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HEPATOPROTECTIVE ACTIVITY OF *VITEX NEGUNDO LINN* AGAINST CCL

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ABSTRACT

Vitex negundo are used traditionally as an anti–hepatoprotective herbal medicine. To study the hepatoprotective effect of *V.negundo*, we checked methanol leaves extract against carbon tetrachloride (CCL_4). CCL_4 is a well characterized hepatotoxin and inducer of CYP 2E1 which induced toxicity in HepG2 liver cells. In the present study, we showed the inhibitory effect of *V.negundo* against CCL_4 toxicity, by decreasing the apoptotic gene expression in HepG2 cells and protect the liver cells against toxicity.

KEYWORDS: Apoptosis, *Vitex negundo*, CYP 2E1, HepG2 cells.

INTRODUCTION

Liver is one of the largest organs in human body that plays an important role in metabolism and excretion. Toxins from food, alcohol, drugs, and diseases weaken and damage liver, causes fatty liver and cirrhosis (Treadway, 1998). Approximately 25,000 Americans die each year from chronic liver diseases and cirrhosis. More than 300,000 people are hospitalized each year due to cirrhosis. Thus liver ailments remain a serious health problem (Day, 2007).

The readily available conventional hepatoprotective synthetic drugs used in the treatment of liver diseases are not sufficient to control severe liver disorder because of their serious side effects. There is a growing concern to evaluate traditional herbal medicines for hepato protective activity; recent research showed that they are effective, because of the use of natural plant extracts as their active principles (De et al., 1993).

In India numerous medicinal plants and their formulations are used to treat liver disorders as traditional systems of medicine. *Vitex negundo* (VN) is also an important source of such natural drugs and its entire parts have been employed as a traditional medicinal cure in Asian systems of medicine (Indian, Chinese, Malaysian) (Tandon et al., 2008). VN has a number of pharmacological activities such as analgesic, anti–inflammatory, hyper pigmentation, larvicidal, necrosis, nephrotoxicity, enzymes inhibition, nitric oxide scavenging activity, snake venom and scorpion– sting neutralization activity, antifeeding activity, antiradical and antilipoperoxidative, CNS activity, hepato protective activity, antibacterial activity, anti–fungal, antiandrogenic effects, mosquito repellent activity. Past work showed that VN had some promising hepato protective activity (Tasduq et al., 2008). Silymarin is a flavonoid isolated from *Silybum marianum*. Silymarin has a variety of biological effects such as anticarcinogenic, antihepatotoxic, inhibition of lipid peroxidation, antiulcer, antioxidative, antiinflammatory and antiarthritic effects.

As a potent hepatotoxin, carbon tetrachloride (CCL_4) is a class group 2B carcinogen used for the investigation of hepatotoxic induced liver diseases. CCL_4 activates the 2E1 isoform of cytochrome P450 (CYP 2E1), which metabolizes and activates many toxicologically important genes (Koop, 1992) and forms highly reactive CCl_3* and CCl_3OO* reactive oxygen species. These reactive oxygen species (ROS) create oxidative stress in the liver (Tasduq et al., 2008). This oxidative stress and exposure to certain chemicals can also activate NF–κβ, recent studies also showed its impact on the pathogenesis of liver fibrosis (Li X and Stark, 2002). Besides this, CCL_4 also induces the expression of pro–inflammatory cytokines such as interleukin–1 beta (IL–1β) and C/EBPβ at the molecular level, which mediates the process of apoptosis, inflammation, and fibrosis which further leads to the development of liver fibrosis (Jia X et al., 2012). It was therefore, our interest to investigate the effect of CCL_4 on HepG2 cells and also to characterize the hepatoprotective activity of crude extracts of VN on HepG2 cells in the present study.

MATERIALS AND METHODS

Chemicals

DMEM medium, fetal calf serums, Trypsin–EDTA solution, minimal medium, Penicillin, Streptomycin are from Mediatech, USA. Acridine orange and CCL_4 are from Sigma Chemicals Co, USA. Silymarin was a gift from Dr. Nicholas Oberlies, Washington University, USA. RNA kit was from Qiagen, USA.

