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Evaluating the anti-cancer potential of hydro-alcoholic extract of *Allium sativum* L.: An *In vitro* and *In vivo* study

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**Abbreviations:**  
NF-κB: Nuclear factor κB; Brdu: 5-bromo-2'-deoxyuridine; AFP: Alphafetoprotein; DEN: Diethylnitrosomine; ASE: *Allium sativum* L. extract; ROS: Reactive oxygen species; CPCSEA- Committee for the Purpose of Control and Supervision on Experiments on Animals; b.w: Body weight.

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**Abstract**

The present study investigates the anti-cancer potential of hydroalcoholic *Allium sativum* L. extract (ASE) through *in vitro* as well as *in vivo* experiments. The hydroalcoholic extract exerted antiproliferative effects on human liver carcinoma cell line (HepG2) in a dose-dependent manner. ASE was orally administered at the concentration of 250 and 500 mg/kg body weight in Diethylnitrosomine (DEN) induced liver cancer rat model. ASE induced apoptosis in both cell lines and cancer induced animals, at the doses of ASE 100 μg/ml in *in vitro* and 500 mg/kg in vivo. The level of anti-oxidant enzymes was also studied in tissue and serum along with biochemical parameters. ASE was able to scavenge reactive oxygen species (ROS) and normalize the altered Alpha fetoprotein (AFP) levels in liver cancer induced rat model. RT-PCR analysis revealed that the mRNA expression of NF-κB was markedly decreased in *in vitro* and in vivo upon treated with the plant extract. Our data suggest the presence of bioactive compounds in ASE, capable of augmenting liver carcinoma cells by induction of apoptosis and altered NF-κB signaling, partially due to antioxidant properties. Further elucidation of bioactive molecules and underlying mechanisms could lead to potential intervention in liver cancer.

**Citation:**  

1. Introduction

Liver cancer is one of the most lethal among the seven leading causes of cancer deaths worldwide. Prognosis is usually poor, and no effective chemotherapeutic treatment is presently available (Hussain et al., 2001). Many of the immunological functions of liver cells are due to high activity of the NF-κB, family of transcription factors. These factors are key regulators of genes involved in immunity, wound healing, proliferation and apoptosis (Karin et al., 2002, 2006; Muriel, 2007). In reference with a pro-oncogenic activity, NF-κB promotes expression of several matrix metalloproteinases (MMPs), including MMP-2, -3 and -9 which are key modulators of many biological processes such as angiogenesis, cellular migration, inflammation and cancer (Lu and Wahl, 2005; Egeblad and Werb, 2002). NF-κB is generally seen as an anti-apoptotic factor since it induces anti-apoptotic genes that block the caspase cascade (Wang et al., 1998; Melisi and Chiao, 2007). Therefore, activation of NF-κB in cancer cells by chemotherapy or radiation...
therapy is often related with the acquirement of resistance to apoptosis. While these data raised an admontory note about the agents that block NF-κB, could represent a new and promising strategy in cancer treatment.

*Allium sativum* L. has been used throughout history for both culinary and medicinal purposes (Block, 1992). Recent studies have validated many of the medicinal properties attributed to *A. sativum* and its preparations have been widely recognized as agents for prevention and treatment of cardiovascular and other metabolic diseases, atherosclerosis, hyperlipidemia, thrombosis, hypertension, dementia, diabetes (Hosseini et al., 2007) and cancer (Amagase and Milner, 1993). The major unique organosulfur compounds in alliums are water-soluble (S-allylcysteine and S-allymercaptocysteine) and lipid-soluble compounds (alliin, diallyl sulfide, triallyl sulfide, diallyl disulfide, diallyl polysulfides) (Lawson et al., 1991; Matsuura, 1997) which have potent antioxidant activity (Awazu and Horie, 1997; Wei and Lau, 1998). The potential targets of the chemotherapeutic agent and plant extract for the treatment of cancer includes inhibition of carcinogen metabolic activity, controlling cell proliferation, cell cycle arrest, induction of apoptosis, anti-oxidative activity, inhibition of angiogenic process and modulation of multidrug resistance (Ren et al., 2003). There is a wealth of evidence suggesting that organosulfur constituents are most likely involved for the beneficial effects of this herb. The anti-cancer potential of hydro-alcoholic extract of *A. sativum* L. (ASE) remains to be determined. The present study was designed to evaluate the in vitro and in vivo anti-cancer potential of ASE. To ascertain the anti-proliferative effects of ASE, MTT assay (in vitro) and BrdU assay (in vivo) was used. Furthermore, expression of anti-apoptotic factor NF-κB was studied at mRNA level by RT-PCR. The levels of MMPs were ascertained using gelatin zymography after treatment with ASE.

