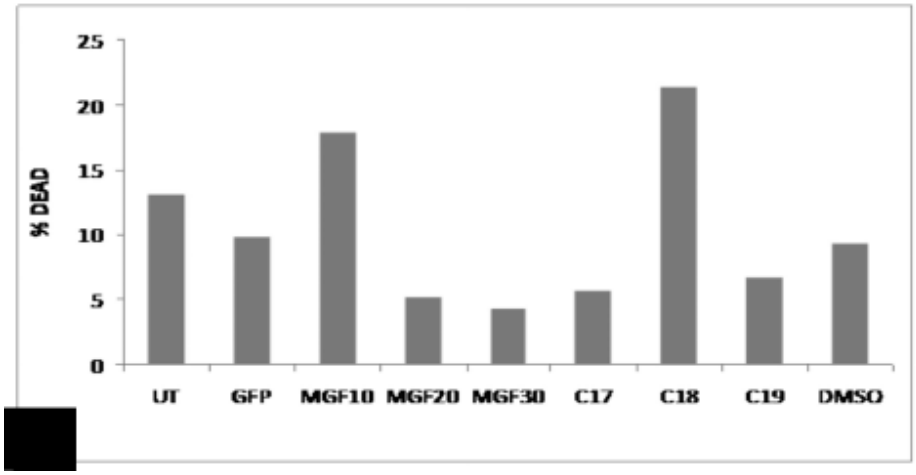


Fig. 1 : Effect of MCF-ET on HIV VPR induced apoptosis in HeLa cells

Table 8: Effect of MCF- Me on VPR transfected HeLa Cells

Cells treated with	% of Dead Cells
Vpr	26.1
D1	21.4
Vpr+D1	23.4
D2	25.4
Vpr+D2	23.1
D3	26.2
Vpr+D3	21.5
D4	21.5
Vpr+D4	17.5

D 1 – MCF-Me EtoAc, D 2 – MCF-Ac oil extract
D 3 – MCF-Me C-1, EtoAc, D 4 – MCF-Me C-5, Me

