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INVESTIGATION OF ANTI-HIV, CYTOTOXICITY AND HEPATOPROTECTIVE ACTIVITIES OF *MORINDA CITRIFOLIA* L (NONI)

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ABSTRACT

Introduction: In recent years all other forms of immunodeficiency syndrome have been overshadowed by an epidemic of severe immunodeficiency caused by a retrovirus called Human immunodeficiency Virus type 1 or HIV-1. Numerous medicinal plants and their formulation are used for liver disorders in ethanomedical practices and in traditional system of medicine. *Morinda citrifolia* (Noni) commonly known as Indian mulberry and enriched with Flavonoids, anthraquinone, steroids, glycosides its fruits has been widely used in tropical regions both as food and folk medicine. Plants are reported to have a broad range of therapeutic effects including antibacterial, anti-viral, anti-tumor, analgesic, anti-inflammatory and immune enhancing properties. **Objective:** Acetone, Ethanol, Methanol extracts and isolated compounds were tested for their Cytotoxicity activity against Hep G2 cell, Hepatoprotective activity against Chang liver cells and anti-HIV activity against replication of HIV-1 (IIIB) and HIV-2 (ROD) in MT-4 cells. **Methodology:** Dried Noni fruit powder was subjected to successive extraction by using Soxhlet extractor with Acetone (MCF-Ac), Ethanol (MCF-Et) & Methanol (MCF-Me) for 72 hours. Noni fruit extracts were tested for Cytotoxicity effect against HepG2 (Human Liver Cancer) cell culture by MTT assay. Ethanol & Methanol extracts were further subjected to Silica gel-column chromatography to identify the active components. Noni extracts and isolated compounds were screened for Hepatoprotection on Human liver derived Chang liver cells against CCl_4 induced damage by using MTT reduction assay and anti-HIV activity against the replication

of HIV-1 (III_B) and HIV-2 (ROD) in MT-4 cells. **Results:** The results revealed that the MCF-Ac, MCF-Et, MCF-Me extracts showed cytotoxicity against HepG2 cells with CTC_{50} values of 200 µg/ml, 220 µg/ml & 246 µg/ml. MCF-Ac and MCF-Me extracts treated with different concentrations and showed a dose dependent increase in Hepatoprotection percentage ranged between 72 – 84% at 100 – 150 µg/ml. MCF-Ac, MCF-Et and isolated compounds from MCF-Me extracts (C-9, Me- I, II & III) were tested against anti-HIV, MCF-Ac extract exhibited anti-HIV-1 & 2 replication in MT-4 cells with maximum protection 2 - 76% IC_{50} values of 157.0 and 37.04 µg/ml, respectively. **Conclusion:** This result strongly supports the basis for the use of extracts and isolated compounds were exhibits significant cytotoxicity activity against Human liver cancer cells. Acetone and Methanol extracts showed promising hepatoprotective activity against Chang liver cells and Acetone extract of Noni fruit exhibit anti viral activity against replication of HIV-1 (III_B) in MT-4 cells.

KEYWORDS: *Morinda citrifolia* Fruit, Anti-HIV, Cytotoxicity, Hepatoprotective activity**.**

INTRODUCTION

Acquired Immunodeficiency Syndrome (AIDS) is a life-threatening and debilitating disease state caused by HIV, a retrovirus. 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 this field, no effective vaccine is available till now to combat HIV. The only available option is chemotherapy that can reduce the viral load and improve the quality of life of HIV patients. Present therapeutic agents experience emergence of resistance and thus demanding large proportion of potent molecules and novel targets to sustain the treatment and enhance the life span of the infected population. At this juncture, designing simple and novel molecules with broad-spectrum antiretroviral activity against HIV, which is cheaper and affordable by the patients is essential. With the growing drug-resistant strains coupled with the increasing failure of synthetic drugs, it is of interest to screen for HIV-1 and HIV-2 inhibitors from natural sources.[4]

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 the fight against disease, nutrient supply, energy provision and reproduction. The major functions of the liver are to metabolize and detoxify carbohydrates, proteins and vitamins. Liver diseases are a serious health problem and absence of reliable liver-protective drugs in allopathic medical practices has resulted in the use of herbs as an alternative treatment mode. Herbs play a vital

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 systems of medicine in India.[12]

