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ANTIBACTERIAL ACTIVITY OF *VITEX NEGUNDO* LINN

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ABSTRACT

Vitex negundo Linn is a large aromatic shrub used as a traditional medicine for the treatment as antimicrobial, anticancer and diuretic properties belongs to the family Verbenaceae. In the present study an attempt had been made to study the antibacterial and hepatoprotective activity of *V. negundo* (VN) against LPS. The antibacterial activity of leaves, bark and seeds of VN were tested against different commonly occurring human pathogenic bacteria's such as *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, and *Klebsiella pneumonia*. Among the different extracts and parts of plant, methanolic extract of leaves showed much more antibacterial activity against these different bacterial strains especially *E.coli.* VN was checked on HepG2 cells against the toxicity induced by LPS to prove the hepatoprotective activity. We showed that the VN induced ROS using JNK and MAPK pathways, decreased the apoptotic gene expressions such as COX2, IL 1 β, NFκβ and iNOS in HepG2 cells to protect the liver cells against LPS toxicity.

Keywords: antibacterial activity, hepatoprotective activity, JNK, LPS, *Vitex negundo*

INTRODUCTION

Sepsis is defined as a systemic inflammatory reaction syndrome (SIRS) against infection which frequently leads to tissue damage and fatal multiple organ dysfunction syndrome (MODS) **(Bone *et al.*, 1992)**. Sepsis is one of the most important causes of death in intensive care units and every year causes 215,000 deaths, thus accounting as 10th most mortality in United States **(Angus *et al.*, 2001)**. Lipopolysaccharide (LPS), the outer membrane of Gram-negative bacteria such as *Escherichia coli*, peptidoglycan and lipoteichoic acid in the cell wall of Gram positive bacteria such as *Staphylococcus aureus* are possible sepsis inducing materials **(Wang *et al.*, 2000)**. Antibiotics are used to prevent and cure bacterial infections. However, in recent years, the heavy use of antimicrobial drugs increased new antibiotic-resistant bacteria and inevitable nosocomial infections **(Wheeler and Bernard, 1999)**. So, there is a need to search alternative drugs and one of such alternative way is using new compounds, not based on existing synthetic antimicrobial molecules.

Liver is one of the largest organs in human body plays an important role in sepsis, besides metabolism and excretion. It is also constantly flushed with toxins from environment, food, alcohol, drugs diseases such as hepatitis A, B, C and E, fatty liver, cirrhosis. These varied disorders damage and weaken the liver, thus liver ailments remain a serious health problem. Moreover the readily available conventional hepatoprotective synthetic drugs used in the treatment of liver diseases are sometimes inefficient and caused severe side effects **(Treadway, 1998)**.

In India, different parts of plants are used to cure various diseases from ancient times. *Vitex negundo* is one such plant belongs to the family Verbenaceae, is a large aromatic shrub distributed throughout the India. The shrub is one of the important plants used in Indian medicine. Almost all parts of the herb are used and believed to be effective against rheumatism, as insecticidal, antimicrobial, hepatoprotective, anticancer, tranquillizer, tonic, febrifuge, expectorant and diuretic properties **(Tasduq *et al.*, 2008)**.

Preliminary studies showed that VN leaf exhibited antioxidant properties and contains natural antioxidants. It was, therefore, our interest to investigate in the present study, to test antibacterial activity of VN, effect of LPS and to characterize the hepatoprotective activity of crude extracts of VN against LPS on HepG2 cells.

MATERIALS AND METHODS

Fresh leaves of VN were collected during the months of May to June, seeds and barks were collected during 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 remove dirt's, dried under shadow and powdered in a grinder. The powdered leaves, barks and fruits were extracted separately with different solvents viz., water, acetone, hexane, chloroform, ethanol, methanol, water: methanol (20:80) extracts and soaked in them separately for 24 h. After 24 h, the extraction was filtered and further concentrated to dryness under reduced pressure with a vacuum evaporator at 40°C, and stored in an air tight container until use at 4°C. Before use, each crude extract was re-suspended in their respective solvent to a concentration of 250 and 50 mg / mL of solvent.