From RNA, cDNA was synthesized using iScript kit, Bio–Rad, USA. DNA as well as RNA concentrations were measured using Nanodrop 1000 spectrophotometer, ThermoScientific, USA. Cell viability was counted using Cellometer from Nexcelom Biosciences, USA. Cell viability assay was measured using cell titer–glo luminescent cell viability, Promega, USA. Lactate dehydrogenase (LDH) assay was done to measure LDH cytotoxicity using LDH Cytotoxicity Assay Kit, Cayman Chemical Company, USA. Caspase– 3 enzyme activity was used to detect the apoptosis using Enzo lifesciences, USA. Tagman probes used for this study were purchased from Life Technologies, USA. 4–12% SDS–PAGE (NuPAGE) was purchased from Life Technologies, USA.

Cell culture

The study was carried out using HepG2 cells lines expressing CYP 2E1. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS), supplemented with minimal medium, 100 units/ml penicillin, 100 mg/L streptomycin in a humidified atmosphere in 5% CO_2 at 37℃, and were sub–culture regularly.

Cell treatment

Experiments were carried out using 3–5 × $10^4$of cells in 5 % FBS culture medium for 24 h. Stock solutions of CCL4, VN extract and silymarin were prepared fresh to avoid oxidation. For CCL_4 toxicity experiments, test substances such as crude extract of VN, silymarin were added to the cell cultures 2 h prior to CCL_4 treatment. Cells were then collected and various parameters were assayed.

Cytotoxicity assays

Cytotoxicity is defined as the potential of a compound to induce cell death. Cell viability is an index of dead cells. To measure the viable as well as dead cells, the cells were seeded on to 96–well plates, after corresponding treatment, 20 µL of the sample were mixed with 20 µL of AO/PI staining mix of cellometer viability dyes. Cell viability was further confirmed using MTT assay. Cells were seeded on to 96–well plates, after corresponding treatment, the medium was removed and cell viability was evaluated to measure the number of viable cells in culture based upon the quantification of the ATP present which signals the presence of metabolically active cells. The cell titer–glo assay was designed for cell proliferation; cytotoxicity assay was determined by ELISA reader at 565 nm (Multiskan Spectrum; Thermo Electron Corporation, USA).

Lactate dehydrogenase (LDH) assay

LDH release assay was done to determine the effect of methanol extract of VN on membrane permeability in HepG2 cells. The cells were seeded in a 96–well plate at a density of 10^4 cells/ well. After corresponding treatment, LDH cytotoxicity assay kit measures the presence of LDH enzyme in the cell culture medium at 490–520nm. The amount of formazan produced is propotional to the amount of LDH released into the culture medium as a result of cytotoxicity.

Caspase3 enzyme activity to detect apoptosis

To measure the caspase–3 activity, cells were plated in 60 mm dishes at a density of approximately 3 X 10^5 cells, after corresponding treatment, the cells were lysed using the lysis buffer provided in the kit. Supernatant from the cell lysates was collected and measured calorimetrically.

RNA extraction, Reverse transcription and Real–time quantitative PCR

When cell count reached about 85–90% confluence in 5% FCS culture medium, the crude extract of VN, silymarin, $DMSO_4$ were added to the cell cultures two h prior to CCL_4 treatment. After 24 h of treatment, cells were collected, washed twice with ice cold PBS and total RNA was isolated using RNeasy Qiagen column. Total RNA concentrations were measured using NanoDrop ND–1000 spectrophometer. Reverse transcription reactions were carried out using 1 µg of total RNA with an iScript cDNA Synthesis kit. mRNA was reverse transcribed into cDNA with a mixture of Oligo (dT) and Random Hexamers using modified MMLV–derived reverse transcriptase in 20 µL script reaction mix. Realtime qPCR was performed with the Roche Light Cycler 480 RT PCR Instrument (Roche, USA) using Taqman master mix (Life technologies). The 18S ribosomal RNA (rRNA) gene was used as housekeeping gene.

