Original Article

Influence of limonin on Wnt signalling molecule in HepG2 cell lines

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Abstract

Objective: The role of limonin as potent anti carcinogenic, apoptosis and chemotherapeutic agents has been supported by limited studies. **Materials and Methods:** In this study, limonin is identified as a potent anti proliferative agent against human hepatoma HepG2 cells based on the cell viability study, LDH leakage assay. Induction of apoptosis in HepG2 cells by limonin was evidenced by western blot analysis of Bax, Cyclin D1, Caspase 3 and Caspase9. **Results:** Since Wnt signalling is involved in the initiation and sustaining of hepatocellular carcinoma we studied differential expression of LRP5, LRP6 and DKK wnt players. **Conclusion:** Limonin found to down regulate these players which forms a rationale for further investigation on effect on limonin in cancer therapy.

Key words: Apoptosis, caspases, hepatocellular carcinoma, limonin, Wnt, β-catenin

INTRODUCTION

Primary liver cancer is the sixth most common cancer in which Hepatocellular carcinoma (HCC) contributes 85% that results in Hepatocellular carcinoma as third most common cause of cancer related deaths.[1] Eighty percent of the cases occur in developing countries with age standardized incidence rates in males exceeding 20 per 100,000 per year in eastern Asia and sub-Saharan Africa.[2-4] Globally, chronic infections with hepatitis B virus (HBV) or hepatitis C virus (HCV) and prolonged dietary exposure to aflatoxin are responsible for about 80% of all HCC in human.[5,6] Other risks factors include primary hemochromatosis and cirrhosis of different etiologies, such as alcoholic cirrhosis and cirrhosis associated with genetic liver diseases, but the principal risk factor varies among countries.[7]

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Because of limited impact on the prognosis of hepatocellular carcinoma patients by systemic therapies, we are in a position to identify novel molecular target to effectively intervene the carcinoma progression. This requires the complete understanding of molecular scenario behind the pathophysiology responsible for hepatocellular carcinoma. Though involvements of p53, Rb, TGF ß were identified by mutation analysis, Wnt/ß-catenin pathway seems to be a central mediator in the pathophysiology of hepatocellular carcinoma.[8] While investigating the effect of limonin in inducing apoptosis in HepG2 cell line, an *in vitro* model for hepatocellular carcinoma, we were intrigued in identifying the influence of limonin on players of Wnt signalling such as LRP5, LRP6 and the negative regulator DKK.

Limonin is a bitter white crystalline substance found in orange and lemon seeds, which is the bitter principle of citrus fruits. It is also known as limonoate D-ring-lactone and limonoic acid di-delta-lactone. Limonin belongs to a group of bioactive triterpenoid aglycone derivatives named limonoids which contain a furan ring attached to the D-ring at C-17 as well as oxygen containing functional groups at C-3, C-4, C-7, C-16 and C-17 and an epoxide group at C-14, C-15. Limonin has been shown to possess anti carcinogenic properties in both cell culture and *in vivo* rodent models.[9] In this context, the present investigation

attempts to evaluate the anticancer property of limonin and its influence in Wnt signalling pathway using HepG2 cell line.

MATERIALS AND METHODS

Drug and materials

Dimethyl sulfoxide (DMSO), ethidium bromide and limonin were purchased from sigma, St Louis, MO, USA. RPMI-1640 and sodium pyruvate were purchased from Biochrom, Berlin, Germany. Penicillin-streptomycin and fetal bovine serum were purchased from Gibco, Germany. Trypsin-EDTA was obtained from Hi media Laboratories Pvt. Ltd., Mumbai. Cell culture plates and dishes were purchased from TPP, Switzerland. Primary antibodies were purchased from Abcam Laboratories, USA and Novocastra Laboratories Ltd., Newcastle, UK respectively. Rabbit antimouse IgG was purchased from Bangalore Genei, India. Nitrocellulose membrane was obtained from Millipore, Bedford, USA. All other chemicals including solvents were of highest purity and of analytical grade.

Drug preparation

Limonin was dissolved in Dimethyl sulfoxide (DMSO) (final concentration of the DMSO was not exceeded 0.1% (v/v) and did not affect the cell survival) prepared in serum free RPMI medium and filtered by 0.045 mm syringe filter and stored at 4ºC. Cultured cells were starred, for 24 hours trypsinized (0.05% trypsin and 0.02% EDTA), seeded at a density of $1 \times 10^{[4]}$ cells per well in 96 well plate for MTT assay or at a density of 0.5×10^{6} cells per well for LDH leakage assay, GSH assay, gene expression analysis, western blot analysis and incubated with or without 60 μM or 80 μM limonin for 24 hours. After the treatment, cells were trypsinized and centrifuged at 2500 rpm for 5 minutes.