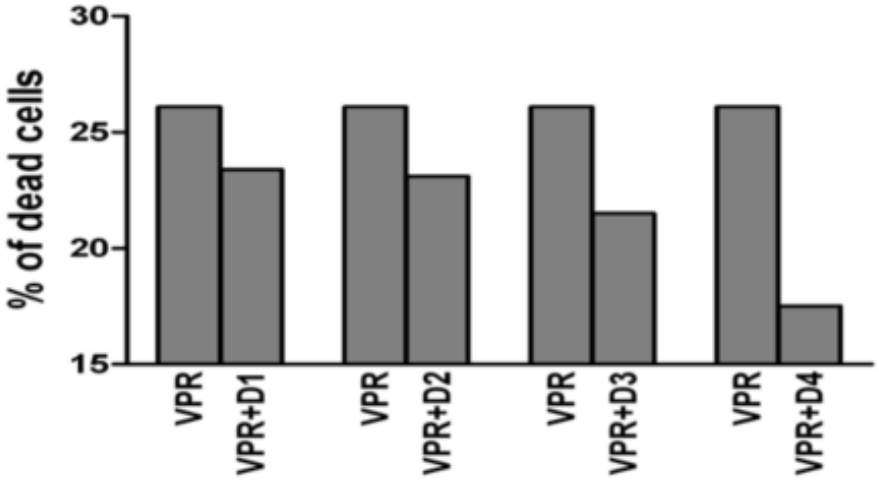


Fig 2 : Effect of MCF-Me on HIV VPR induced apoptosis in HeLa cells

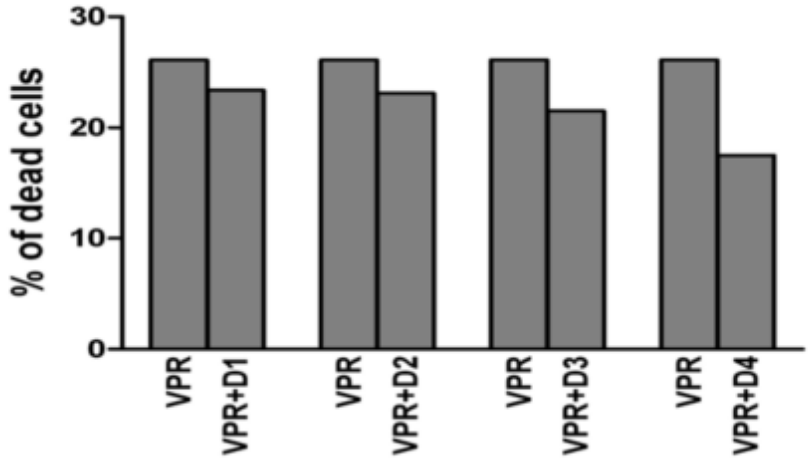
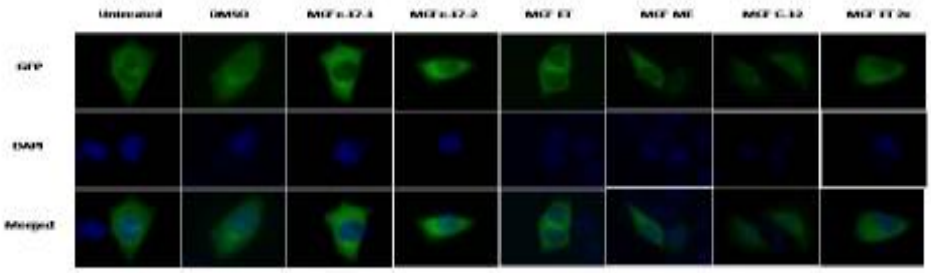


Fig 3 : Effect of MCF-Me on HIV VPR induced apoptosis in HeLa cells

Image analysis shows that there is no difference of Vpr localization pattern in the inhibitor treated and untreated Hela cells and also on DMSO treated Hela cells. Therefore we could say that the Vpr inhibitors have no effect on its localization. Though not much difference was observed in the localization pattern of Vpr but a clear diffusion was observed in case of D 4 (Fig…). Further experiment with various drug concentration treatments is needed to determine any conclusive effect on Vpr localization. Although drug treatment may not cause any change in Vpr localization yet it can affect Vpr function by means of other pathways. Hence further experiments like apoptosis assay is to be done to completely elucidated these drugs on Vpr function.



Drug treatment on Vpr localization

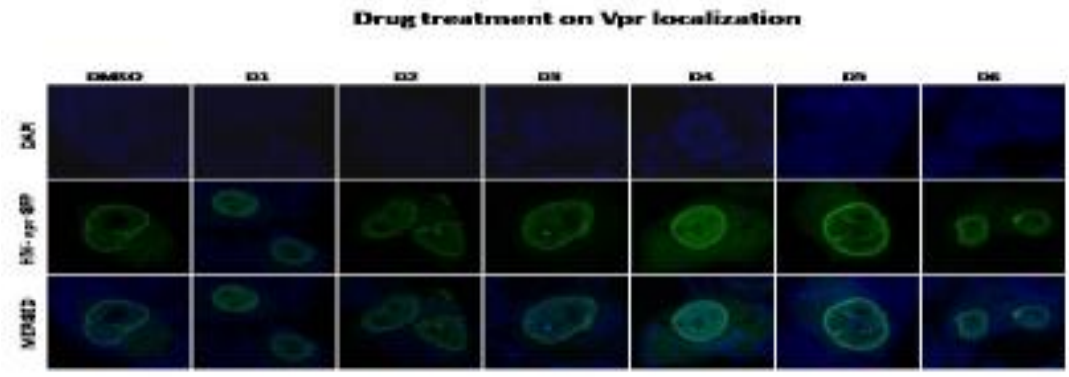


Fig. 4 and 5: Effect of MCF-ET and MCF-Me in HIV Vpr in HeLa Cells

HIV Integrase and HIV In/Ledgf Inhibitory Activity: The ethanol, methanol and acetone extracts of *Morinda citrifolia* were prepared and this extracts were selected for further studies on Phytochemical analysis (Isolation and characterization of active constituents and studies of antiviral activity), since above extracts found to have significant activity against both steps of HIV Integrase enzymatic activity. (Selvam *et al.,* 2010). From the results of the present investigation ethanolic fractions (MCF F1-6) and isolated compounds MCF-ET (C-10,12,14,15,16) inhibited HIV Integrase/LEDGF protein-protein interaction (Table 9; 10) essential for HIV virus integration. Ethanolic fractions (MCF F1-6) also inhibited both steps of HIV Integrase enzymatic activity, which is essential for replication of HIV virus.