Morinda citrifolia L (Noni) commonly known as Indian mulberry, is enriched with flavonoids, anthraquinone, steroids, and glycosides. Its fruits have been widely used in tropical regions as both food and folk medicine. Plants are reported to have a broad range of therapeutic effects including antibacterial, anti-viral, anti-tumor, analgesic, anti-inflammatory and immune-enhancing properties.[15] Recently, much attention has been devoted for searching the effective anti-HIV agents from medicinal plants and *M citrifolia* reported to posses the broad-spectrum pharmacological activities.[10] Leaf and stem extracts of *M. citrifolia* tested for anti-HIV activity and cytotoxicity in MT-4 cells exhibited significant activity[9]. Literature review revealed that only one study was available for anti-HIV activity of *M. citrifolia*. The compound isolated from *M. citrifolia* roots named 1-methoxy-2-formyl-3-hydroanthraquinone suppressed the cytopathic effect of HIV-infected MT-cells, without inhibiting cell growth[14]. 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 or hepatoprotective activities of drugs.[5,7] CCl_4-mediated hepatotoxicity was chosen as the experimental model for this study. Antioxidant or free radical generation inhibition is important in protection against CCl_4-induced liver lesions.[3] In this study, Silymarin is used as a standard hepatoprotective compound since it is reported to have a protective effect on the plasma membrane of hepatocytes.[11] The objective of the present work was to investigate the Cytotoxic activity against HepG2 (Human liver cancer) cells, Hepatoprotective activity against CCl_4-induced toxicity and cytotoxicity using Chang liver cells, and anti-HIV activity against the replication of HIV-1 (III_B) and HIV-2 (ROD) in MT-4 cells.

MATERIALS AND METHODS

Extraction and Isolation

M. citrifolia fruits were collected from Noni Research cum Demonstration Centre, Wadakkencherry (Thrissur, Kerala, India). Fresh fruits of *M. citrifolia* were cut into small pieces, shade dried at room temperature and then powdered. Dried fruit powder (500 g) was subjected to hot continuous extraction in Soxhlet extractor with acetone, ethanol and methanol for 72 hours. After extraction, the solvent was distilled out and syrupy extract was

concentrated *in vaccuo* and kept in a desiccator. Active constituents were separated from ethanol (MCF-Et) and methanol (MCF-Me) extracts by using open column chromatography having a solvent system of $CHCl_3$–MeOH (100, 95:5, 90:10 and 85:15). All fractions were monitored by Thin Layer Chromatography (TLC) and spots were visualized through iodine chamber. The combined fractions F-17, 18 and 19 were separated from ethanolic extract by column chromatography and further purified by preparative TLC. Fraction-17 (300 mg) on purification through preparative TLC ($CHCl_3$: MeOH 85:15) provided a dark brownish semisolid single compound (80 mg). While fraction-18 (220 mg) on preparative TLC ($CHCl_3$: MeOH 90:10) furnished brownish semisolid single compound (55 mg). Fraction-19 was also subjected to preparative TLC ($CHCl_3$: MeOH 85:15), which furnished yellowish semisolid compound (40 mg). The further spectral studies are in the progress. Acetone, ethanol and methanol extracts (MCF-Ac, MCF-Et and MCF-Me) and isolated compounds (MCF-Et C-1 and MCF Me C-9) were studied for cytotoxicity against HepG2 (Human liver cancer) cells by using 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl Tetrazolium bromide (MTT) assay methods. Hepatoprotection on human liver-derived Chang liver cells against CCl_4-induced damage was assessed by estimating mitochondrial synthesis and anti-HIV activity against the replication of HIV-1 (III_B) and HIV-2 (ROD) in MT-4 cells.

***Morinda citrifolia* L (Noni fruit)**

***In-vitro* Cytotoxicity Activity**

Preparation of Suspensions

MCF-Ac, MCF-Et and MCF-Me and isolated compounds of *M. citrifolia* fruits (MCF-Et-C-1 and MCF-Me-C-9) were dissolved in DMSO and the volume was made up to 10 ml with

DMEM/MEM to obtain stock solution of 1 mg/ml concentrations ranging from 1000 to 62.5 µg/ml with respective media and used for *in-vitro* investigations.

Cell lines and Growth Media

HepG2 (Human Liver Cancer) cells were cultured in Minimum Essential Medium (MEM) and Dulbecco's Modified Eagles Medium (DMEM) respectively. The medium also contained 10% fetal calf serum (FCS), Penicillin (100 IU) and Streptomycin (100 µg).