Maintenance of HepG2 cell line culture and limonin treatment

Human liver-derived hepatoma G2 (HepG2) cells was obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were grown as monolayers in Dulbecco's Modified Eagle's medium (DMEM) with 10% v/v heat inactivated fetal bovine serum (FBS) and antibiotics. Cells were incubated at 37 under 5% CO_2 in a humidified atmosphere. Upon reaching confluence, the cells were trypsinized, plated in flask/plate and were incubated for 24 hours for attachment.

Cell proliferation assay by MTT

3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) method was performed as described.[10] In brief, the viability of the cells was assessed by MTT assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Amount of formazan was determined by measuring the absorbance at 540 nm using an ELISA plate reader and the percentage of viable cells were calculated.

Lactate dehydrogenase leakage assay and estimation of glutathione

Lactate dehydrogenase (LDH) leakage assay was performed in cultured media of limonin (with or without 60 and 80 μM) treated cells by the method.[11] LDH activity was expressed as μ moles of NADH liberated/minute. Reduced glutathione (GSH) was determined in limonin treated (with or without 60 and 80 μM limonin) cells by the method.[12] The amount of GSH was expressed as n moles/10^{6} cells.

Cell morphology by light microscopy

Light microscopic examination of the cells was performed to observe the morphological changes after the treatment with or without 60 and 80 μM of limonin for 24 hours. After the treatment period, the morphological changes were observed under inverted microscope (Nikon, Tokyo, Japan).

Preparation of cell lysate for western blot and RT-PCR analysis

The cells were plated in 100 mm petri plates at a concentration of 1×10^{6} cells/plate and treated with or without 60 and 80 μM of limonin for 24 hours. After the treatment, cells were washed thrice with ice-cold PBS and collected for western blot and RT-PCR analysis.

Expression of P53, Bcl2, Bax, caspase 9, caspase 3 and Cycli D1 protein analysis by western blot

The collected cells were sonicated with RIPA buffer and the content of protein concentration of supernatants (cell lysate) was quantified by the method[13] by using bovine serum albumin (BSA) as the standard. Then the protein concentration was calculated to load equal concentration of protein on all lanes of the gel. 50 μg of total protein was mixed with 2x sample buffer and boiled for 5 minutes. The sample was run on 12% SDS-PAGE gel in 1x running gel buffer at 100 V for 2.5 hours. On the other hand, PVDF membrane was immersed in 10% methanol for a minute. Then the proteins were electro transferred from gel to a polyvinylidene difluoride (PVDF) membrane (Millipore, Germany) at 30 V for 7 hours. The membrane was blocked in blocking buffer containing 5% skimmed milk powder for overnight. After overnight, the blocked membranes were incubated with rabbit anti-p53, anti-bcl2, anti-Bax (Novocastra Laboratories, UK) and mouse anti-caspase-9 and caspase-3 (Transduction Laboratories, UK), mouse anti-β-actin (Sigma-Aldrich Corporation, St. Lois, MO, USA) antibodies with blocking buffer. After primary antibodies incubation, the membranes were washed thrice with TBS buffer. Washed membranes

were incubated with horse radish peroxidase-conjugated secondary antibodies [Mouse anti-rabbit Ab (1:10000) and rabbit anti-mouse Ab (1:5000)] (GENEI, Bangalore, India). Following two intermittent washes with 1X T-TBS and TBS, band intensity on membrane was quantified by using enhanced chemiluminescence (ECL) (Perkin Elmer, USA) method. Immunoblot for β actin was used as an internal control for equal loading in the gel.

Real time reverse transcription–polymerase chain reaction (qRT-PCR) analysis

After quantitation, cDNAs were synthesized using cDNA synthesis kit (Qiagen) from RNA isolated from different groups. Real-time PCR was performed using an ABI 7000 (PE Applied-Biosystems) in the presence of SYBR-green for LRP5, LRP6 and DKK. The optimization of the real time PCR reaction was performed according to the manufacturer's instructions but scaled down to 20 μl per reaction. The PCR conditions were standard (SYBR-Green from Invitrogen) after optimization (see results section), nucleotide primers were used at various concentrations for the detection and quantification of 18s rRNA signal.