2. Material and Method

2.1. Plant Material
Fresh *A. sativum* bulb was purchased from the local market of Ahmedabad, India and was authenticated by Dr. Vasant A. Patel, Department of Botany, Smt. S.M.P. Science College, Hemachandracharya North Gujarat University, Gujarat, India, voucher no. ISNU/AS/CN-120422/01. The bulb consists of about 10 daughter bulbs (clove) arranged roughly in a circle around a central axis. Each daughter bulb has a tough, white or reddish skin around a fleshy tubular leaf, investing a more or less rounded elongated cone of leaf primordia and vegetative apex.

2.2. Preparation of hydro-alcoholic *Allium sativum* L. Extract (ASE)
*A. sativum* L. bulbs were grinded in 50% ethanol. The obtained hydro-alcoholic A. sativum extract was stirred overnight at 50°C, followed by filtration under sterile conditions. The filtrate was vacuum dried at 50°C to remove the solvent completely, weighed and reconstituted in double distilled water to form a final concentration of 50 mg/ml ASE. The yield of the extract was 11.8% (w/w). It was stored at −20°C in 1 ml aliquots until further use.

2.3. Cell culture
HepG2 (human liver) cell line were obtained from NCCS, Pune, India. The cell line was maintained on Dulbecco’s modified Eagle’s minimal essential medium (DMEM) supplemented with streptomycin (100 U/ml), gentamycin (100 mg/ml), and 10% FBS at 37°C and humid environment containing 5% CO₂.

2.4. Experimental animals
56 adult healthy male Wistar rats weighing 250-350 g were procured from Zydis Research Centre, Ahmedabad, India. Compliance with CPCSEA guidelines. The Guidance for Care and Use of Animals for Scientific Research (Indian National Science Academy, 2000) was strictly followed. The animals were acclimatized for five days prior to the experiments. The animals were monitored every day for their body weight, food and water intake and any visible symptoms, during the whole experiment. Hepatocarcinogenesis was induced with a single intraperitoneally injection of DEN (Sigma Chemical Co., St. Louis, MO, USA) at a concentration of 200 mg/kg body weight (b.w). 2-Acetaminofluorene (2-AAF) was given orally in the concentration of 20 mg/kg body weight 5 days a week, for 3 weeks to enhance the effect of DEN in the induction of liver cancer. Autopsy of animals was performed after induction and treatment. The animals were divided into 4 groups: control, DEN treated group 1 (DEN+2-AAF induced), Reversal group 2 (DEN+ASE250 mg/kg b.w) and Reversal group 3 (DEN+ASE500 mg/kg b.w) respectively. The experimental design is shown in (Table 2.4).
Table 2.4: Autopsy schedule indicated number of animals in each of four groups.

<table>
<thead>
<tr>
<th>Days of dissection</th>
<th>Control</th>
<th>DEN treated</th>
<th>DEN+ASE treated 250mg/kg</th>
<th>DEN+ASE treated 500mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 days</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
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<tr>
<td>40 days</td>
<td>4</td>
<td>4</td>
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<td>4</td>
</tr>
<tr>
<td>60 days</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

2.5. Cytotoxicity assay (in vitro)
HepG2 cells were seeded in a 96-well plate at a density of 2.0×10^4 cells/ml as described by Mauceri et al. (1998). Extract of different concentrations in two fold dilution (1.55, 3.12, 6.25, 12.5, 25.0, 50.0, 100, 200, 400, 800, 1600 µg/ml) were added to each well and cultured for 72 h, was assessed following our previous study (Chaudhary et al., 2011). The medium of control culture was treated with vehicle (0.02 % ethanol) in fresh media, followed by incubation with 0.5 mg/ml MTT for 4 h. Finally, 100 µl of DMSO was added and absorbance at 595 nm wavelength was measured.