Collection and identification of test material

Fresh leaves of VN were collected in the month of May to June, fruits and barks were collected in the month of July to August from Chennai, India. They were authenticated by the Department of Botany, M.O.P. Vaishnav College for Women, Chennai, Tamilnadu. The leaves, barks and fruits were cleaned with sterile distilled water to make it free from dust and other contaminants; they were then dried under shade and powdered. The powdered leaves; bark and fruits were extracted separately with different solvents viz., water, acetone, hexane, chloroform, ethanol, methanol, water: methanol (20:80) and kept as search for 24 h. After 24

h, the extract was filtered and further concentrated to dryness under reduced pressure using a vacuum evaporator at 40°C, they were then stored in an air tight container maintained at 4°C. Before use, each crude extract was re-suspended in their respective solvent to yield 50 mg extract residue per ml solvent.

Statistical analysis

Results are expressed as mean ± SD. Comparisons were made between control and treated groups data indicated using unpaired Student's t–test and P values < 0.01 were considered statistically significant.

RESULTS AND DISCUSSION

Liver cells are very useful as an *in vitro* model system to study the biochemical and toxicological effect of various chemicals, this attempt was made to study the toxicological effect of CCL_4 and the possible protection using VN. VN was known to be used traditionally for its medicinal properties. Among the different crude extractions of leaves, bark and seeds, leaves were found to show better activity than seeds and bark in the present study, this proved the fact that seeds of VN were used as antiandrogenic in dogs (Bhargava, 1989), anticancer activity (Suresh et al., 2011). Both bark and seeds showed antimicrobial and anti–inflammatory activities (Nyiligira et al., 2004). Several phytochemical studies showed the presence of volatile oils, lignans, flavonoids–like flavones, luteolin–7–glucoside, glycosides and phenols in VN (Tasduq et al., 2008). These phenols decreased the production of ROS and lipid peroxidation (Kadir et al., 2013), thus protecting the liver against CCL_4–induced liver toxicity and oxidative stress. When the crude extract of leaves, bark and seeds were checked for cytoprotective effect, crude extract of leaves were found to show effective activity followed by seeds and bark on HepG2 cells. Different solvents such as water, acetone, hexane, chloroform, ethanol, methanol, water: methanol (20:80) was used to measure cell viability activity on HepG2 cells. Methanol was found to be more effective followed by water: methanol, water, and ethanol, water: ethanol, chloroform, acetone and hexane (Table 1). Among the different solvents, methanol extraction of VN showed high hepatoprotective activity (Vasanth et al., 2008) also showed that 250 mg/L of methanol extract of VN showed the maximum percentage of cell viability against CCL_4 in HepG2 cells. Methanol extract of VN also possesses flavonoids and phenols (Sassa et al., 1987).

Table 1: Cell viability of different extractions using different solvents on HepG2 cells

Plant parts	Methanol	Water: Methanol	Water	Ethanol	Water: Ethanol	Chloroform	Acetone	Hexane
Leaves	+++	++	++	++	++	++	+	+
Seeds	++	++	+	++	+	++	+	+
Bark	++	++	+	++	+	+	+	+

+++ cell viability more than 125%

++ cell viability more than 75%

+ cell viability more than 50%

Dose response experiments were conducted and studied using different concentration of crude extract of VN leaves and silymarin. The crude extract of VN above 500 mg/L was found to be toxic, while 250 mg/L was found to be a protective effect (Fig1).

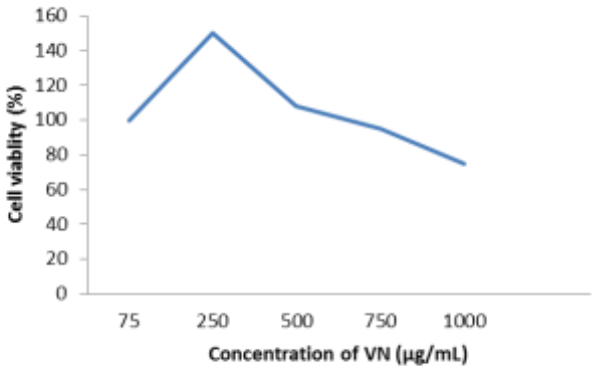


Figure 1: Cytoprotective effect of VN on CCL_4 induced cytotoxicity

For silymarin a concentration above 50 mg/L showed loss of cell viability (Fig 2).