Real time PCR–data analysis

Efficiency of amplifications was determined by running a standard curve with serial dilutions of cDNA. For each measurement, a threshold cycle value (CT) was determined. This was defined as the number of cycles necessary to reach a point in which the fluorescent signal is first recorded as statistically significant above background. In this study, the threshold value was determined with a baseline set manually at 100 relative fluorescence units (RFU). Results were analyzed using the comparative critical threshold (ΔΔCT) method in which the amount of target RNA is adjusted to a reference (internal target RNA). The fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method as previously described.[14]

RESULTS

Preliminarily, the anti-proliferative effect of limonin on HepG2 cells was tested by MTT method, which is reliable to detect proliferation of cells. Figure 1 shows the exposure of different concentrations of limonin (20, 40, 60, 80 and 100 μM) for 24 and 48 hours, which resulted in decreased cell proliferation in a dose and time dependent manner when compared with control cells.

Lactate dehydrogenase activity

The levels of lactate dehydrogenase (LDH) released into the medium of control and limonin treated (60 and 80 μM) HepG2 cells are presented in Figure 2. From this Figure it was observed that LDH activity found to be significantly elevated after 24 hours of exposure of 60 and 80 μM limonin in the cultured medium when compared to the control.

GSH content

It is well known that the toxicity of anti-tumor drugs may largely depend on the intracellular level or reduced glutathione (GSH).[15] It is well known that, glutathione plays an important role in protecting cells and cellular components against oxidative stress and in detoxification. It is often found that GSH levels are increased in the drug resistant cancer cells when compared to the drug sensitive cells. Inhibition of glutathione synthesis or modulation of glutathione storages in tumors to reduce anticancer drugs resistance may comprise a novel anticancer strategy.[16] The levels of GSH content in control and limonin treated HepG2 Cells were presented in Figure 3. The significant ($P < 0.05$) depletion of GSH was observed in limonin treated HepG2 cells at the concentration of 60 and 80 μM/ml when compared to the control cells.

Microscopic observation

Figure 4 showed the morphological changes of control and limonin treated HepG2 cells at the concentration of 60 and 80 μM for 24 hours of exposure. In drug treated HepG2 cells, destruction of monolayer was observed. This was not seen in HepG2 cells in the absence of limonin treatment. On the contrary, control HepG2 showed swelling and rounded morphology of the cells with condensed chromatin and their membrane. This may lead to crooked and vesicle shaped. Progressive structural alterations and reduction of HepG2 cell populations were observed in both the concentrations.

Gene expression of p53 and Cyclin D1

A key assumption in studying mRNA expression is that it is informative in the prediction of protein expression.[17] The gene expression level of p53 in the HepG2 cells was depicted in Figure 5. The expression level was

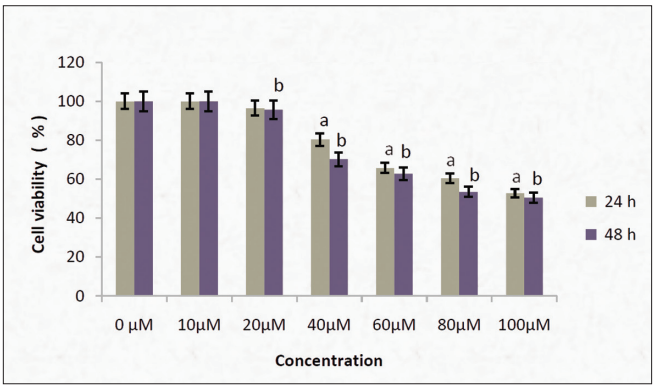

Figure 1: Effect of Limonin on HepG2 cells viability - MTT assay Each Bar represents mean ±SD of six observations, a - Control Vs DMSO, 20, 40, 60, 80 and 100 μM 24 h, b - Control Vs DMSO, 20, 40, 60, 80 and 100 μM for 48 h

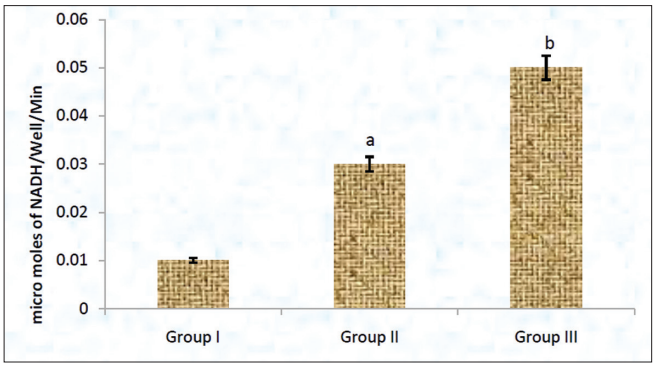

Figure 2: Viability and lactate dehydrogenase leakage in control and limonin treated HepG2 cells after 24 hours of exposure, Each bar represents mean SD of six observations, a - Group I Vs Group II & Group III, b - Group II Vs Group III