Table 9: HIV Integrase Inhibitory Activity of MCF Fractions (F1-F6)

Compounds	3'P [a] (µg/ml)	ST [b] (µg/ml)	LEDGF-IN (µg/ml)
MCF-F1	>100	>100	57% at 30
MCF-F2	49 ± 15	4 ± 1	50
MCF-F3	72 ± 25	9 ± 4	>80
MCF-F4	30	27 ± 18	80
MCF-F5	63 ± 18	35 ± 21	60% at 70
MCF-F6	73 ± 39	56 ± 33	60

The results are IC50 ±S.D, *n* = 3 for HIV-1 IN inhibitory activity, [a]Concentration required to inhibits 3' processing reaction, [b]Concentration required to inhibits 3' processing reaction[a]Concentration required to inhibits HIV IN/LEDGF interaction,

Table 10: HIV Integrase and HIV In/Ledgf Inhibitory Activity

Compound	LEDGF IC_{50} (μg/mL)	3'P : IC_{50} (μg/mL)	ST : IC_{50} (μg/mL)
MCF-ET-C-10-ME	14	>100	>100
MCF-ET-C-12-ME	10	>100	>100
MCF-ET-C-14-ME	0.53	94	58
MCF-ET-C-15-ME	36	>100	>100
MCF-ET-C-16-ME	31	>100	>100
MCF-ET-C-17-ME	>50	>100	>100
MCF-ET-C-18-ME	>50	>100	>100

The results are IC_{50} ±S.D, n = 3 for HIV-1 IN inhibitory activity. [a]Concentration required to inhibits 3' processing reaction, [b]Concentration required to inhibits 3' processing reaction, [a]Concentration required to inhibits HIV IN/LEDGF interaction.

6. Summary and conclusion

Anti-HIV activity and cytotoxicity of *Morinda citrifolia* L was evaluated in the present study.

Ethanol, methanol and acetone crude extracts of *morinda citrifolia* L noni were prepared by hot continues extraction process by using Soxhlet apparatus. Compounds were isolated from ethanol and methanol extracts by using column chromatography technique. Acetone extract also gives oil on distillation. Isolated compounds were characterized by TLC and spectral analysis. Acetone and methanol extracts were investigated for hepatotoxicity and hepatoprotective activity for the assessment of safety of Noni in human liver cells. Crude extracts and isolated compounds were investigated for cytotoxicity in human liver cancer cells for evaluating of anticancer activity. Noni extracts and isolated compounds were tested for antiviral against HIV -1 & -2 and cytotoxicity in MT-4 cells. Crude extracts and isolated compounds were also investigated for inhibition of HIV Integrase, HIV Integrase/LEDGF, HIV Viral protein R (Vpr) localization and HIV Vpr induced cell apoptosis for understanding the mechanism of antiviral action of Noni. Crude extracts and compounds exhibits significant anticancer activity against human liver cancer. Noni extracts having hepatotoxicity at high concentration and also shows promising Hepatoprotective activity against CCl_4 induced hepatotoxicity in human liver cells. Acetone extract of Noni inhibits the replication of HIV -1 (IIIB) and cytotoxicity only at high concentration in MT-4 cells. Ethanolic fractions prevent the both HIV Integrase

enzyme and HIV Integrase/LEDGF activity, compounds isolated from ethanolic extract also inhibits the HIV IN/LEDGF activity, which is essential for HIV replication. Extracts and isolated compounds inhibit the HIV VPR induced cell apoptosis in HeLa Cells. Acetone extract (MCF-AC) identified for anti-HIV activity and potential inhibitors of HIV Integrase activity, also exhibits Hepatoprotective activity.

References

Al-Mawsawi, L.Q. and Neamati, N. 2007. Blocking interactions between HIV-1 integrase and cellular cofactors: an emerging antiretroviral strategy. *Trends Pharmacol Sci*., 28:526–535.

Andersen, J.L. and Planelles, V. 2005. Role of Vpr in HIV-1 pathogenesis. *Curr HIV Res*., 3:43-51.

Anthony, N. J. 2004. HIV-1 integrase: a target for new AIDS chemotherapeutics. *Curr. Top. Med. Chem*., 4:979–990.

Bachand, F., Yao, X.J., Hrimech, M., Rougeau, N. and Cohen E, A. 1999. Incorporation of Vpr into human immunodeficiency virus type 1 requires a direct interaction with the p6 domain of the p55 gag precursor. *J Biol Chem*., 274:9083-9091.

Bina, S., Siddiqui, F.A., Sattar, S.B., Tahsin, G. and Fayaz, A. 2007. Chemical Constituents from the Stems of *Morinda citrifolia* Linn. *Arch Pharm Res*., 30: 793-798,

Bishayee, A., Sarkar, A. and Chatterjee, M. 1995. Hepatoprotective activity of carrot (Daucus carota L) against carbon tetrachloride intoxication in mouse liver. *J Ethnopharmacol*., 47(2): 69-74.