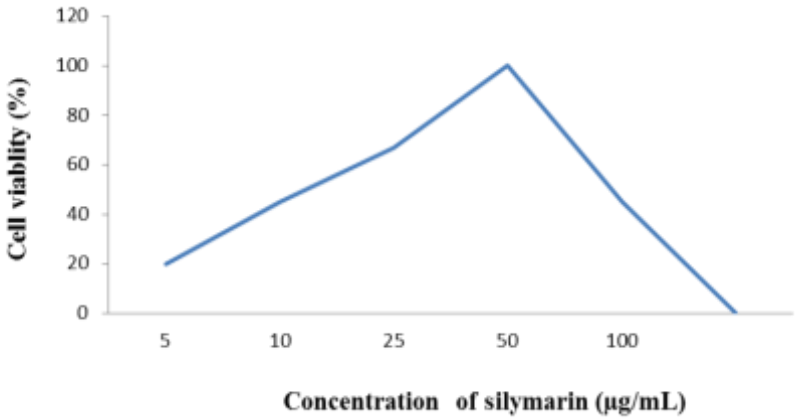


Figure 2: Cytoprotective effect of Silymarin on CCL_4 induced cytotoxicity

Cytotoxicity test by the cell titer–glo assay helped to evaluate the cell viability using CCL4 in HepG2 cells. Among the different concentrations of CCL_4 checked on HepG2 cells for cytotoxicity, 2mMol/L was found effective to generate oxidative stress and also to study the cytoprotective effect of silymarin and crude extract of VN leaves (Fig 3).

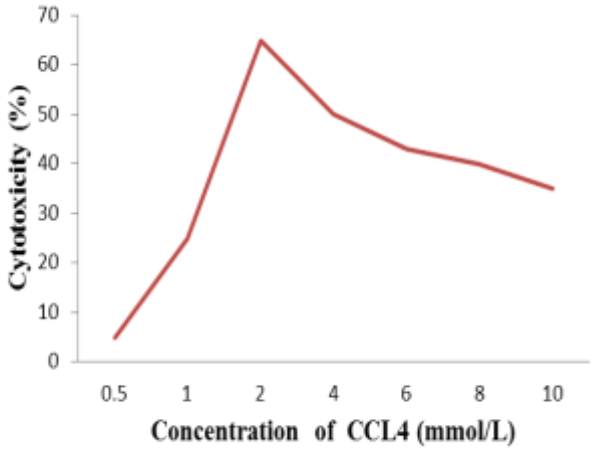
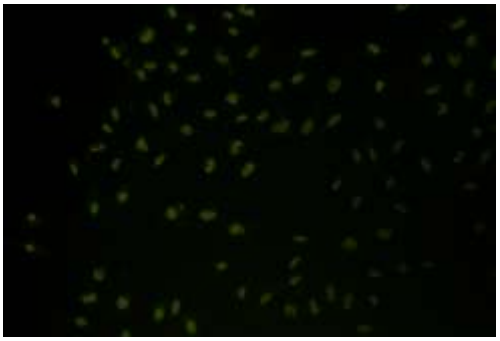


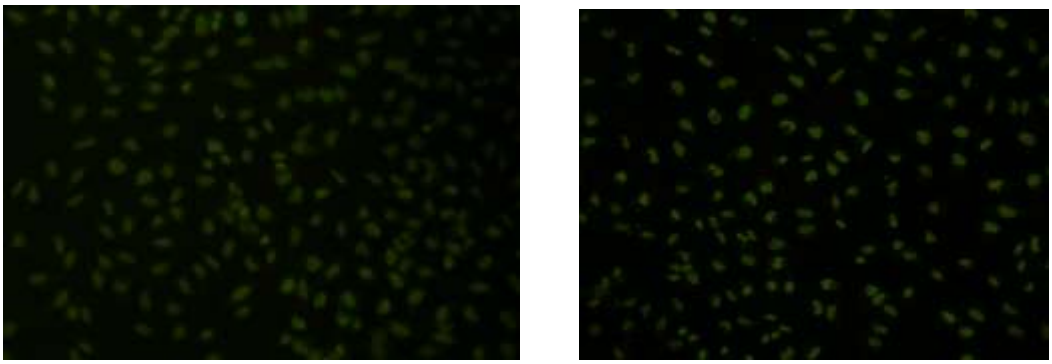
Figure 3: Effect of CCL_4 on cell viability