Cytotoxic Screening

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0×10^5 cells/ml using medium containing FCS 10%. To each well of the 96-well microtiter plate (Nunclon), 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, partial monolayer was formed. The supernatant liquid was flicked off, washed the monolayer once and 100 ml 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. Microscopic examination was carried out and observations were recorded every 24 h. After 72 h, the drug solutions in the wells were discarded and MTT assay was performed.[13]

Hepatoprotective Effect in Chang Liver Cells

The Methanol and Acetone extracts investigated for cytotoxicity in human liver cell line (Chang liver cells) to assess hepatotoxicity. The screening for 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[2]. 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 microtiter plates and incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2[13]. The cells were then exposed to toxicant (medium containing 15 mM CCl_4) along with/without various concentrations of MCF-Ac and MCF-Me or the medium alone. Later, 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 50 µl 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 50 µl of propanol

was added and the plates were gently shaken to solubilise the formed Formosan. The absorbance was measured using a microplate reader at 540 nm.

Anti-HIV Activity

The extracts and isolated compounds were tested for anti-HIV activity against the replication of HIV-1 (IIIB) and HIV-2 (ROD) in MT-4 cells. The cells were grown and maintained in PRMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM-glutamine, 0.1% sodium bicarbonate and 20 µg/ml Gentamicin (culture medium). HIV-1 (HTVL-IIIB/LAI) strain and HIV-2 (LAV-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 with MT-4 cells (6×10^5 cells/ml). After 5 days of incubation at 37°C, the number of viable cells was determined by MTT method[10]. Cytotoxicity of the compounds for mock-infected MT-4 cells was assessed by the MTT method. Anti-HIV activity of standard Azidothymidine was also performed by a similar method in MT-4 cells.

RESULTS AND DISCUSSION

Cytotoxicity Activity

M. citrifolia is known to be enriched with flavonoids, anthraquinone, steroids, glycosides and the plant has also exhibited anticancer activity[8]. The aim of the present work was to study the Cytotoxic effect of MCF-Ac, MCF-Et, MCF-Me and isolated fraction of *M. citrifolia* fruits on HepG2 (Human liver cancer) cells. MCF-Et and isolated fraction (MCF-C-10) of *M. citrifolia* fruit showed potent cytotoxicity against HepG2 cells with CTC_{50} (50% cytotoxicity) values of 200 µg/ml, 220 µg/ml, 246 µg/ml and 213 µg/ml, respectively. MCF-Me and isolated fractions (MCF-Me-C-1 and C-5) showed significant cytotoxicity against HepG2 cells with CTC_{50} values of 200 µg/ml, 222 µg/ml, 232 µg/ml and 205 µg/ml (Table 1). 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 of *M. citrifolia* against liver cancer cells. Hence, these extracts merit further investigation to screen their anticancer activity using *in-vivo* model.

Hepatoprotective Activity

Methanolic and acetone extracts of *M. citrifolia* demonstrated Hepatoprotective activity. Both extracts were exhibit cytotoxicity in human liver cells only at higher concentration, more than 220 µg/ml (Table-2). To our knowledge, this is the first study that reveals the hepatoprotective activity of the acetone and methanolic extracts of *M. citrifolia* fruit against CCl_4-induced toxicity in Chang liver cells. The CCl_4 exposed Chang liver cells showed a percentage viability of 42%. These exposed cells when treated with different concentrations of acetone and methanolic extracts of *M. Citrifolia* showed a dose-dependent increase in percentage viability and the results were highly significant ($P < 0.0001$, when compared to CCl_4-intoxicated group). The percentage viability ranged between 72% and 84% at 100–150 µg/ml concentration of acetone and methanolic extracts. The increase in percentage viability of the Chang liver cells treated with *M. citrifolia* extracts was significant ($P < 0.01$ when compared to standard). The result proves the hepatoprotective activity of *M. citrifolia* L (Table 2). Further studies for isolation of active constituents and *in-vivo* models for hepatoprotective activity have to be investigated.

Anti-HIV Activity

MCF – Ac extract and their Oil fraction, MCF-Et extract and isolated compounds (MCF-Et-C-10 & C-12), MCF-Me extract and isolated compound (MCF-Me-C-9, I, II, IIII), MCF-Ac) were investigated for antiviral activity against HIV-1 (III_B) and HIV-2 (ROD) virus replication in MT-4 cells. The cytotoxicity also tested against uninfected MT-4 cells (C-type adult T leukaemia cells) by MTT assay for the study of toxicity profile. MCF-Ac inhibits the replication of HIV-1 (III_B) in MT-4 cells and cytotoxicity was found to be only at very high concentration in MT-4 cells (C-type adult T leukaemia cells). All the extracts and isolated compounds showed 2% to 76% (Table 3) maximum protection against HIV-1 and 2 replication at sub-toxic concentrations and cytotoxicity against MT-4 cells only at a higher concentration (CC_{50} more than 125 µg/ml).