Screening for antibacterial activity of crude extracts

The crude extracts were screened initially for their antibacterial activity against the four bacterial species by employing agar-well-diffusion **(Patel *et al.*, 2007)** and agar disc-diffusion methods **(Bauer *et al.*, 1966)**.

Bacterial strains

Four bacterial strains were used for this study. It includes two gram positive bacterial strains such as *Bacillus subtilis* and *Staphylococcus aureus* and two gram negative bacterial strains *Klebsiella pneumonia* and *Escherichia coli*. These bacterial strains were sub-cultured periodically and maintained on Nutrient Agar (NA) medium for further experiments at room temperature at 28±2 °C.

Effect of crude extract on growth of bacteria by agar well diffusion method

In the preliminary screening, the effect of crude extract of leaves, seeds and barks on bacterial growth was determined by agar well diffusion method **(Patel *et al.*, 2007)**. About 1 ml of each bacterial inoculum was spread on the agar surface using sterile spreader. Then a well of 0.5 cm was made in the agar medium using a sterile cork borer. About 100 µL of each crude extract in different solvent was transferred into well and plates were incubated at 37°C for 24 h. $DMSO_4$ was served as a control. The development of inhibition zone around the well was measured. Each experiment was repeated as triplicates and the mean value was taken for each sample.

Effect of crude extract on the growth of bacteria by agar disc diffusion method

The antibacterial activity of the extracts of leaves, bark and seeds of VN were performed using a modified agar disc diffusion method **(Bauer *et al*., 1966)**. About 250 mg/mL of crude extract of leaves, barks and fruits in different solvents were loaded on a sterile filter paper disc of 6 mm diameter. The petriplates containing nutrient agar medium were spread with 100 µL of actively growing bacterial broth culture using spreader and allowed to dry for 10 minutes. Then the impregnated discs were placed on the surface of inoculated agar medium and incubated for 37°C for 24 h. Discs loaded with small volume of $DMSO_4$ was served as a control. The development of inhibition zone around the loaded disc was recorded. Each experiment was repeated for three times and the mean value was taken for each sample.

SDS-PAGE analysis

Polypeptide profiles of control as well as VN extract treated cultures were analyzed using 4-12 % SDS-PAGE followed by coomassie blue staining. Bacterial culture was harvested after 24 h treatment with 250 µg of methanolic extract of VN and $DMSO_4$.Cells were centrifuged and washed twice with PBS, lysed in sample buffer, heated at 95°C for 5 minutes.

Chemicals

DMEM medium, fetal calf serums, Trypsin-EDTA solution, Penicillin, Streptomycin were purchased from Mediatech, USA. Acridine orange and LPS were purchased from Sigma Chemicals Co, USA. Silymarin was a gift from Dr. Nicholas Oberlies, Washington University, USA. RNA kit was purchased from Qiagen, USA. From RNA, cDNA was synthesis using iScript kit, Bio-Rad, USA. DNA as well as RNA concentrations were measured using Nanodrop 1000 spectrophotometer, Thermo Scientific, USA. Cell viability was counted using Cello meter 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 life sciences, 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

HepG2 cells were maintained on Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum (FCS), supplemented with 100 units/mL penicillin, 100 mg/L streptomycin in a humidified atmosphere in 5% CO_2 at 37°C, and were sub-cultured regularly. For all the experiments, cells were plated at a density of 3×10^4 cells/cm^2 in 5% FCS.

Cell treatment

Stock solutions of LPS, VN extract and silymarin were prepared every time fresh to avoid oxidation. For LPS toxicity experiments, test substances such as crude extract of VN, silymarin were added to the cell cultures two h prior to LPS treatment for 24 h. Cells were then collected for studying various parameters.