Acridine orange staining was used to separate and identify dead cells from live cells under fluorescence microscopy. The number of dead cells obtained from acridine orange staining was in correlation with CCL_4 induced cell deaths as observed by cell titer–glo assay. $DMSO_4$ treated control cells were round (Fig 4a), while treatment with 2mMol/L of CCL_4 for 24 h showed swelled, detached, distorted cells with membrane blebbing and cytoplasmic shrinkage (Figure 4b). These structural changes were prevented to a maximum extent by treating with crude extract of VN (250 mg/L) and silymarin at 50 mg/L (Fig 4c and 4d). Besides CCL_4 treated cells for 24 h showed increase in nuclei condensation, apoptotic bodies and cellular debris.

a. $DMSO_4$



b. CCL_4 for 24



c. Cells with Silymarin **d. Cells with VN**

Figure 4: Effect of CCL_4 on VN and silymarin

Nearly 80–85% of cells treated with CCL_4 for 24 h showed cell death which was quantified by MTT assay, cellometer fluorescent cell viability counters and by the LDH assay. Therefore, we can assume that the VN methanolic extract showed stronger apoptosis against the CCL_4 induced apoptotic changes (Fig 5).

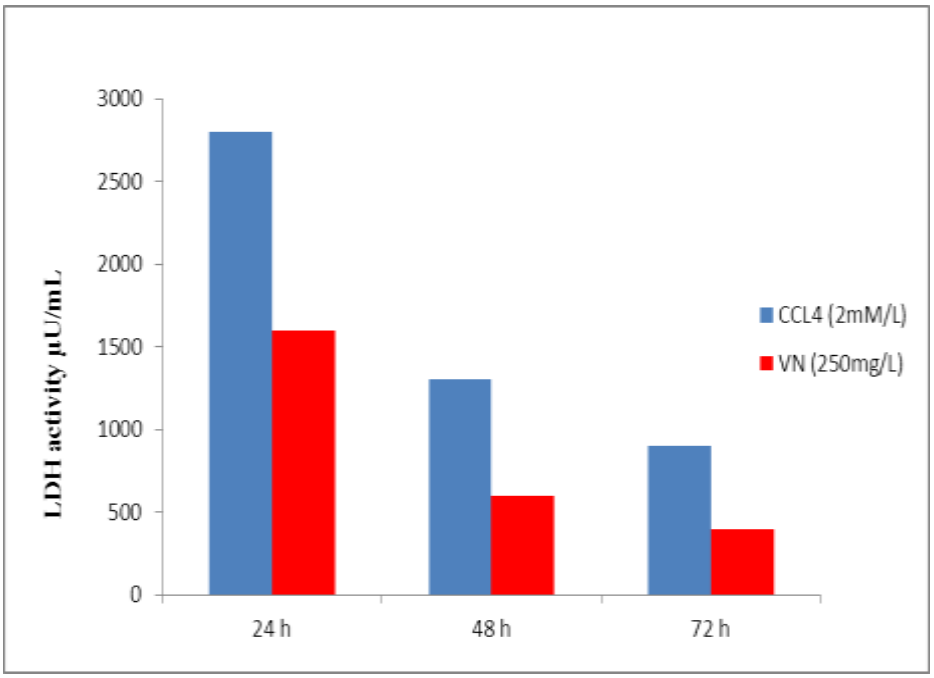


Figure 5: Effect of CCL_4 and VN treatment on LDH release LDH assay

Caspase–3 activation is another important element in the apoptotic signaling cascade pathway. Caspase–3 colorimetric assay confirmed the induction of apoptosis by CCL_4 for 24 h in HepG2 cells. Treatment of VN for 2 h reduced the caspase 3 activation (Fig 6).