2.6. Antiproliferative study (in vivo)
The intraperitonial injection of BrdU of 100 mg/kg body weight was given at 8 to 10 h prior to their autopsy. After the animals were sacrificed, liver tissues were fixed in 10% formalin and embedded in paraffin. Slides were stained overnight with the primary mouse anti-BrdU antibody. Subsequently, peroxidase-conjugated rabbit antimouse IgG was applied and finally stained with DAB (Muskhelishvili et al., 2003).

2.7. DNA fragmentation assay
HepG2 cells were treated with 50 and 100 µg/ml extract concentrations based on MTT assay results and 250 and 500 mg/kg b.w were given to liver cancer induced animals. The cells were pelleted by centrifugation at 1000xg for 5 min at room temperature. Aliquots from cells were resuspended in 500 µl of digestion buffer (10 mM EDTA, pH 8.0, 50 mM Tris, 0.5 % SDS) containing 0.5 mg/ml and for in vivo study, liver tissues were chopped and suspended in lysis buffer (2 % Sodium lauryal sulphate, 10 mM EDTA, 10 mM Tris-HCl, pH 8.5) and proteinase K. After incubation at 55° C for 1 h, phenol:chloroform washes were given and RNase A was added for an additional 1 h and DNA was precipitated with chilled ethanol. The DNA was separated in 1 % agarose gels and visualized by UV illumination after ethidium bromide staining.

2.8. Gelatinase zymography of MMP-2 and MMP-9
Matrix metalloproteinases (MMP) released into conditioned media was determined by gelatinase zymography according to the method of Newcomb et al. (2005), for in vitro study and Patricia et al. (2005), for in vivo study with minor modifications. Briefly, gelatinases present in the tissue extracts degrade the gelatin matrix, leaving a clear band after staining the gel for protein. Proteins were electrophoresed under non-reducing conditions on 10% polyacrylamide gels containing 1 mg/ml gelatin (Sigma–Aldrich). After electrophoresis, the gels were renatured in 2.5 % Triton X-100 (2×15 min), then incubated overnight at 37° C in development buffer (50 mM Tris–HCl, pH 7.6; 10 mM CaCl_2; 50 mM NaCl; 0.05 % Brij35 (Invitrogen). The gels were stained with 0.5 % Coomassie Brilliant Blue R-250.

2.9. RT-PCR
Total RNA from HepG2 cells and liver tissue was isolated using the TRIzol reagent (Sigma–Aldrich) and reverse transcribed according to the manufacturer’s instructions. Briefly, the cDNA was amplified in a 50 µl reaction containing primer pairs (each 1.0 µl): β-actin (forward primer 5’ TCACCCCACTGTGCCCATCTACGA3’), reverse primer 5’ CAGCGGAACCGCTCATTGCCAATGG3’) and NF-κB (forward primer 5’ CCCACGAGCTTTGTAGAAAG3’), reverse primer 5’ CCAGGTTCGAAACTGTTGAT3’), 10X buffer (5.0 µl), cDNA (2.0 µg), 25 mM MgCl_2 (3.0µl), 10 mM dNTPs (1.0 µl), and Taq polymerase (2.5 U). PCR amplification cycles consisted of denaturation at 94° C for 1 min, primer annealing at 57° C for 45 s and extension at 72° C for 45 s for a total of 30 cycles followed by final extension at 72° C. The PCR product was separated by electrophoresis on 2 % agarose gels.
2.10. Estimation of antioxidants in rats

2.10.1. Preparation of Sample
Following each autopsy schedule, the liver of each group was removed immediately and homogenized in 10 volumes of chilled homogenizing buffer containing 250 mM Sucrose, 12 mM Tris-HCl, 0.1 mM DTT, at pH 7.4. Homogenates was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was subsequently transferred to chilled micro centrifuge tubes and used for below mentioned estimations.