Cytotoxicity assays

Cytotoxicity is defined as the potential of a compound to induce cell death. So cell viability is an index of cytotoxicity. To measure the viable as well as dead cells, the cells were seeded onto 96-well plates, and after the corresponding treatment, 20 µL of the sample was mixed with 20 µL of AO/PI staining mix of cello meter viability dyes, was used to calculate the number of viable as well as dead cells. Cell viability was further confirmed using MTT assay. Cells were seeded onto 96-well plates, and after the corresponding treatment, the medium was removed and cell viability was evaluated to measure the number of viable cells in culture based upon the quantitation of the ATP present, which signals the presence of metabolically active cells. The cell titer-glo assay is designed for cell proliferation and cytotoxicity assays and 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 methanolic 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 proportional to the amount of LDH released into the culture medium as a result of cytotoxicity.

Caspase- 3 enzyme activity to detect apoptosis

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

DNA laddering

HepG2 cells both treated and untreated were collected, centrifuged at 1500 × g for 5 minutes. Washed twice with ice cold PBS and gDNA was isolated using phenol/chloroform extraction and eluted in TE buffer. Nearly 1.5 µg of DNA was stained with ethidium bromide and visualized by 2.0% TAE to analyze DNA fragmentation **(Arumanayagam *e t al*., 2015).**

RNA extraction, reverse transcription and real-time quantitative PCR

When cells were nearly 85-90% confluence in 5% FCS culture medium, crude extract of VN, silymarin, $DMSO_4$ were added to the cell cultures two h prior to LPS 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 (Bio-Rad). 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. Real-time 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 **(Arumanayagam *e t al*., 2015).**

Statistical analysis

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

RESULTS

When the crude extract of leaves, bark and seeds were checked for cytoprotective effect in HepG2 cells, crude extract of leaves were found to be most effective 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, ethanol, water: ethanol, chloroform, acetone and hexane.

Table 1 Agar well diffusion method using methanolic extract of VN

Strains	Leaves (mm)	Seeds (mm)	Bark (mm)
Staphylococcus aureus	14.0	9.9	8.6
Escherichia coli	22.5	17.11	14.13
Bacillus subtilis	11.16	9.3	6.23
Klebsiella pneumonia	8.50	5.8	4.4

In both agar well (Table 1) as well as agar cup (Table 2) diffusion method, methanolic crude extract of leaves showed maximum inhibition to *E.coli* followed by *S. aureus, B. subtilis* and *K. pneumonia.* In both methods, there was no inhibition zone around the control DMSO4.

Table 2 Agar disc diffusion method using methanolic extract of VN

Strains	Leaves (mm)	Seeds (mm)	Bark (mm)
Staphylococcus aureus	14.1	10.9	8.8
Escherichia coli	22.8	17.5	14.22
Bacillus subtilis	11.05	9.21	6.8
Klebsiella pneumonia	8.22	6.0	4.26

SDS-PAGE study showed that methanolic extract of VN leaves on *E.coli* showed a strong proteolytic activity, while the control did not show any proteolytic activity (Fig 1).

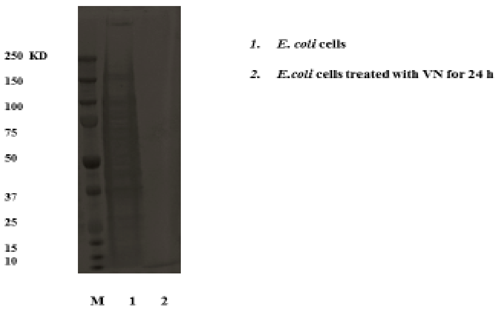

Figure 1 Proteolysis activity of VN on 4-20 % tris-HCl gel

Different concentrations of crude extract of VN leaves and silymarin were used to study the dose response effects. The crude extract of VN at a concentration of 250 mg/L was found to be a protective (Fig 2), but a concentration above 500 mg/L was found to be toxic. But for silymarin, a concentration above 50 mg/L was showed the loss of cell viability (Fig 3).