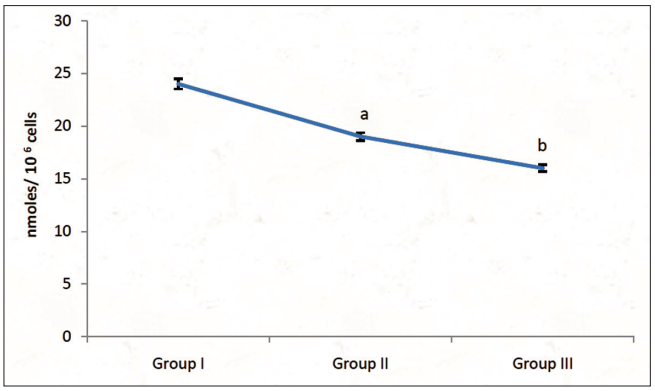

Figure 3: Levels of GSH in control and limonin treated HepG2 cells, Each bar represents mean SD of six observations, a - Group I Vs Group II & Group III, b - Group II Vs Group III

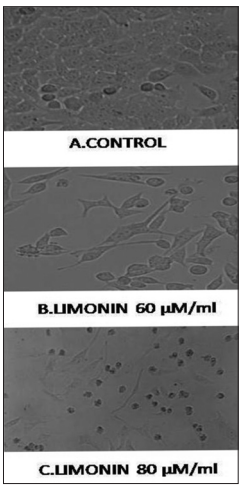

Figure 4: Microscopic image of control and limonin treated HepG2 cells

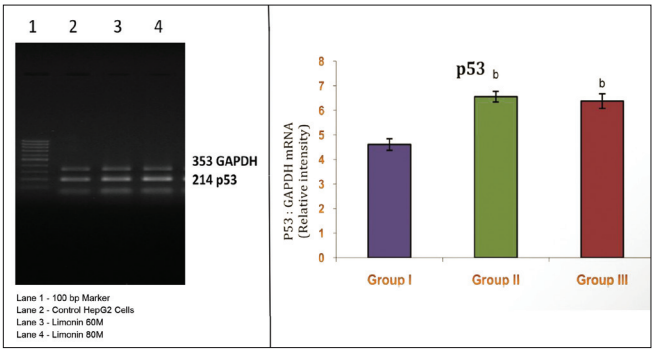

Figure 5: Differential mRNA expression of p53 in HepG2 cells

significantly increased ($P < 0.05$) in group II (60 µM) and even in group III (80 µM) when compared with control HepG2 cells where the p53 expression was significantly high. GAPDH in (lane 1) serve as an internal control. The gene expression level of Cyclin-D1 in the HepG2 cells was depicted in Figure 6. The expression level was significantly decreased ($P < 0.05$) in group II (60 µM) and even in group III (80 µM) when compared with control HepG2 cells where the Cyclin-D1 expression was significantly low. GAPDH in (lane 1) serve as an internal control.

p53, Bcl2, Bax, Caspase-9, Caspase-3 and Cyclin D1 proteins expression

Figure 7a shows the expression of p53 protein in control and limonin treated (60 µM and 80 µM) HepG2 cells by western blotting method. Administration of limonin increase the band intensity of 53 kDa protein compared to the control. The accumulation of p53 protein indicates the expression of tumor suppressor protein-induced apoptosis in limonin treated HepG2 cells. The Bcl-2 family consists of about 20 homologues of important pro- and anti-apoptotic regulators of programmed cell death. Bcl-2 represents the founding member of the new and growing class of cell death inhibiting oncoproteins.[18] Figure 7b display the expression of Bcl-2 protein (25 kDa) in HepG2 control and drug treated (60 µM and 80 µM) cells. The Figure 8a shows Bax protein expression (20 kDa) seems to be up regulated in both limonin treated (60 µM and 80 µM) HepG2 cells.