2.10.2. Anti-oxidant assays
The tissue homogenate was then subjected to anti-oxidant studies of Catalase (Aebi, 1984), Superoxide dismutase (Kono, 1978), Glutathione content (GSH) (Sedlak and Lindsay, 1968), lipid peroxidation level (Beuge and Aust, 1978) and Protein carbonyl content (Levine et al., 1990).

2.10.3. Serum Profiling
Serum was separated from the blood collected at the time of autopsy. The serum was used for the biochemical estimation of Serum glutamate oxaloacetate transferase (SGOT), Serum glutamate pyruvate transferase (SGPT), γ-Glutamyl-Transferase (GGT) and bilirubin assay. These assays were performed with the help of Qualigens Diagnostics kits.

2.10.4. AFP assay
PATHOZYME® ALPHA-FETOPROTEIN (Omega) kit was used for the detection of AFP level in the serum of experimental animals following the manufacturer’s protocol.

2.11. Preliminary phytochemical screening
One gram of the extracts was dissolved in 100 ml of its solvent to obtain a stock of concentration 1% (v/v). The extract thus obtained was subjected to preliminary phytochemical screening.

2.11.1. Screening procedure
The extracts were tested for the presence of alkaloids, amino acids, antraquinones, flavonoids, glycosides, phytosterol, saponins, steroids, tannins and triterpenoids following the methodology of Harborne (1998) and Kokate (2001).

2.11.2. HPLC analysis
The analysis was performed with a flow rate of 0.50 mL min⁻¹, using mobile phase 0.05 M KH₂PO₄ buffer of pH 3 and acetonitrile (50:50 v/v) for 60 min. The hydro-alcoholic extract and each solvent were filtered on qualitative circle, Whatman filter paper No. 1 under vacuum. Analysis by high-performance liquid chromatography (HPLC) system consisting of Photodiode array detector (Waters Alliance model: 2695 separation module with Waters 2996 Photodiode Array Detector, Waters Corporation, Milford, MA, USA) was operated at 210 nm and a reverse-phase C18 column (Phenomenex® make Luna C18 (2) HPLC column with Length=25 cm, ID= 4.6 mm, Particle size=5.0μm, Phenomenex Inc, Torrance, CA, USA) was used for the process.

2.12. Statistical analysis
Values were expressed as mean ± SEM. A one-way analysis of variance (ANOVA) was used to determine the statistical significance, which were assumed to be different when the comparison showed a significance level of P<0.05.

3. Results and Discussion
3.1. Antiproliferative effects of ASE
The ASE was evaluated for its antiproliferative potential using MTT assay. The extract inhibited the proliferation of HepG2 cells in a dose dependent manner. IC₅₀ value was calculated from the growth curve obtained by MTT assay and was found to be 100±7.9 μg/ml, so we selected two doses 50 µg/ml and 100 µg/ml for further testing (Fig. 3.1A). The phase contrast micrographs supported the MTT results as there was decrease in cell density when treated with different doses of extract (Fig. 3.1B). Significant numbers of cells were found to be round, detached and floating at higher concentrations of the extract. DEN induced in vivo model for hepatocarcinogenesis is widely studied and model system. It is known to cause perturbations in the nuclear enzymes involved in DNA repair and replication (Bhosale et al., 2002) and has been suggested to cause cellular injury due to the enhanced formation of free radicals (Ramakrishnan et al., 2006). To measure DNA synthesis /cell proliferation, BrdU immunostaining was used. Earlier studies have demonstrated that incorporation of BrdU in the DNA indicates highly proliferative activity of the cell (Muskhelishvili et al., 2002) and has been suggested to cause cellular injury due to the enhanced formation of free radicals (Ramakrishnan et al., 2006). To measure DNA synthesis /cell proliferation, BrdU immunostaining was used. Earlier studies have demonstrated that incorporation of BrdU in the DNA indicates highly proliferative activity of the cell (Muskhelishvili et al., 2003). BrdU readily gets incorporated into nuclei during the DNA synthetic phase of the cell cycle, and is detected with an anti-BrdU antibody. Analysis of the number of BrdU-labeled cells demonstrated that DEN induction significantly increased the number of BrdU-positive cells in the rat liver as compared to control. ASE treatment is shown to lower the proliferation rate as indicated by lower
number of BrdU-positive cells. Furthermore, the number was significantly lowered in animals group treated with ASE 500 mg/kg than ASE 250 mg/kg body weight. ASE 500 mg/kg b.w. was able to reduce significantly the number of BrdU positive cells and tissue histology appeared to be closer to normal. In present study, number of BrdU positive cells was significantly higher in DEN induced animals as compared to control animals. ASE extract administration decreased the number of positive cells significantly indicating its anti-proliferative activity (Fig. 3.1C). Uda and colleagues (2006) reported that aged garlic extract inhibited the development of putative preneoplastic lesions in rat hepatocarcinogenesis by slowing in the proliferation rate of liver cells after partial hepatectomy. In concordance, our study further establishes the anti-proliferative role of ASE.