Table 1: Determination of Cytotoxic 50% concentration (CTC_{50}) by using MTT assay in HepG2 Cells.

Extracts	Method	CTC_{50} value (in µg/ml)[a]
MCF-Ac	MTT	223.89 ± 3.24
MCF-Me	MTT	220.09 ± 5.96
MCF-Et	MTT	200.42 ± 4.31
MCF-Et	MTT	220.2 ± 11.93
MCF-C-10	MTT	246 ± 5.88
MCF-C-10	MTT	213 ± 7.74
MCF-Me-C-1	MTT	232.04 ± 4.63
MCF-Me-C-5	MTT	205.83 ± 4.56
Cisplatin (Std)	MTT	11.09 ± 0.59

[a]*Cytotoxic 50% concentration (**CTC_{50}**) is the average of 6 independent determinations; the values are expressed as mean ± SEM.*

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

Extract	CTC_{50} value (µg/ml)
MCF – Me	223.89 ± 3.24
MCF - Ac	245.09 ± 2.33

**Cytotoxic 50% concentration*

Table 3: Hepatoprotective effect of *Morinda citrifolia* MCF-Me and MCF-Ac extracts on CCl_4-induced toxicity in Chang liver cells.

Treatment	Concentration	% Viability
Control	-	100
CCl_4	15µ M	42.38 ± 0.94[a]
MCF-Me	150 µg/ml	72.54 ± 1.09[b]
	100 µg/ml	77.53 ± 1.15[b]
MCF-Ac	150 µg/ml	82.46 ± 0.94[b]
	100 µg/ml	83.85 ± 1.01[b]
Silymarin	150 µg/ml	85.29 ± 0.65[b]
	100 µg/ml	86.16 ± 0.98[b]

Values are expressed as mean ± SEM; average of 6 independent determinations is considered. [a]P < 0.001, when compared to untreated cells; [b]P < 0.001, when compared to CCl_4-intoxicated cells.

Table 4: Anti-HIV activity and cytotoxicity of *Morinda citrifolia* in MT-4 cells.

Sample	HIV Strain	IC_{50}[a] (µg/ml)	CC_{50}[b] (µg/ml)	Maximum protection (%)
MCF-Ac	III_B	157.0 ± 37.04	771.67 ± 44.69	76
	ROD	>771.67 ± 44.69	771.67 ± 44.69	6
MCF-Ac-Oil	III_B	>534.75	534.75 ± 226.36	22
	ROD	>534.75	534.75 ± 226.36	22
MCF-Et	III_B	>125.00	>125.00	5
	ROD	>125.00	>125.00	4
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-C9-Me-I	III_B	>125.00	>125.00	4
	ROD	>125.00	>125.00	5
MCF-C9-Me-II	III_B	>92.70	>92.70	2
	ROD	>92.70	>92.70	2
MCF-C9-Me-III	III_B	>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

III_B = HIV-1, ROD = HIV-2. All the values of SD are of 2 independent experiments.

[a]Effective concentration of compound achieving 50% protection of MT-4 cells against cytopathic effect of HIV. [b]Cytopathic concentration of compounds required to reduce the viability of mock-infected MT-4 cells by 50% protection.

CONCLUSION

MCF-Ac, MCF-Et, and MCF-Me from *M citrifolia* L (Noni) were prepared by hot continuous extraction process by using Soxhlet apparatus. Active constituents were isolated from ethanol and methanol extracts by using column chromatography technique. MCF-Ac also gives oil on distillation; the isolated compounds were characterized by TLC and spectral analysis. Crude extracts and isolated compounds were investigated for cytotoxicity in HepG2 cells for evaluating anticancer activity. MCF-Ac and MCF-Et were investigated for hepatotoxicity and Hepatoprotective activities for the assessment of safety of *M. citrifolia* in human liver cells. *M. citrifolia* fruit extracts and isolated compounds were tested for anti-viral against HIV-1 and 2 and cytotoxicity in MT-4 cells. This result strongly supports the basis for the use of extracts and isolated compounds exhibit significant cytotoxicity activity against HepG2 cells. Acetone and methanol extracts showed promising hepatoprotective activity against Chang liver cells and acetone extract of *M. citrifolia* fruit exhibit anti-viral activity against replication of HIV-1 (III_B) in MT-4 cells.

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