Bolton, D.L. and Lenardo, M.J. 2007. Vpr cytopathicity independent of G2/M cell cycle arrest in human immunodeficiency virus type 1- infected CD4+ T cells. *J Virol*., 81:8878-8890.

Castro. J.A., De Ferreyra, E.C., De Castro, C.R., De Fenos, O.M., Sasame, H. and Gillette, J.R. 1974. Prevention of carbon tetrachloride-induced necrosis by inhibitors of drug metabolism further studies on their mechanism of action. *Biochem Pharmacol.,* 23(2): 295-302.

Clawson, G.A. 1998. Mechanisms of carbon tetrachloride hepatotoxicity. *Pathol immunopathol Res*., 8 (2): 104-112.

Clavel, F., Guyader, M., Guetard, D., Salle, M., Montagnier, L., Alizon Pauwels, R., Balzarini, J., Baba, M., Snoeck, R., Schols, D.J. and Herdewijin, H. 1986. Molecular cloning and polymorphism of the human im munodeficiency virus type 2. *Nature*, 324, 691–695.

Cohen, J. 2002. Therapies. Confronting the limits of success. *Science,* 296: 2320-2324.

De Rocquigny, H., Caneparo, A., Delaunay, T., Bischerour, J., Mouscadet, J.F. and Roques, B.P. 2000. Interactions of the C-terminus of viral protein R with nucleic acids are modulated by its N-terminus. *Eur J Biochem.*, 267:3654-3660.

De Rijck, J., Vandekerckhove, L., Gijsbers, R., Hombrouck, A., Hendrix, J., Vercammen, J., Engelborghs, Y., Christ, F. and Debyser, Z. 2006. Overexpression of the lens epithelium-derived growth factor/p75 integrase binding domain inhibits human immunodeficiency virus replication. *J Virol.*, 80:11498–11509.

Di Marzio. P., Choe, S., Ebright. M., Knoblauch, R. and Landau, N.R. 1995. Mutational analysis of cell cycle arrest, nuclear localization and virion packaging of human immunodeficiency virus type 1 Vpr. *J Virol.*, 69:7909-7916.

Evering, T. H. and Markowitz, M. 2007. Raltegravir (MK-0518): an integrase inhibitor for the treatment of HIV-1. *Drugs Today*, 43: 865–877.

Francis, D. and Rita, L. 1986. Rapid colorimetric assay for cell growth and survival modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *Journal of Immunological Methods,* 89: 271-277.

Fouchier, R.A., Meyer, B.E., Simon, J.H., Fischer, U., Albright, A.V., Gonzalez-Scarano, F. and Malim, M.H. 1998. Interaction of the human immunodeficiency virus type 1 Vpr protein with the nuclear pore complex. *J Virol.*, 72: 6004-6013.

Hou, Y., Mc Guinness, D. E., Prongay, A.J., Ingravallo, P., Lunn, C.A. and Howe, J.A. 2008.

Screening for antiviral inhibitors of the HIV integrase - LEDGF/p75 interaction using the AlphaScreen (TM) luminescent proximity assay. *J. Biomol Screening*, 13:406–414.

Jacotot, E., Ferri, K.F., El Hamel, C., Brenner, C., Druillennec, S., Hoebeke, J., Rustin, P., Metivier, D., Lenoir, C. and Geuskens, M., 2001. Control of mitochondrial membrane permeabilization by adenine nucleotide translocator interacting with HIV-1 viral protein rR and Bcl-2. *J Exp Med.*, 193:509-519.

Kondo, E., Mammano, F., Cohen, E.A. and Gottlinger, H.G. 1995. The p6gag domain of human immunodeficiency virus type 1 is sufficient for the incorporation of Vpr into heterologous viral particles. *J Virol.*, 69:2759-2764.

Lavallee, C., Yao, X.J., Ladha, A., Gottlinger, H., Haseltine, W.A. and Cohen, E.A. 1994. Requirement of the Pr55gag precursor for incorporation of the Vpr product into human immunodeficiency virus type 1 viral particles. *J Virol.*, 68:1926-1934.

Le Rouzic, E., Mousnier, A., Rustum, C., Stutz, F., Hallberg, E., Dargemont, C. and Benichou, S. 2002. Docking of HIV-1 Vpr to the nuclear envelope is mediated by the interaction with the nucleoporin hCG1. *J Biol Chem.*, 277:45091-45098.