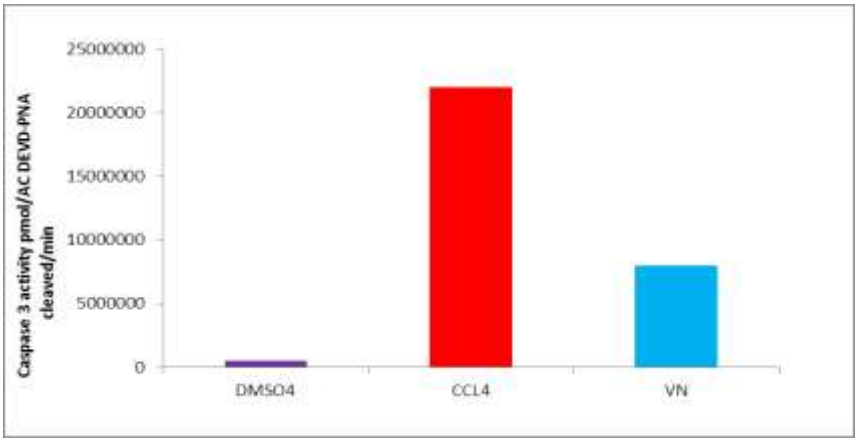


Figure 6: Caspase 3 activity of HepG2 cells treated with CCL_4 and VN

Treatment with CCL_4 for 24 h markedly increased the proportion of apoptotic cells to 58% in cell cycle analysis. But crude extract of VN and silymarin had anti–apoptosis effect as VN reduced the percentage of apoptotic cells to 22%, while silymarin to 34% respectively.

Among the different inflammatory genes, we noticed the expression of few genes during CCL_4 treatment. After CCL_4 treatment for 24 h, we observed an increase in mRNA gene expression of CYP 2E1, NFKβ, IL1β and C/EBPβ in HepG2 cells.

The mRNA expression of proinflammatory factor IL 1β was increased in CCL_4 treated cells for 24 h. IL-1 β comprises a major proinflammatory family of cytokines which acts through the IL-1 receptor (IL-1R), it played an important role in both acute and chronic inflammation specially in the inflammatory response. The mRNA expression of proinflammatory factor IL 1β was increased almost 11 fold in CCL_4 treated cells than the control. Pretreatment with silymarin and VN followed by CCL_4 treatment decreased the mRNA expression of IL 1β not to the basal level but to 6.0 and 4.0 fold higher than the control (Fig 7). Stimulants that induce as well as suppress the expression of IL 1 β have recently gained therapeutic and clinical interest especially in cancer research (Son et al., 2006).