3.2. DNA fragmentation assay in rat model
Cleavage of chromosomal DNA into oligonucleosomal size fragments is a biochemical hallmark of apoptosis. The apoptosis resistance of hepatic cells was reported to be one of the significant factors for

Fig 3.1: (A) MTT assay of HepG2 cell lines treated with different concentration of ASE.

Fig 3.2: (A) Induction of DNA fragmentation by ASE in HepG2 cell lines. The cells were Lane L: 100 bp DNA ladder marker; Lane 1: Vehicle-treated cells; Lanes 2 and 3: Cells treated with 50 and 100 µg/ml of ASE, respectively. (B) Induction of DNA fragmentation by ASE in liver tissue having Lane 1: Control; Lane 2: Treated group 1 (DEN+2AAF); Lane 3: Reversal group 2 (DEN+ASE250 mg/kg b.w) and Lane 4: Reversal group 3 (DEN+ASE500 mg/kg b.w).

Fig 3.3: (A) Gelatin zymography of MMP-2 and MMP-9 in HepG2 cell lines as a Lane 1-Control, Lanes 2 and 3: Cells treated with 50 and 100 µg/ml of ASE, respectively. (B) Gelatin zymography of MMP-2 and MMP-9 in liver tissue of Lane 1: Control; Lane 2: Treated group 1 (DEN+2AAF); Lane 3: Reversal group 2 (DEN+ASE250 mg/kg b.w) and Lane 4: Reversal group 3 (DEN+ASE500 mg/kg b.w).

Fig 3.4: (A) Reverse transcription-polymerase chain reaction (RT-PCR) showing NFkB expression in HepG2 cell lines as, Lane 1-Control, Lanes 2 and 3: Cells treated with 50 and 100 µg/ml of ASE, respectively. (B) RT-PCR of NFkB expression in liver tissue as, Lane 1: Control; Lane 2: Treated group 1 (DEN+2AAF); Lane 3: Reversal group 2 (DEN+ASE250 mg/kg b.w) and Lane 4: Reversal group 3 (DEN+ASE500 mg/kg b.w).
hepatocarcinogenesis or tumor progression in hepatocellular carcinomas (HCCs) (Ikeguchi et al., 2001). DNA fragmentation reflecting the endonuclease activity characteristic of apoptosis, was analyzed. ASE was able to induce DNA fragmentation in HepG2 cells and experimental animals more prominent at 100 µg and 500 mg/kg body weight respectively (Fig. 3.2 A and B). There was ladder formation of low molecular weight in the treated group as compared to control group. DNA isolated from liver cancer induced animals and HepG2 did not show any fragmentation because of apoptosis resistance potency of HCC cells.

3.3. Gelatinase zymography of the MMPs
Matrix metalloproteinase (MMP) activation is associated with basement membrane remodeling that occurs in injured tissues and during tumor invasion (Osaki et al., 1996). High levels of MMP2 and MMP9 were reported in hepatocellular carcinomas (HCCs) and liver metastases, compared with their low levels of expression in normal liver (Musso et al., 1997). Gelatin zymography is mainly used for the detection of the gelatinases, MMP-2 and MMP-9. After zymography, two gelatinolytic bands could be observed of MMP-2 and MMP-9. The expressions of these proteases were observed to be elevated in HepG2 and cancer induced animals, the enzymatic activity of both the MMP-2 and MMP-9 enzyme was decreased following the extract treatment with maximal effect in 100 µg/ml treated group in the media from HepG2 cells (Fig. 3.3 A) and ASE500 mg/kg b.w treated DEN induced rats (Fig. 3.3 B). ASE was able to reduce the levels of MMPs significantly implicating its anti-invasive role in cancer.