Le Rouzic, E. and Benichou, S. 2005. The Vpr protein from HIV-1: distinct roles along the viral life cycle. *Retrovirology,* 2:11.

Lin, S.C., Lin, C.H., Lin, C.C., Lin, Y.H., Chen, C.F., Chen, I.C. and Wang, L.Y. 2002. Hepatoprotective effects of *Arctium lappa Linne* on liver injuries induced by chronic ethanol consumption and potentiated by carbon tetrachloride. *J Biomed Sci.*, 9(5): 401-409.

Llano, M., Saenz, D.T., Meehan, A., Wongthida, P., Peretz, M., Walker, W.H. and Poeschla, E.M. 2006. An essential role for LEDGF/p75 in HIV integration. *Science*, 314:461–464.

Li, G., Elder, R.T., Qin, K., Park, H.U., Liang, D. and Zhao, R.Y. 2007. PP2A dependent and independent pathways for ATR phosphorylation of Chk1. *J. Biol. Chem.*, 282(10):7287-7298.

Li, M.S., Garcia-Asua, G., Bhattacharyya, U., Mascagni, P., Austen, B.M. and Roberts, M.M. 1996. The Vpr protein of human immunodeficiency virus type 1 binds to nucleocapsid protein p7 in vitro. *Biochem Biophys Res Commun.*, 218:352-355.

McClatchey, W. 2002. From Polynesian healers to health food stores: changing perspectives of *Morinda citrifolia* (Rubiaceae). *Integr Cancer Ther.*, 1:110-20.

Malet, I., Delelis, O., Valantin, M.A., Montes, B., Soulie, C., Wirden, M., Tchertanov, L., Peytavin, G., Reynes, J., Mouscadet, J.F., Katlama, C., Calvez, V. and Marcelin, A.G. 2008. Mutations associated with failure of raltegravir treatment affect integrase sensitivity to the inhibitor in vitro. *Antimicrob Agents Chemother.*, 52:1351–1358.

Masakazu, K., Raymond, P., Dong, S.A., Jonathan, P., Robert, D. and Irvin, S.Y. 2006. Cell-Based Chemical Genetic Screen Identifies Damnacanthal as an

Inhibitor of HIV-1 Vpr Induced Cell Death. *Biochem Biophys Res Commun*., 348: 1101–1106.

Miyoshi, I., Taguchi, H., Kubonishi, I., Yoshimoto, S., Ohtsuki, Y., Shiraishi, Y. and Akagi, T. 1982. Type C virus-producing cell lines derived from adult T cell leukemia. Gann Monograph. *Cancer Res.,* 28, 219–228.

Periyasamy Selvam, Narayanan Murugesh and Myriam witvrouw. 2007. Studies of Comparative Anti-HIV activity and cytotoxicity of *Morinda citrifolia* L. *Noni Clinical Research Journal*, 1 (1-2): 25-27.

Periyasamy Selvam and Christophe pannecuoque. 2008. Studies on anti-HIV and cytotoxicity of leaf extract of *Morinda citrifolia* L. *Noni Clinical Research Journal,* 2 (1-2): 25-28.

Periyasamy Selvam, Christophe pannecuoque and Erik De Clercq. 2009. Studies on anti-HIV activity and cytotoxicity of stem extracts of *Morinda citrifolia* L. (Noni). *Noni Clinical Research Journal*, 3 (1-2): 47-50.

Periyasamy Selvam, Kasthuraiah Maddali and Yves Pommier. 2009. Studies on HIV Integrase inhibitory activity of Morinda citrifolia L. Noni fruit extracts. Noni search 2009-proceedings of fourth National Symposium on Noni for Empowerment and prosperity, Chennai, October 24-25, 193-201.

Paxton, W., Connor, R.I. and Landau, N.R. 1993. Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of gag and mutational analysis. *J. Virol*., 67:7229-7237.

Periyasamy Selvam. 2012. Studies on HIV integrase-LEDGF/p75 interaction inhibitory activity of isatine derivative using the alpha screen luminescent proximity assay. *BMC Infectious Diseases*, 12 (1): 1-2.

Pawlus, A.D. and Kinghorn, D. 2007. A Review of the Ethnobotany, Chemistry, Biological Activity and Safety of the Botanical Dietary Supplement *Morinda Citrifolia* (Noni). *Journal of Pharmacy and Pharmacology*, 59 (12): 1587-92.

