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 in 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 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) and agar disc-diffusion methods (Bauer et al., 1966).

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, ethanal, 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. DMSO2 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.

Keywords: antibacterial activity, hepatoprotective activity, JNK, LPS, Vitex negundo
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 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. 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, ThermoScientific, USA. Cell viability was counted using Cello meter from Nexcemol 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% CO₂ at 37°C, and were sub-cultured regularly. For all the experiments, cells were plated at a density of 3 × 10⁴ cells/cm² 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. 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 spectrophotometer. 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 M-MLV-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 et 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 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 spectrophotometer. 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 M-MLV-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 et 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: chloroform, acetone and hexane.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Leaves (mm)</th>
<th>Seeds (mm)</th>
<th>Bark (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>14.0</td>
<td>9.9</td>
<td>8.6</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>22.5</td>
<td>17.11</td>
<td>14.13</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>11.16</td>
<td>9.3</td>
<td>6.23</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>8.50</td>
<td>5.8</td>
<td>4.4</td>
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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 DMSO.

Table 2 Agar disc diffusion method using methanolic extract of VN

<table>
<thead>
<tr>
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<th>Leaves (mm)</th>
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<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>14.1</td>
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<td>8.8</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>22.8</td>
<td>17.5</td>
<td>14.22</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>11.05</td>
<td>9.21</td>
<td>6.8</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>8.22</td>
<td>6.0</td>
<td>4.26</td>
</tr>
</tbody>
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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).

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 10⁴, 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 et al., 2015).

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 10⁴ 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.

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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 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).

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.

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 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).

LPS triggers the secretion of pro-inflammatory cytokines in many cell types, through the nuclear factor-kappa B (NF-κB) / COX-2 pathway in macrophages. Both the NFκB 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, NFκB, 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).
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.

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).

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 ILβ, pro-inflammatory genes such as JNK, iNOS and cyclooxygenase (COX-2) by activating several types of transcription factors including the nuclear factor NF-κB during as well as sepsis (Hack et al., 1997).

**NFκB**

NFκB is a family of closely related protein dimers which bind to a common sequence motif in a DNA called the κB site. During resting condition, the NFκB 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 NFκB (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 cells most likely through the suppression of NFκB (Ippoushi et al., 2003).

**COX2**

Cyclooxygenases are prostaglandin H synthase, which convert arachidonic acid into prostaglandins. Several studies confirmed the impacts of polyphenols from plant products are found to be a potent inhibitor of COX2 (Aggarwal and Shishodia, 2006).

**MAPK**

In addition to NFκB and Akt pathways, MAPK pathway also received increasing attention as a target molecule for cancer. The MAPK pathway cascade, MAPK1 activates MAP2K which in turn activates a MAPK (ERK, JNK, and p38), resulting in the activation of NFκB, cell growth, and cell survival (Seger and Krebs, 1995). Recently many studies have demonstrated the role of pyrrolopyrimidines in anti-inflammatory activity through down regulation of NFκB and MAPK pathway (Su et al., 2008). Higher concentration of polyphenol from green tea catechins 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, NFκB, 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 NFκB and JNK pathway. Therefore, the inhibition of these inflammation genes is an important target in the treatment of disease with anti-inflammatory components (Surl et al., 2001). Inhibition of NFκB activation by VN shows that it can be useful as a nontoxic, pharmacological active compound especially as antibacterial and hepatoprotective agent.

**REFERENCES**