The expression of Caspase-9 and caspase-3 in HepG2 cells is presented in Figure 8b and Figure 9a respectively. From this, it was observed that treatment of HepG2 cells with limonin reduced the intensity of 40 kDa and 32 kDa band respectively and appearance of low molecular weight protein below the 32 kDa in 60 µM and 80 µM. These results strongly suggest that drug treatment stimulated the proteolytic cleavage of caspase-3 protein being processed through caspase-3 and helps to initiate the apoptosis. The Figure 9b shows the expression of cyclin D1 protein expression. Similarly, the cyclin D1, (33 kDa) presents with decreased expression in both 60 µM and 80 µM treated limonin which is statistically significant than control cells.

mRNA expression of LRP5, LRP6 and DKK

Figure 10, 11, 12 shows the effects of Limonin on mRNA expression of LRP5, LRP6 and DKK respectively.

Limonin (80 μM) down regulates the LRP5 mRNA expression at 3 fold change which was significant than the limonin at 60 μM. Similarly the down regulation of LRP6 was also observed at 2.5 fold change. The mRNA expression of DKK, a negative regulator of Wnt signalling, was found to be strongly up regulated at 4 fold change when compared to control and Limonin (60 μM).

DISCUSSION

Use of *in vitro* assay systems for the screening of potential anticancer agents has been common practice almost since the beginning of chemotherapy in 1946, following the discovery of the antineoplastic activity of nitrogen mustard. HCC is an aggressive tumor associated with dismal prognosis. Currently, there is no effective systemic chemotherapy for HCC, whereas alternative treatment strategies such as transcatheter arterial chemoembolization, percutaneous intratumoral ethanol injection and radiofrequency ablation are mainly for palliation and are applicable only to patients with tumors localized in the liver.[19] HCC is clearly a disease for which alternative therapeutic modalities must be developed. A thorough understanding of the pathogenesis of HCC thus holds the promise of finding an effective chemoprevention and treatment for this cancer.[20]

The role of limonin as potent anti carcinogenic and apoptosis and chemotherapeutic agents has been supported by limited studies. In *in vitro* studies, limonin, nomilin and limonoid glucosides were proved to have a significant ability to inhibit proliferation of human breast cancer. Limonin and nomilin are the most prevalent citrus limonoids.[21] Limonin belongs to a group of bioactive triterpenoid aglycone derivatives named limonoids which contain a furan ring attached to the D-ring at C-17 as well as oxygen containing functional groups at C-3, C-4, C-7, C-16 and C-17 and an epoxide group at C-14, C-15.[9] Limonin was studied as an abortifacient agent,[22] amoebicidal substance,[23] chemopreventive agent and as inhibitors of oral carcinogenesis.[24] Limonin has been shown to possess anti carcinogenic properties in both cell culture and *in vivo* rodent models.[25] Limonin, nomilin and

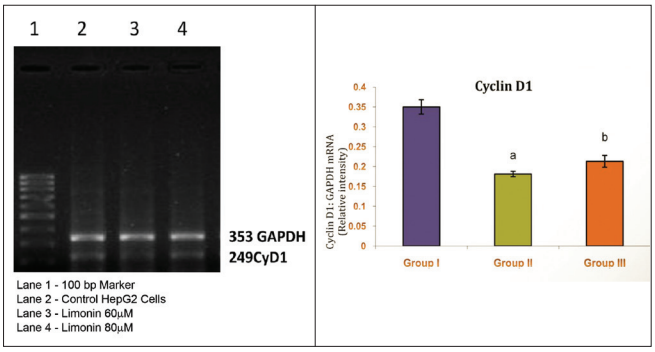

Figure 6: Differential mRNA expression of cyclin D1 in HepG2 cells

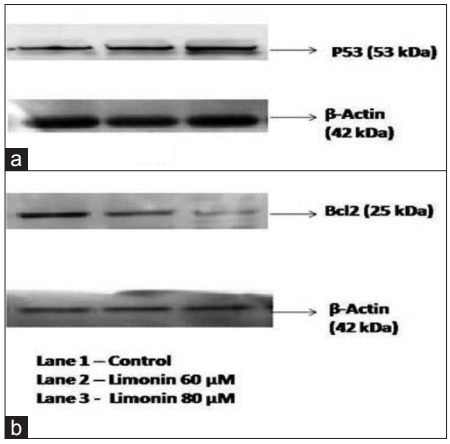

Figure 7: Western blotting analysis of p53 and Bcl2 protein expression in control and limonin treated HepG2 cells