Pauwels, R., DeClercq, E., Desmyter, J., Balzarini, J., Goubau, P., Herdewijn, P., Vandeputte, M. and Vanderbaeghe, H. 1987. Sensitive andrapid assay on MT-4 cells for the detection of antiviral compounds against the AIDS virus. *J. Virol. Methods,* 16, 171–185.

Poeschla, E. M. 2008. Integrase, LEDGF/p75 and HIV replication. *Cell Mol. Life Sci.,* 65:1403–1424.

Popov, S., Rexach, M., Ratner, L., Blobel, G. and Bukrinsky, M. 1998. Viral protein R regulates docking of the HIV-1 preintegration complex to the nuclear pore complex. *J Biol Chem*, 273:13347-13352.

Richman, D.D. 2001. HIV chemotherapy. *Nature,* 410: 995–1001.

Popovic, M., Sarngadharan, M.G., Read, E. and Gallo, R.C. 1984. Detection, isolation and continuous production of cytopathic retroviruses (HTLVIII) from patients with AIDS and pre-AIDS. *Science*, 224, 497–500.

Ramellini, G. and Meldolesi, J. 1976. Liver protection by Silymarin: *in vitro* effect on dissociated rat hepatocytes. Arzneim Forsch. *Drug Research*, 26(1): 69-73.

Rubinstein, D. 1962. Epinephrine release and liver glycogen levels after carbon tetrachloride administration. *Am J Physiol.,* 203: 1033-1037.

Thabrew, M.I., Hughes, R.D. and Mcfarlane, I.G. 1997. Screening of hepatoprotective plant components using a HepG2 cell cytotoxicity assay. *Pharm-Pharmacol*., 49(11): 1132-1135.

Umezawa, K. 1992. Isolation of 1-methoxy-2-foremyl-3-hydroxyanthraquinone from *Morinda citrifolia* and neoplasm inhibitors containing the same. Japan Kokai Tokyo Koho JP 06 87, 736 (94-87, 736) Appl; 92/264, 311 07.

Van Maele, B., Busschots, K., Vandekerckhove, L., Christ, F. and Debyser, Z. 2006. Cellular co-factors of HIV-1 integration. *Trends Biochem Sci*., 31:98–105.

Vanegas, M., Llano, M., Delgado, S., Thompson, D., Peretz, M. and Poeschla, E. 2005. Identificatio of the LEDGF/p75 HIV-1 integrase interaction domain and NLS reveals NLS-independent chromatin tethering. *J Cell Sci*., 118:1733–1743.

Vodicka, M.A., Koepp, D.M., Silver, P.A. and Emerman, M. 1998. HIV-1 Vpr interacts with the nucear transport pathway to promote macrophage infection. *Genes Dev.,* 12:175-185.

Yan Hou, Debra, E., Mcguinness, Andrew, J., Prongay, Boris feld, Paul Ingravallo, Robert, A., Ogert, Charles, A., Lunn and John, A. Howe. 2008. Screening for antiviral inhibitors of the HIV integrase–LEDGF/p75 interaction using the Alphascreen luminescent proximity assay. *J Biomol Screen*, 13: 406

Yao, X.J., Subbramanian, R.A., Rougeau, N., Boisvert, F., Bergeron, D. and Cohen, E.A. 1995. Mutagenic analysis of human immunodeficiency virus type 1 Vpr: role of a predicted N- alpha-helical structure in Vpr nuclear localization and virion incorporation. *J Virol.,* 69:7032-7044.

Waldhuber, M.G., Bateson, M., Tan, J., Greenway, A.L. and McPhee, D.A. 2003. Studies with GFP-Vpr fusion proteins: induction of apoptosis but ablation of cell-cycle arrest despite nuclear membrane or nuclear localization. *Virology*, 313:91-104.

Wang, M.Y., West, B.J., Jensen, C.J., Nowicki, D., Su, C., Palu, A.K. and Anderson, G. 2002 *Morinda citrifolia* (Noni) a literature review and recent advances in Noni research. *Acta Pharmacologica Sinica*, 23: 1127-41.

West, B.J., Jensen, C.J. and Westendorf, J. 2006. Noni Juice is Not Hepatotoxic. *World Journal of Gastroenterology*, 12 (22): 3616-20.

Zhao, L.J., Wang, L., Mukherjee, S. and Narayan, O. 1994. Biochemical mechanism of HIV-1 Vpr function. Oligomerization mediated by the N-terminal domain. *J. Biol. Chem.*, 269:32131-32137.