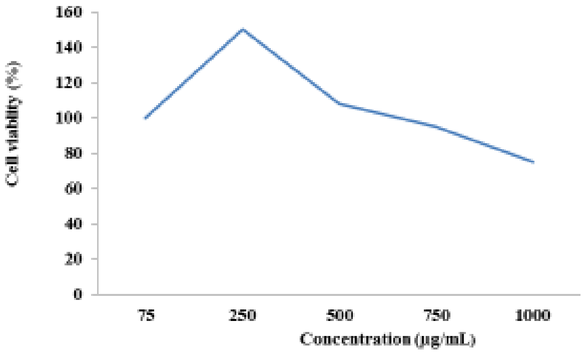

Figure 2 Cytoprotective effect of VN on LPS induced cytotoxicity

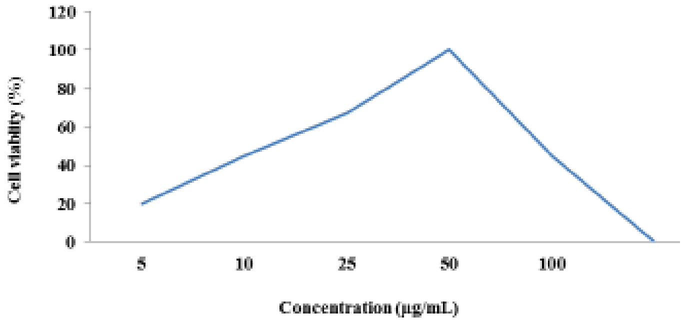

Figure 3 Cytoprotective effect of silimarin on LPS induced cytotoxicity

Among the different concentrations of LPS checked on HepG2 cells, a concentration of 200 µg per mL was found to be effective to generate oxidative stress (Fig 4). The same concentration was used to study the cytoprotective effect of silymarin and crude extract of VN leaves.

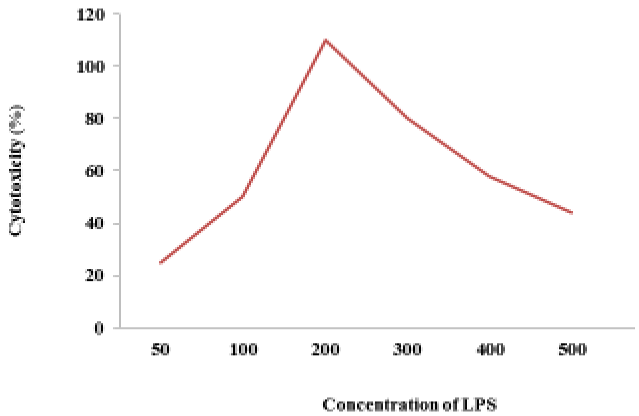

Figure 4 effect of LPS on cell viability

Acridine orange staining was used to identify the dead cells under fluorescence microscopy. Under light microscope, these cells were easily identified by their morphological changes. LPS at a concentration of 200 µg/mL for 24 h caused cells to lose their normal round structure, membrane blebbing and cytoplasmic shrinkage. Besides LPS treated cells were swelled, detached, distorted and their monolayer was disturbed. These structural changes were prevented to a maximum extent by treating with crude extracts of VN (250 mg/L) and silymarin at 50 mg/L. $DMSO_4$ treated control cells showed round shape nuclei, while LPS treated cells for 24 h showed morphological alterations such as increase in nuclei condensation, apoptotic bodies and cellular debris (Fig 5). Nearly 85-87% of cells treated with LPS for 24 h showed cell death, using cello meter fluorescent cell viability counters. Even the LDH assay confirmed the above acridine orange staining conclusions. Therefore, we can assume that stronger apoptosis is associated with higher concentration of VN methanolic extract against the LPS induced apoptotic changes (Fig 6).