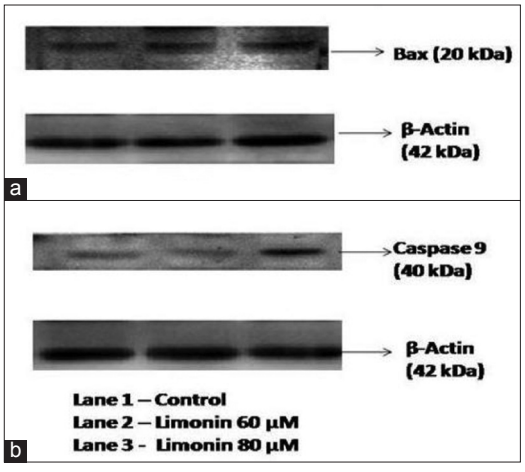

Figure 8: Western blotting analysis of Bax and caspase-9 protein expression in control and limonin treated HepG2 cells

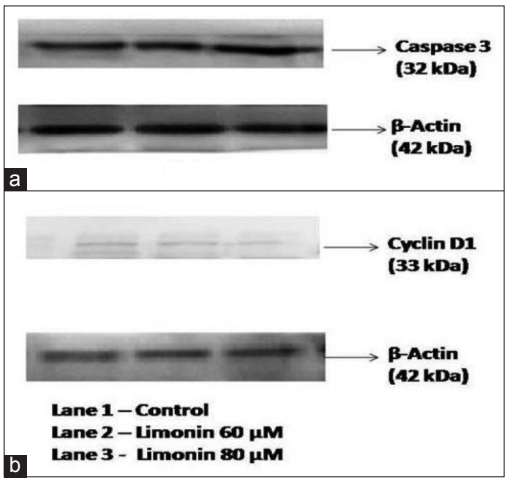

Figure 9: Western blotting analysis of Caspase-9 and Cyclin D1protein expression in control and limonin treated HepG2 cells