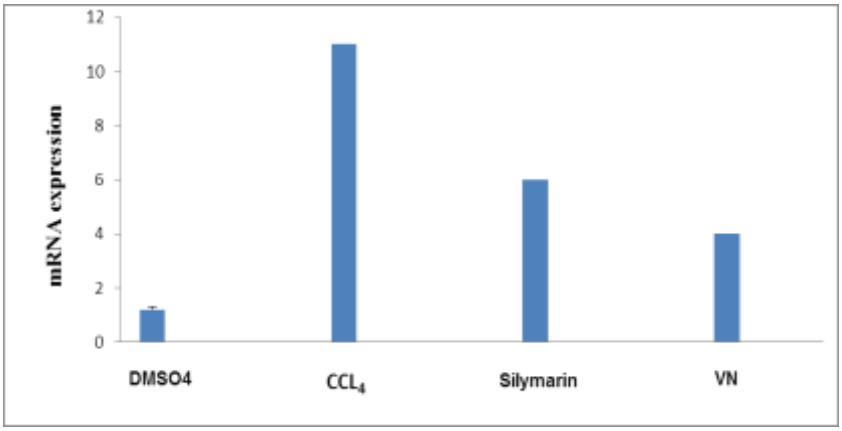


Figure 7: Effect of IL 1β on HepG2 cells

24 h treatment with CCL_4 increased C/EBPβ expression, while VN and silymarin decreased C/EBPβ expression. C/EBPβ (CCAAT/enhancer-binding protein) proteins comprise a family of transcription factors form which homo and hetero dimers played an important role in cellular proliferation, differentiation, inflammation and metabolic processes. C/EBPβ is another mechanism involved in the suppression of NF–κβ by ER stress. In the liver and hepatic cells, C/EBPβ mRNA levels are up regulated during the response to LPS (An et al., 1996). In monocytes, along with nuclear factor-kβ (NF–κβ), C/EBP β is also required for the induction of IL-1β transcription in response to different stimuli (Yang et al., 2010). The mRNA expression of CEBP β was increased to 25 fold in CCL_4 treated cells while in silymarin and VN treated cells, it was significantly reduced to 18 and 12 fold respectively (Fig 8).

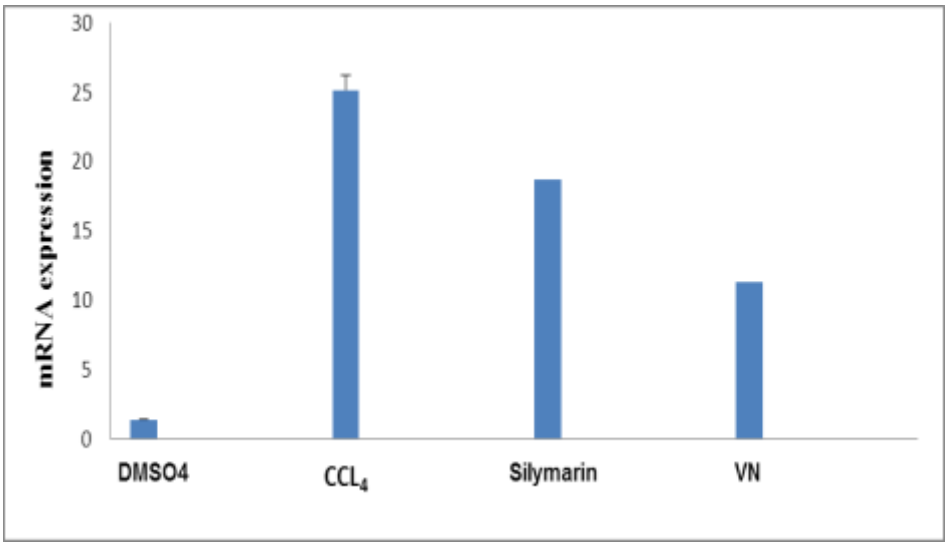


Figure 8: Effect of CEBP β on HepG2 cells

CYP2E1 found in liver are expressed mainly in the hepatocytes of the liver, is one of the important genes in the production of oxidative stress and ROS pathway during CCL_4 injury, alcohol liver disease and xenobiotic toxicity. It is known that through the induction of CYP 2E1, CCL_4 toxicity induced necrosis and inflammation, increased ROS production, triggers transcription of genes involving inflammation, proliferation and cell death (Recknagel and Glande, 1973).