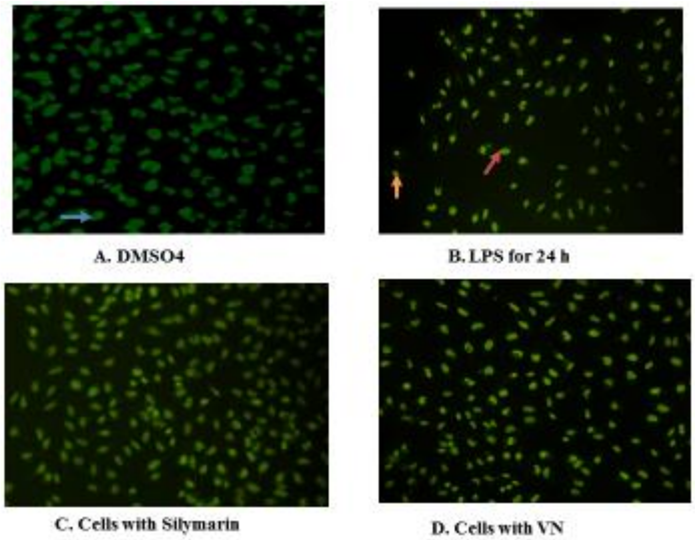

Figure 5 Effect of LPS on VN and silimarin. Blue arrow showed normal nuclei (Fig A), while orange arrow showed condensation and red arrow showed fragmented nuclei (Fig B).

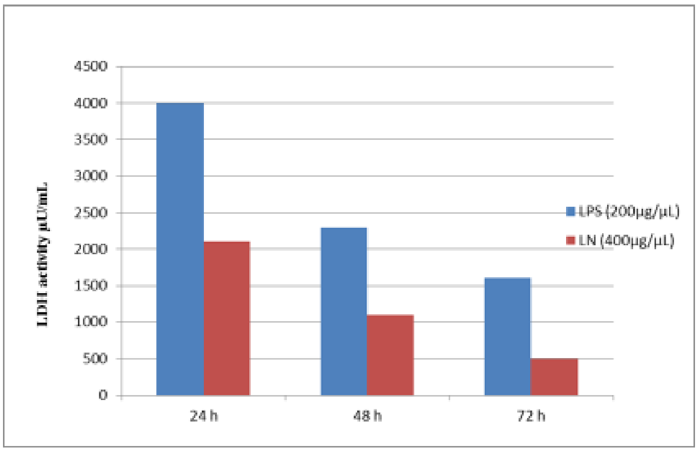

Figure 6 Effect of LPS and VN treatment on LDH release

LPS triggers the secretion of pro-inflammatory cytokines in many cell types, through the nuclear factor-kappa B (NF-κβ) / COX-2 pathway in macrophages. Both the NFκβ and MAPK pathways are critical for the induction of numerous immune response genes including cytokines and chemokines. Among the different inflammatory genes, we noticed the expression of few genes during LPS treatment. After LPS treatment for 24 h in HepG2 cells, we observed an increase in mRNA gene expression of JNK, NFκβ, iNOS and COX2. The mRNA expression of iNOS was increased 78 fold higher in LPS treated cells but decreased 57 and 22 fold respectively in silymarin and VN treated cells (Fig 7).

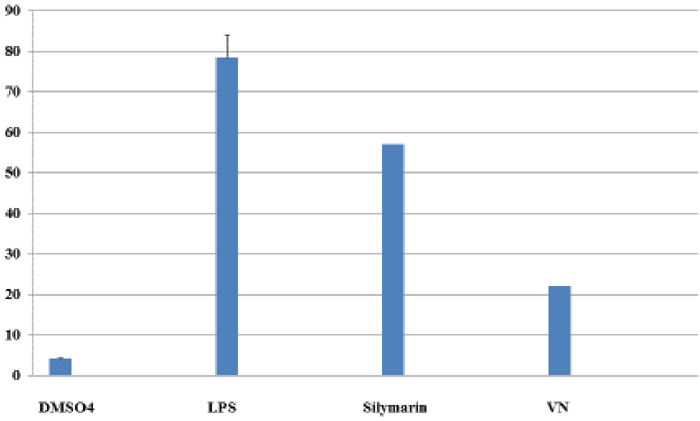

Figure 7 Effect of iNOS on HepG2 cells

The mRNA expression of NFκβ was 17.5 fold higher in LPS treated cells. But the mRNA expression of treated cells with silymarin and VN reduced to 13.9 and 9.6 fold respectively (Fig 8). The mRNA expression of JNK was increased 18.3 fold higher in LPS treated cells but decreased 8.7 and 4.05 fold respectively in silymarin and VN treated cells (Fig 9). The mRNA expression of COX 2 was 69.7 fold higher in LPS treated cells. But the mRNA expression of treated cells with silymarin and VN reduced to 55 and 31 fold respectively (Fig 10). The mRNA expression of all these genes was much lower in silymarin treated cells than VN treated cells thus confirmed the rescuing and hepatoprotective properties of silymarin and VN.