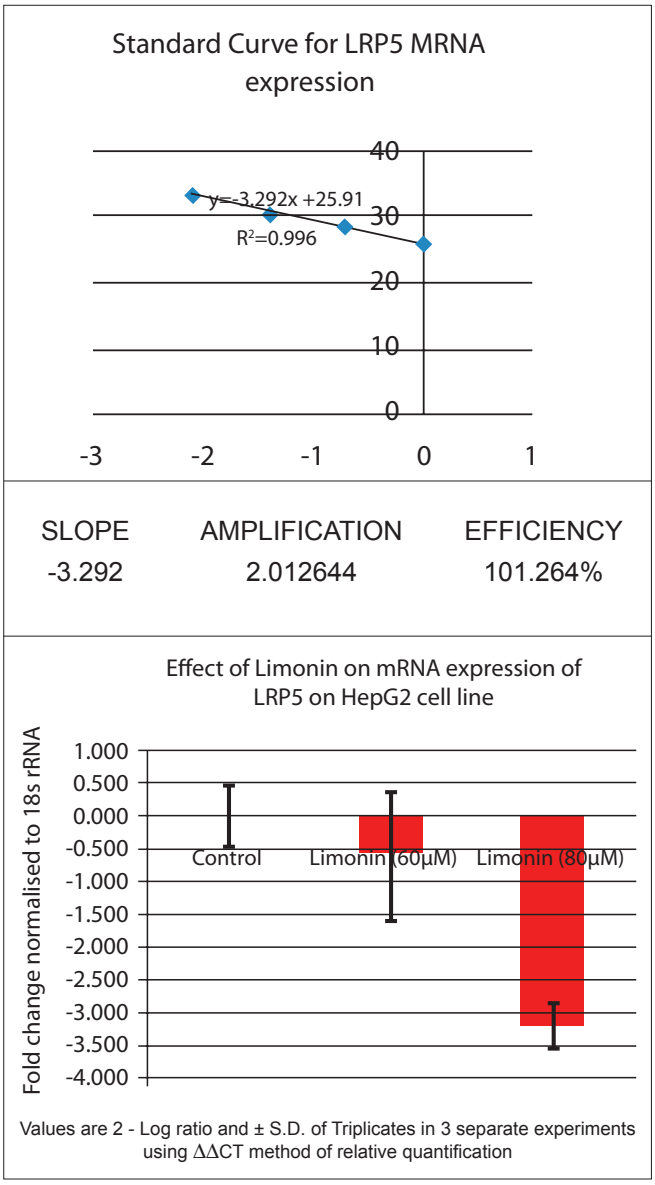

Figure 10: Real Time Quantification of LRP5 mRNA expression

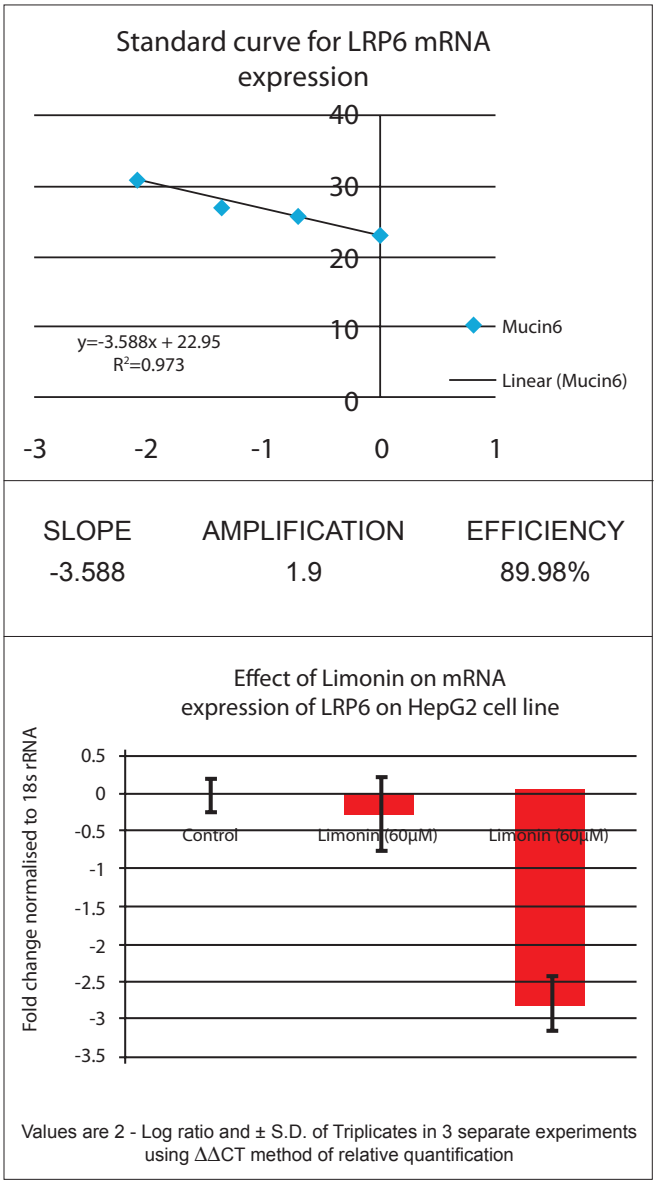

Figure 11: Real Time Quantification of LRP6 mRNA expression

limonin glucosides were tested for their ability to inhibit proliferation of MDA-MB-435 estrogen receptor-negative human breast cancer cells, by the incorporation of [3H] thymidine.[26]

In the present study, limonin significantly reduced the cell viability in a concentration dependent manner. From the results it is inferred that the exposure of different concentration of limonin (20, 40, 60, 80 and 100 µM/ml) for 24 hours resulted decrease of cell proliferation in a dose dependent manner. Limonin nearly inhibits 50% cell population at the concentration of 60 µM for 24 hours when compared to control. Thus the inhibitory effect on HepG2 cells by limonin strongly proves the anti-proliferation property of limonin. The lactate dehydrogenase leakage was increased significantly in limonin treated HepG2 cells when compared with the control cells which suggest that the lactate dehydrogenase leakage in HepG2 cells may be due to cytotoxic nature of limonin and confirm its anti tumor activity. The ratio of reduced glutathione to oxidized glutathione within cells is used scientifically as a measure of cellular toxicity.[27] Decrease in GSH levels in limonin treated HepG2 cells at the concentration of 60 and 80 µM limonin indicates inhibition of HepG2 cells growth and cause apoptosis. The phenolic compounds are generally known to show not only their antioxidant effects but also pro-oxidant actions under the *in vitro* conditions.[28] In the present investigation, it seemed possible that treatment with limonin in HepG2 cells deplete the GSH levels and potentiates to some extent of oxidation induction, which switching the mode of death via apoptosis. Therefore,