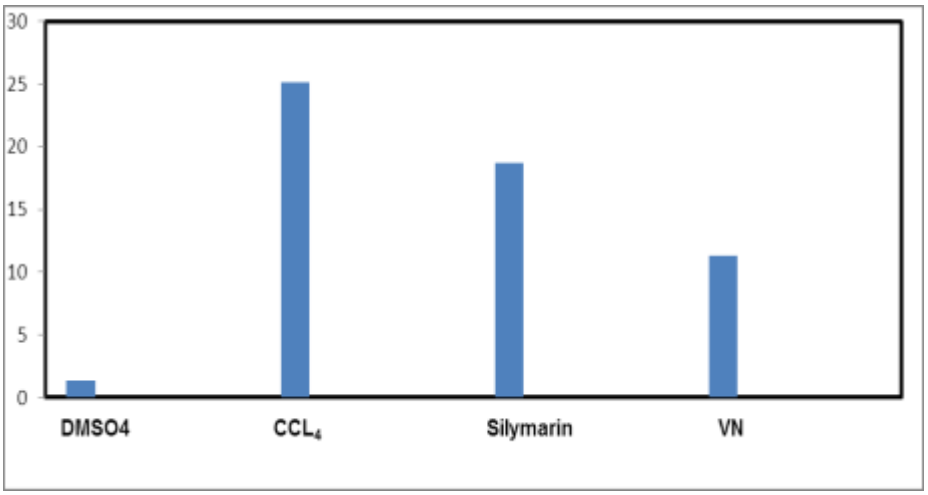


Figure 9: Effect of CYP 2E1 on HepG2 cells

NF-kβ is a highly regulated transcription factor activated by different external stimuli such as the tumor necrosis factors (TNFs), interleukin-1 (IL-1), ROS, ER stress which controls the expression of several genes involved in the immune response, inflammation, apoptosis and cell survival. NF-kβ activation genes expressed due to the inflammatory responses has a great impact on the pathogenesis of liver fibrosis. These changes associated with CCL_4 induced liver damage are similar to that of acute viral hepatitis (Rubinstein, 1962). When cells treated with CCL_4 for 24 h showed the mRNA expression of NF-kβ to18 fold higher as a result of inflammation. But the mRNA expression of treated cells for 2 h prior to treatment with CCL_4 for 24 h with silymarin and VN reduced to 14 and 9 fold respectively (Fig 10). This showed that VN as a potent inhibitor of NF-kβ. Several phytochemicals were known to inhibit NF-kβ by suppressing cellular proliferation and induce apoptosis. Inhibiting NF-kβ signaling has potential therapeutic application especially in cancer and inflammatory diseases (Aggarwal and Shishodia, 2006).

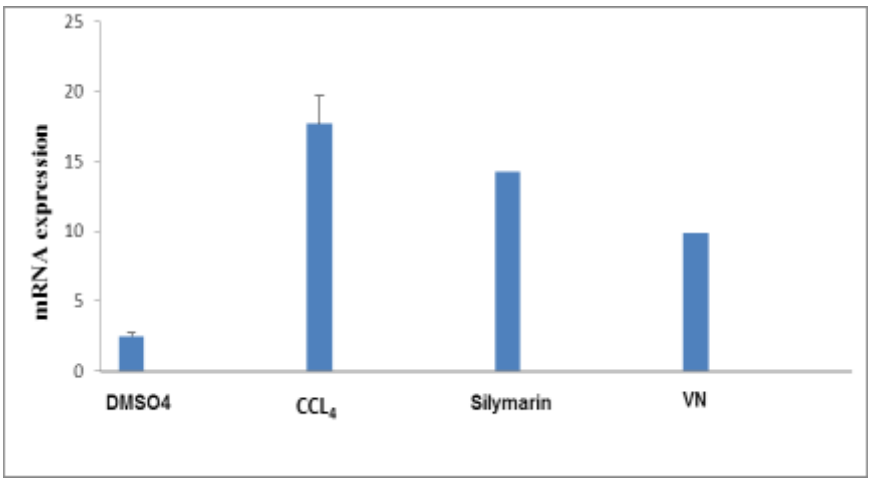


Figure 10: Effect of NFKβ on HepG2 cells

The mRNA expression of all these genes was much lower in silymarin treated cells than VN treated cells, confirming the rescuing and hepatoprotective properties of VN.

Treatment with CCL_4 for 24 h increased CYP 2E1, NF-kβ, IL 1β and C/EBPβ. But when cells treated 2 h prior with VN and silymarin decreased the mRNA expression of these genes confirmed VN inhibits CCL_4 induced ROS production by expressing CYP 2E1, CEBP β and blocking NF-kβ, iL 1β pathway. Inhibition of NF-kβ as well as iL 1β activation by VN showed that it can be used as a non toxic, pharmacological active compound in anti–inflammatory agent.

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