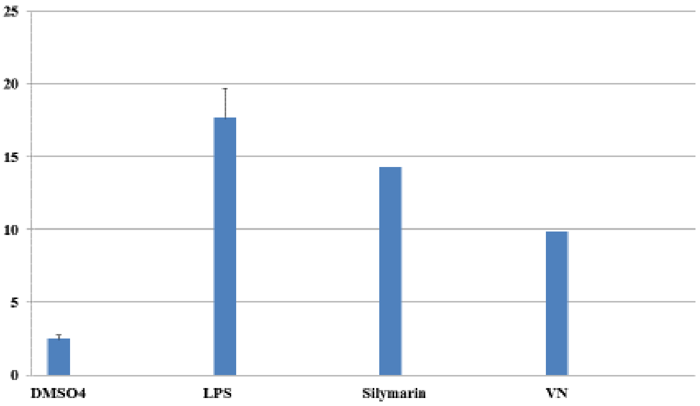

Figure 8 Effect of NFKβ on HepG2 cells

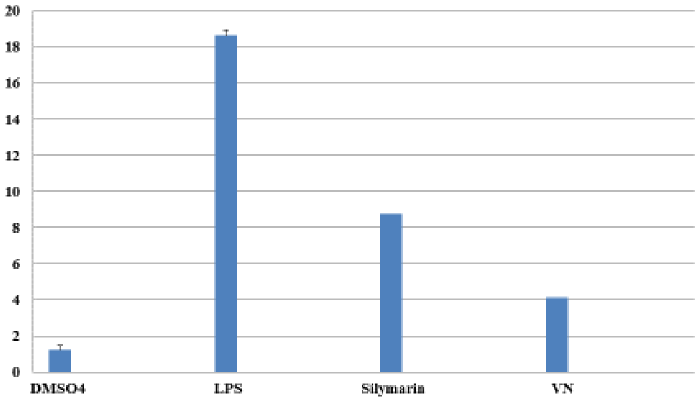

Figure 9 Effect of JNK on HepG2 cells

Caspase-3 activation is an important element in the apoptotic signaling cascade pathway. Caspase-3 colorimetric assay confirmed the induction of apoptosis by LPS for 2 h in HepG2 cells. Treatment of VN for 24 h reduced the caspase 3 activation (Fig 11).

Apoptosis nature of cells treated with LPS for 24 h was confirmed by gDNA fragmentation. Cells treated with LPS for 24 h showed a DNA ladder kind of formation. Apoptosis nature of cells treated with LPS for 24 h was confirmed by gDNA fragmentation. Treatment with crude extracts of VN and silymarin for 2 h showed some kind of protection against LPS induced DNA fragmentation (Fig 12).