3.4. Expression of NF-κB
NF-κB plays important roles in processes, including development, cell growth and survival, and proliferation and are involved in many pathological conditions (Hayden et al., 2008). Thus the role of NF-κB was looked into in the present study. As the extract was able to exert antiproliferative effects in HepG2 cells and DEN induced rats, the expression of transcription factor NF-κB was analysed using RT-PCR. The extract was able to inhibit the expression of NF-κB at transcriptional level as evident by RT PCR results at 100 µg/ml on HepG2 cells (Fig. 3.4A) and ASE500 mg/kg in cancer induced rats (Fig. 3.4B). Results indicated that the level of expression increased in liver cancer induced animals because of activation of NF-κB signaling pathway. There is a decrease in the expression level of the mRNA level in ASE treated HepG2 cells and DEN induced animals in a dose dependent manner.

3.5. Tissue Biochemistry
While certain NF-κB-regulated genes play a major role in regulating the amount of reactive oxygen species (ROS) in the cell, ROS have various inhibitory or stimulatory roles in NF-κB signaling (Wyllie et al., 1980). ASE reduces the ROS levels by its potential antioxidant activity and thus deactivates NF-κB signaling pathway. It is well known that A. sativum contains natural inhibitor of NF-κB (Keiss et al., 2003). In our study the levels of antioxidants such as catalase and SOD were reduced in DEN treated animals. The increased levels of ROS are unable to be controlled by cellular antioxidants leading to damage to the liver tissues. In tissue biochemistry of liver total protein, catalase activity, superoxide dismutase (SOD) activity, reduced glutathione (GSH), lipid peroxidation (LPx) levels and protein oxidation (PO) levels were determined. ASE 250 mg/ml and 500 mg/ml contain many antioxidants that control the levels of ROS and thus increase the catalase and SOD activity of cancer induced animals almost close to the control group animals. Further, protein carbonyl content, the most commonly used marker for protein oxidation due to ROS formation, was analyzed (Stadtman and Oliver, 1991). Redox cycling cations can bind to cation binding locations of protein and with the aid of further attack of ROS can transform side chain of several amino acids to carbonyl groups. LPx levels are directly associated with intracellular ROS levels and increases with elevation in the ROS levels. In present study LPx and PO levels were found to be significantly increased after 60 days in DEN treated group as compared to control group. ASE treated group showed decrease in the LPx as well as PO levels when administered for 40 days and were significantly lowered in the 60 days treated group as compared to DEN induced animals. Results showed that higher PO results from oxidative stress of ROS in DEN induced animals which were reduced by ASE treatment in dose dependent manner. ASE treatment was also able to revert back the damage to phospholipids in the membrane as less LPx was observed in ASE treated group as compared to DEN treated animals. Cellular damage is prevented by another mechanism in which high concentrations of cellular nucleophiles float around the nucleus. GSH is one of the important molecules. It plays an important role providing reducing equivalents to protect cells from peroxidative damage
Table 3.5: Anti-oxidant assay from liver tissue of Control, Control animals, Treated group 1 (DEN+2AAF), Reversal group 2 (DEN+ASE250 mg/kg b.w) and Reversal group 3 (DEN+ASE500 mg/kg b.w)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>40 days plant extract</th>
<th>60 days plant extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>DEN</td>
</tr>
<tr>
<td>SOD (units/min/g)</td>
<td>112.6 ± 5.13</td>
<td>85.4 ± 6.7**</td>
</tr>
<tr>
<td>CAT (H₂O₂/min/mg)</td>
<td>0.057 ± 0.004</td>
<td>0.032 ± 0.005**</td>
</tr>
<tr>
<td>GSH (mmol/gm)</td>
<td>4.1 ± 0.18</td>
<td>2.2 ± 0.12**</td>
</tr>
<tr>
<td>LPx (mmoles MDA/mg)</td>
<td>35.92 ± 1.7</td>
<td>53.84 ± 2.6**</td>
</tr>
<tr>
<td>PO (nmol/ml)</td>
<td>40 ± 2.0</td>
<td>48.9 ± 2.4**</td>
</tr>
</tbody>
</table>

Values are mean ± SEM for 4 animals in each observation. *<0.05; **<0.01; ***<0.001; as compared with control group.