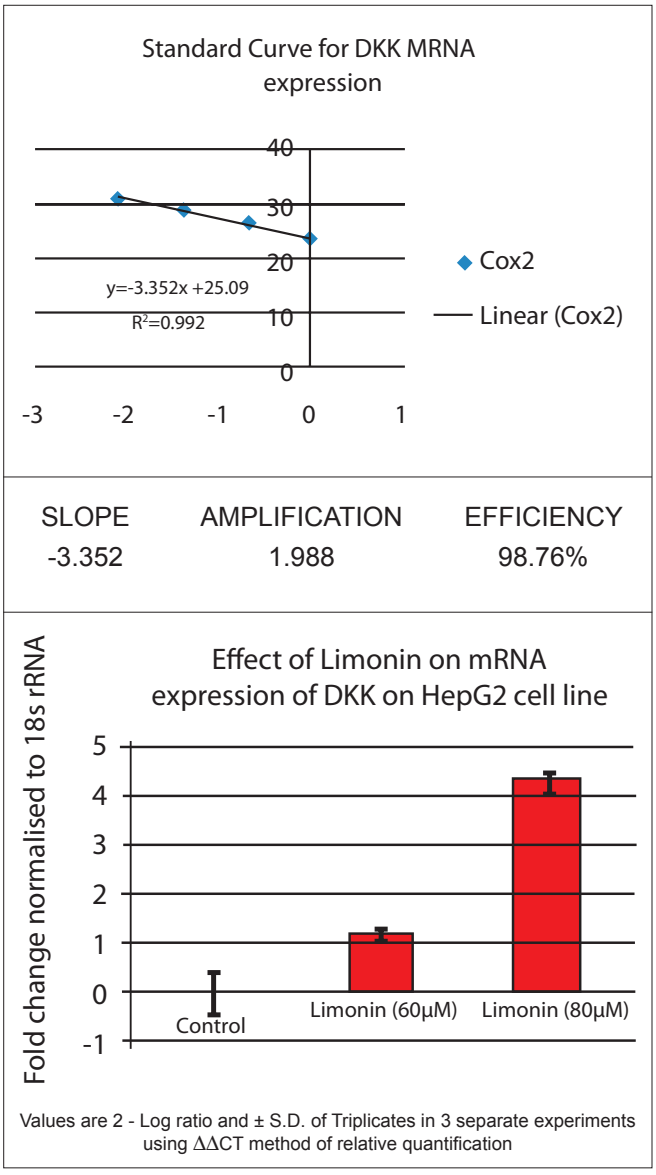

Figure 12: Real Time Quantification of DDK mRNA expression

the cytotoxic action of this drug may be attributed to its pro-oxidant action on the cells.

The light microscopic observations showed morphological changes like reduction in cell volume, cell shrinkage, reduction in chromatin condensation and formation of cytoplasmic blebs as a mean of apoptosis progression in HepG2 cells. Apoptosis can be induced by diverse stimuli including some cell damaging agents and cancer therapy.[29] p53, being a master regulator of cell cycle, regulated by MAPK and AKT pathway helps in the DNA repair due to the conformational change in its kinase domains.[30] This guardian of the genome was found to be up regulated in both 60 µM and 80 µM dose of limonin. This shows the Cell cycle regulating potential of limonin in order to repair the DNA damage caused in the HCC due to various consequences. Cyclin D1 forms a complex with cdk in order to proceed in different phases of cell cycle.[31,32] The mRNA expression and Protein expression was found to be low which helps to speculate that the key molecules for cell division was kept at low level in limonin treated groups. Thus, outcome of the result for cyclin D1 shows the anti proliferative effect of limonin by regulating the molecules like cyclin D1 for the continuation of cell cycle. In cumulative interpretation, with mRNA expression and protein expression analysis, the results obtained shows the down regulation of p53, cyclin D1, anti-apoptotic protein Bcl2 with increased expression of Bax, Caspase-3 and Caspase-9. This clearly helps to speculate the protective effect of limonin over hepatocellular carcinoma as there is no interplay from external factors in HepG2 cell system to confuse with results. Hence anti-proliferative nature of limonin is doubtless in hepatocellular carcinoma.

An extensive study of HCC resulting from three of the main etiological factors HCV infection, HBV infection and chronic alcohol intake indicates common molecular/genetic changes with Rb1, p53 and Wnt the main pathways affected. Due to mutation originated from several etiological factors, there will be an anomalous activation of Wnt signaling that leads to accumulation of ß-catenin in cytoplasm. Normally, the ß–catenin undergoes ubiquitination process. We studied the mRNA expression of key players of Wnt signaling LRP5, LRP6 and negative regulator DKK in which we found this anomalous activation was found to be suppressed at mRNA level. Further studies are warranted to confirm its suppression of Wnt signaling and to identify the key molecule on which the limonin acts. Clinical studies of limonin in patients with hepatocellular carcinoma may help to determine additional therapies.

CONCLUSION

In conclusion, our results support the efficacy of limonin against the hepatocellular carcinoma and we also speculate that its efficacy is by stabilizing the Wnt signalling pathway. This may be because of activating the negative regulation of Wnt signalling through DKK. Future studies will identify the potency of DKK as novel target for cancer therapy.

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