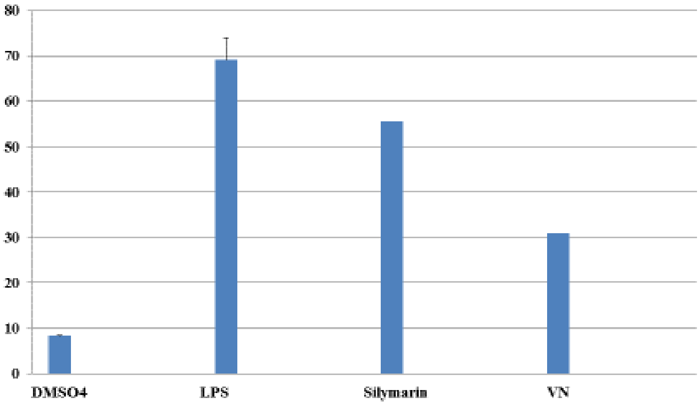

Figure 10 Effect of COX2 on HepG2 cells

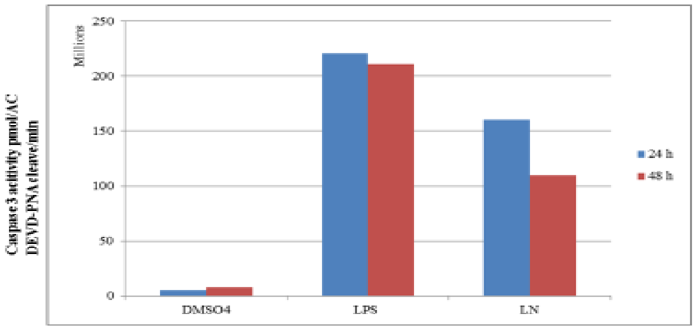

Figure 11 Caspase 3 activity of HepG2 cells treated with LPS on VN

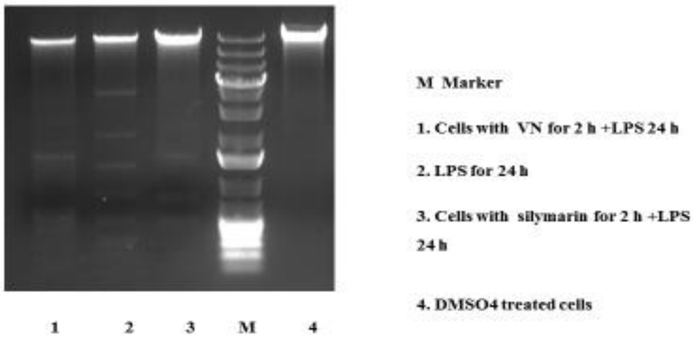

Figure 12 Effect of NG and silimarin agaisnt CCL4 induced DNA fragmentation

Treatment with LPS for 24 h markedly increased the proportion of apoptotic cells significantly to 60% in cell cycle analysis. But crude extract of VN and silymarin had anti-apoptosis effect as VN reduced the percentage of apoptotic cells to 28%, while silymarin to 41% respectively.

DISCUSSIONS

Due to liver's integral role in metabolism and host defense mechanism, liver is also the key organ responsible for the initiation of multiple organ failure during sepsis. So an attempt was made to study liver cells as an *in vitro* model to study toxicological effect of LPS and the possible protection using plant extracts. Research showed that plant extracts produced potential physiological activities, because of their polyphenols. These polyphenols act as anti-inflammatory, cytoprotective and hepatoprotective agents, thus forms an important source for human health research. VN was used traditionally throughout the world for its medicinal properties.

Among the different crude extracts of leaves, barks and seeds are used in this study, leaves are found to be most efficient than seeds and bark. Bhargava, 1989 showed that seeds of VN were used as antiandrogenic in dogs. Seeds of VN showed anticancer activity **(Awale *et al*., 2011)**. Both bark and seeds are used as antimicrobial and anti-inflammatory activities **(Nyiligira *et al*., 2004)**.

Among the different solvents used, methanolic extract of VN leaves was found to be most hepatoprotective activity. **Arumanayagam *et al*., (2015)** also showed that 250µg/mL of methanolic extract of VN leaves showed the maximum percentage of viability against CCL4 induced toxicity in HepG2 cells. Several phytochemical studies showed that methanolic extract of VN had high antioxidant activity due to its high phenolic and different flavonoid contents **(Zargar *et al*., 2011)**. Various studies have shown that antioxidant activity of different flavonoids was reported to be better than that of individual flavonoid