Table 3.6: Anti-oxidant assay from serum of Control, Control animals, Treated group 1 (DEN+2AAF), Reversal group 2 (DEN+ASE250 mg/kg b.w) and Reversal group 3 (DEN+ASE500 mg/kg b.w)

<table>
<thead>
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<th>Parameters</th>
<th>40 days plant extract</th>
<th>60 days plant extract</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>DEN</td>
</tr>
<tr>
<td>SGOT (U/L)</td>
<td>24.9±1.34</td>
<td>38.9±1.44**</td>
</tr>
<tr>
<td>SGPT (U/L)</td>
<td>35.8±1.79</td>
<td>51.6±2.58**</td>
</tr>
<tr>
<td>Bilirubin (g%)</td>
<td>0.33±0.016</td>
<td>1.1±0.045**</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>5.2±0.2</td>
<td>10.6±0.5**</td>
</tr>
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Table 3.7: HPLC data showing peak values

<table>
<thead>
<tr>
<th>Peak</th>
<th>RT (min)</th>
<th>Area</th>
<th>% Area</th>
<th>Height</th>
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</thead>
<tbody>
<tr>
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<td>327581</td>
<td>0.26</td>
<td>23438</td>
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<tr>
<td>Peak 2</td>
<td>1.704</td>
<td>18640377</td>
<td>14.91</td>
<td>963404</td>
</tr>
<tr>
<td>Peak 3</td>
<td>2.000</td>
<td>8518909</td>
<td>6.81</td>
<td>64577</td>
</tr>
<tr>
<td>Peak 4</td>
<td>2.951</td>
<td>97490863</td>
<td>77.98</td>
<td>3003346</td>
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<tr>
<td>Peak 5</td>
<td>6.099</td>
<td>40720</td>
<td>0.03</td>
<td>2183</td>
</tr>
</tbody>
</table>

Fig 3.6: AFP levels in control, DEN induced, ASE treated with 250mg/kg b.w and 500 mg/kg

Fig 3.7: HPLC chromatogram showing different peaks for some of unknown compounds from ASE.
GSH was found to decrease in DEN induced animals as compared to control group in 40 days and 60 days treated group. In ASE treated animals with 250 and 500 mg/kg administration for 60 days lead to significant increase in GSH levels as compared to DEN treated animals (Table 3.5).

3.6. Serum profiling

In serum analysis, Gamma glutamate transferase (GGT), Serum glutamate oxaloacetate transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT) and bilirubin were estimated. SGPT, SGOT and GGT are important enzymes present in the liver. Liver damage lead to elevated levels of these enzymes in serum of DEN induced animals as compared to control group. The activities of the enzymes were increased indicating the toxic effect of DEN on liver. ASE was able to normalize the level of these enzymes upon treatment for 60 days. DEN induction leads to an increase in bilirubin levels. ASE was to significantly bring down the level of bilirubin in time and dose dependent manner (Table 3.6). AFP screening, at present, is the most sensitive, convenient and economical methods for detecting early liver cancers (Liu et al., 2011). AFP values were observed to be significantly increased in 40 days and 60 days DEN induced animals as compared to control group. The values were noted to decline significantly following the plant extract treatment towards to control group animals (Fig. 3.6).

Conclusion

This study showed that ASE limits the proliferation of HepG2 cells and its oral administration for sixty days to male Wistar rats protected the hepatic tissues from damage by increasing the anti-oxidant levels, induction of apoptosis, lowering the MMP level and down regulation of NF-κB against DEN induced hepatocarcinogenesis. Further investigation of the bioactive component(s) in the extract for their anti-proliferative properties and their mode of action would lead to a therapeutically important intervention for liver cancer.

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development and progression. Nature, 441, 431-
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