(Pekkarinen *et al.*, 1999). These antioxidants decreased the production of ROS thus protect the liver against liver toxicity and oxidative stress.
In our report *E. coli* was completely inhibited, followed by *S. aureus* using methanolic extract of VN leaves. **Panda *et al.*, *(*2009)** also reported that *E. coli* was completely inhibited by leaf and bark extracts of VN followed by *S. aureus*. He further noticed that ethanol and methanol extracts of the leaves were most active inhibiting agent against both gram-positive and gram-negative bacteria. In our study methanolic extract was most efficient. So the plant VN can be used as a source which could yield drugs that could improve the treatment of infection caused by this organism.
Among different cell lines, HepG2 cells were used because of their morphological and biochemical characters are similar to that of normal hepatocytes, many hepatoprotective chemical have also been studied on HepG2 cells **(Sassa *et al.*, 1987)**.
LPS is one of the highly conserved pathogen-associated molecular patterns (PAMPs) as well as an endotoxin, upon entering the blood circulation, it stimulating the host cells to produce a large amount of pro-inflammatory cytokines such as tumor necrosis factor TNF α and interleukin IL1β, pro-inflammatory genes such as JNK, iNOS and cyclooxygenase (COX-2) by activating several types of transcription factors including the nuclear factor NF κβ during inflammation as well as sepsis **(Hack *et al*., 1997)**.

NFkβ

NFkβ is a family of closely related protein dimers which bind to a common sequence motif in a DNA called the kβ site. During resting condition, the NFkβ dimers reside in the cytoplasm, while upon activation by endotoxins it translocate into the nucleus, induces the expression of more than 200 genes which showed to suppress apoptosis and induce inflammation **(Lentsch and Ward, 1999)**. Several studies confirmed the impacts of polyphenols from plant products are found to be a potent inhibitor of NFKβ **(Aggarwal and Shishodia, 2006)**.

iNOS

LPS is a potent stimulator of nitric oxide (NO). Moreover, large amounts of NO to be generated in the liver during sepsis, which could impair hepatic function by direct injury to hepatocytes **(Roland *et al*., 1996).** Overexpression of iNOS has been implicated in the pathogenesis of septic shock, inflammation, and carcinogenesis. Several polyphenols from turmeric, green tea, and grapes also inhibited iNOS expression in LPS-treated RAW 264.7 cell lines most likely through the suppression of NFkβ **(Ippoushi *et al.*, 2003)**.

COX2

Cyclooxygenases are prostaglandin H synthase, which convert arachidonic acid released by membrane phospholipids into prostaglandins. There are two isoforms of prostaglandin H synthase such as COX-1 and COX-2. COX-1 is expressed in many tissues, while COX-2 is overexpressed in premalignant, malignant conditions and inflammation. COX-2 expression is induced by several transcription factors, cytokines, LPS **(Crofford *et al.*, 2000)**. Several phyto components such as galangin, luteolin, apigenin, 6-hydroxykaempferol, quercetagenin, sasanquol, genistein, green tea catechins, curcumin and resveratrol also showed to suppress COX-2 **(Aggarwal and Shishodia, 2006)**.

MAPK

In addition to NFkβ and Akt pathways, MAPK pathway also received increasing attention as a target molecule for cancer. The MAPK pathway cascade, MAP3K activates MAP2K which in turn activates a MAPK (ERK, JNK, and p38), resulting in the activation of NFkβ, cell growth, and cell survival **(Seger and Krebs, 1995)**. Recently many studies have demonstrated the role of phytochemicals in anti-inflammatory activity through down regulation of NFKβ and MAPK pathway **(Su *et al*., 2008)**. Higher concentration of polyphenol from green tea activates JNK leading to apoptosis in human hepatoma HepG2-C8 cells **(Chen *et al*., 2000)**.

CONCLUSIONS

HepG2 cells when treated with LPS for 24 h increased JNK, NFκβ, iNOS and COX2 mRNA expressions. But when HepG2 cells treated 2 h prior with VN and silymarin followed by LPS for 24 h, decreased the mRNA expression of these genes, confirm VN inhibits LPS induced ROS production and blocked NFκβ and JNK pathway. Therefore, the inhibition of these inflammation genes is an important target in the treatment of disease with anti-inflammatory components **(Surh *et al.*, 2001)**. Inhibition of NFκβ activation by VN shows that it can be useful as a nontoxic, pharmacological active compound especially as antibacterial and hapatoprotective agent.

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